Altering the Temporal Regulation of One Transcription Factor Drives Evolutionary Trade-Offs between Head Sensory Organs

Highlights
- Sensory trade-offs derive from changes in head primordium partitioning
- Changes in temporal patterning of the head primordium lead to changes in eye size
- A SNP in an eyeless enhancer alters temporal regulation and eye size
- The SNP alters the affinity of the Cut repressor to a site in the eyeless enhancer

Authors
Ariane Ramaekers, Annelies Claeys, Martin Kapun, ..., Thomas Flatt, Erich Buchner, Bassem A. Hassan

Correspondence
ariane.ramaekers@curie.fr (A.R.), bassem.hassan@icm-institute.org (B.A.H.)

In Brief
Size trade-offs between head sensory organs (visual versus olfactory), which develop from a common primordium, are pervasive in nature. Ramaekers et al. demonstrate that genetically encoded changes in the temporal regulation of the eye-determination transcription factor Eyeless/Pax6 can explain developmental and evolutionary changes in the relative size of the fly eye.
Altering the Temporal Regulation of One Transcription Factor Drives Evolutionary Trade-Offs between Head Sensory Organs

Ariane Ramaekers,1,9,* Annelies Claeyts,5,3 Martin Kapun,4,10 Emmanuèle Mouchel-Vielf,5 Delphine Potier,6 Simon Weinberger,2,3 Nicola Grillenzi,1 Delphine Dardalhon-Cuménal,5 Jiekyun Yan,2,3 Reinhard Wolf,7 Thomas Flatt,4 Erich Buchner,6 and Bassem A. Hassan1,11,*

1Institut du Cerveau et de la Moelle Épinière (ICM) - Hôpital Pitié-Salpêtrière, Sorbonne Université, Inserm, CNRS, Paris, France
2VIB Center for Brain and Disease, VIB, Leuven, Belgium
3Center for Human Genetics, University of Leuven School of Medicine, Leuven, Belgium
4Department of Biology, University of Fribourg, Fribourg, Switzerland
5Sorbonne Université, CNRS, Laboratoire de Biologie du Développement, Institut de Biologie Paris Seine, LBD-IBPS), Paris, France
6Aix-Marseille Université, CNRS, INSERM, CML, Marseille, France
7Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany
8Institute for Clinical Neurobiology, University Hospital Würzburg, Würzburg, Germany
9Present address: Institut Curie, PSL Research University, CNRS, Sorbonne Université, Nuclear Dynamics Laboratory, Paris, France
10Present address: Department of Cell & Developmental Biology, Medical University of Vienna, Austria, Department of Evolutionary Biology and Environmental Studies, University of Zürich, Switzerland
11Lead Contact
*Correspondence: ariane.ramaekers@curie.fr (A.R.), bassem.hassan@icm-institute.org (B.A.H.)
https://doi.org/10.1016/j.devcel.2019.07.027

SUMMARY

Size trade-offs of visual versus olfactory organs is a pervasive feature of animal evolution. This could result from genetic or functional constraints. We demonstrate that head sensory organ size trade-offs in Drosophila are genetically encoded and arise through differential subdivision of the head primordium into visual versus non-visual fields. We discover that changes in the temporal regulation of the highly conserved eyeless/Pax6 gene expression during development is a conserved mechanism for sensory trade-offs within and between Drosophila species. We identify a natural single nucleotide polymorphism in the cis-regulatory region of eyeless in a binding site of its repressor Cut that is sufficient to alter its temporal regulation and eye size. Because eyeless/Pax6 is a conserved regulator of head sensory placode subdivision, we propose that its temporal regulation is key to define the relative size of head sensory organs.

INTRODUCTION

The senses animals rely on have been shaped during evolution to better navigate and exploit the environment. As a result, even closely related species living in different ecological niches show variation in the sizes and shapes of their sensory structures. Adaptive variation in visual sensory organs is a fascinating case in point and ranges from almost complete loss of the eyes in darkness-adapted animals (Partha et al., 2017; Rétaux and Casane, 2013) to the expansion of visual organs and processing areas in some other groups such as tree-dwelling mammals (Campi and Krubitzer, 2010; Campi et al., 2011) and predator insects (Elzinga, 2003). A striking, yet poorly understood feature of natural variation in eye size is that it often occurs as a trade-off between the visual organs and other head sensory structures such as olfactory organs. This was described in a large variety of animal groups including mammals (Nummela et al., 2013) and fishes (Rétaux and Casane, 2013). In arthropods as well, trade-offs between the size of the eyes and of the antennae, where most olfactory organs are located, are pervasive. Examples include beetle species with different life-styles (nocturnal versus diurnal; visual hunters or not (Bauer and Kredler, 1993); fireflies emitting or not emitting light signals (Stanger-Hall et al., 2018); surface and cave crustaceans (Protas and Jeffery, 2012); and millipedes (Liu et al., 2017). This is also the case between and within species of fruit flies, in which eye size often anti-correlates with the size of the face and/or of the antennae (Posnien et al., 2012; Arif et al., 2013; Norry and Gomez, 2017; Gaspar et al., 2019; Keesey et al., 2019). However, the developmental mechanisms that govern such trade-offs are essentially unknown.

A commonly observed property of sensory organ formation is the shared developmental origin of most head sensory structures—such as eyes and noses—that derive from the subdivision of a single multipotent primordium. In vertebrates, the olfactory and lens placodes derive from the subdivision of the anterior aspect of a multipotent preplacodal ectoderm (Grocott et al., 2012; Singh and Groves, 2016). Similarly, during Drosophila development, the ectodermal eye-antennal imaginal disk (EAD) gives rise to all external sensory, including the visual (compound eyes and ocelli) and olfactory (antennae and maxillary palps) sense organs, and non-sensory head cuticle. In vertebrates and in flies, antagonistic relationships between gene
regulatory networks (GRNs) and signaling pathways that promote different sensory identities regulate the subdivision of the multipotent primordium (Grocott et al., 2012; Singh and Groves, 2016; Wang and Sun, 2012; Weasner and Kumar, 2013). First active in the entire tissue, their expression segregates as the visual and non-visual territories become distinct (Bhattacharyya et al., 2004; Kenyon et al., 2003). In addition to promoting eye fate, the transcription factor (TF) Pax6 and its two Drosophila orthologues Eyeless (Ey) and Twin-of-Eyeless (Toy), play a key role in the growth and the subdivision of the multipotent primordium (Zhu et al., 2017). In Drosophila, at early developmental stages, Ey and Toy are co-expressed in the entire EAD with antennal TFs, such as Homothorax (Hth). The expression of these eye and antenna-promoting TFs progressively segregates along the EAD’s anterior-posterior axis, delineating the posterior eye and anterior antennal compartments. Eye and antennal TFs mutually repress each other: the antennal TFs Hth and Cut (Ct) directly repress ey expression while Sine oculis, another eye promoting TF, represses ct (Anderson et al., 2012; Wang and Sun, 2012; Weasner and Kumar, 2013). Consequently, loss or gain of function of these selector TFs leads to the transformation of most of the head tissue into visual or olfactory organs at the expense of the other sensory structure (Palmer et al., 1995; Czerny et al., 1999; Anderson et al., 2012).

Therefore, the subdivision of a single multipotent primordium into distinct territories through mutual repression by antenna-promoting TFs is a shared step of the development of head sensory organs across animals. It is thus tempting to speculate that evolutionary mechanisms have exploited this process leading to natural sensory size trade-offs between visual and olfactory territories. A hint in that direction comes from studies on Astyanax fishes, which live as cave or surface-dwelling morphs (Rétaux and Casane, 2013). Cave morphs have small lenses and large olfactory placodes, while surface-dwellers show the reciprocal ratio. Chemical manipulation of signaling pathways that regulate the subdivision of the lens versus olfactory territories mimics the differences observed between natural morphs (Hinaux et al., 2016). Whether this is a mechanism of natural variation in sensory trade-offs is unknown. Demonstrating a direct link requires the identification of naturally occurring causal genetic variants and the elucidation of their effect on the GRNs that regulate visual and olfactory sensory organ development. The paucity of model systems amenable to combining comparative, genetic, molecular, and developmental analyses has thus far hindered such an endeavor.

We reasoned that natural variation in eye size between and within Drosophila species may offer precisely such a model. We therefore used comparative analyses combined with developmental, molecular, and genome editing approaches to tackle this question. We show that differential subdivision of the EAD, resulting in different proportions of eye and antennal compartments, underlies eye size variation between and within Drosophila species. In both cases, this is associated with changes in the temporal regulation of the expression of ey during EAD subdivision. We also demonstrate that in D. melanogaster (D. mel.), this is caused by a non-coding single polymorphic nucleotide (SNP) present in most natural populations of D. mel. This SNP is located in a binding site for the antennal factor Ct within the eye enhancer of ey. Using CRISPR/Cas9 genome editing, we show that this SNP is causal to temporal changes in ey expression and to facet number variation. Thus, changes in the subdivision of a multipotent primordium, caused by subtle alterations of the mutual repression between distinct fates, underlies natural variation in sensory trade-offs.

**RESULTS**

**Reciprocal Changes in the Sizes of Visual and Non-visual Head Structures**

The insect compound eye is composed of a crystalline array of small units, named facets or ommatidia. In insects, and specifically in Drosophilids, eye size depends both on the number and diameter of the ommatidia and is often negatively correlated with face and/or antenna size (Posnien et al., 2012; Arif et al., 2013; Norry and Gomez, 2017; Gaspar et al., 2019; Keesey et al., 2019). In this study, we selected four Drosophila species, which presented a larger eye to face width ratio as compared to D. mel. (Figures 1A, 1A’, and S1). All subsequent morphological measurements were performed on females raised in density-controlled conditions. We focused on Drosophila pseudoobscura (D. pse), which had the largest difference in terms of ommatidia number, an increase of 35% as compared to D. mel. (Figures 1B and 1B’) while sharing similar facet diameters (Figures 1C and 1C’). Interestingly, the third antennal segment, which hosts the olfactory sensillae, was thinner in D. pse. as compared to D. mel. (Figures 1D and 1E). As a control, we measured tibia length as a proxy to body size (Posnien et al., 2012; Arif et al., 2013) (Figure S2). In line with previous studies (e.g., Posnien et al., 2012; Arif et al., 2013; Gaspar et al., 2019; Keesey et al., 2019), our results suggest that variation in eye, face, or antennal size cannot be explained solely by variation in body size.

Increased facet number has been associated with higher visual acuity in predator flies (Elzinga, 2003; Gonzalez-Bellido et al., 2011). We thus tested whether a modest variation such as the one observed between D. pse. and D. mel. was potentially relevant to visual function. We measured the minimal angular distance between two successive vertical black stripes resolved by the flies as a read-out of their visual acuity (Figure 1F) (Gott, 1964; Buchner, 1976). D. pse. were able to distinguish between more closely juxtaposed stripes (minimal angle = 7.0′) as compared to D. mel. (minimal angle = 8.51′) (Figure 1F’). Thus, D. pse. have a better visual acuity correlating with an increased number of facets.

**A Trade-Off between Eye and Non-eye Progenitor Fields**

What is the developmental origin of facet number variation between D. mel. and D. pse. and why does it inversely correlate with the size of non-visual structures? All external structures of the head of the adult fly including the sensory organs develop from the EAD (Figure 2A). The eye field occupies most of the posterior EAD compartment and is marked by the expression of Eyes Absent (Eya; Figure 2B) (Roignant and Treisman, 2009). We measured the surface of the eye field in late, fully grown EADs (stage P0) using Eya and found that the eye field was 31% larger in D. pse. than in D. mel. (Figure 2B’). This is very close to the 35% difference in the number of adult eye facets.
We therefore queried the developmental origin of the difference in eye field size between the two species and considered several possibilities. A first possibility is that the initial pool of embryonic cells forming the EAD differs between the two species. In the late embryo (stage 17), the EADs is composed of a few dozen closely juxtaposed cells located anterior to the brain. Using Ey as a marker, we quantified and compared the number of embryonic EAD cells between the two species (Figures 2 D and 2D) but found no significant difference in the number of EAD progenitors, ruling out this first possibility. Variation in eye field size could also originate from different rates of proliferation. However, the similar density of mitotic cells in the proliferating eye field in EADs of the two species did not support this hypothesis (Figures 2F and 2F'). In addition, the density of ommatidia progenitor cells in the eye field, characterized by the expression of the proneural factor Atonal, was similar between the two species (Figures S3A–S3A'). Finally, variation in eye field size could also derive from a change in the subdivision of the EAD between eye and non-eye fields. To test this possibility, we compared the proportion of the EAD occupied by the eye field in early L3 imaginal disks, after the subdivision between the fields is completed (Figure 2E). The total EAD size was similar between the two species, confirming that it underwent similar growth during prior larval development (Figure 2E). In contrast, already at this early stage, the eye field was proportionally larger in D. pse. than in D. mel. (Figure 2E). Thus, the two species differ by the proportion of the multipotent EAD dedicated to the eye versus non-eye tissues, resulting in different proportions of the head structures in the adult. Therefore, the species variation in eye size involves a developmental trade-off between eye and non-eye primordia.

**Temporal Regulation of EAD Subdivision Governs the Trade-Off between Eye and Non-eye Fields**

What are the regulatory mechanisms governing this developmental trade-off? EAD subdivision requires the temporally progressive restriction of selector TFs expression to the anterior “antennal” or posterior “eye” compartments, a process completed by mid to late second instar larval stage (L2) (Kenyon et al., 2003). At this developmental time point, the mutually exclusive expression domains of antenna and eye selectors define the relative sizes of the compartments. In D. mel., a 3.2 kb cis-regulatory intron governs ey expression during eye development (Figure 3A). We cloned the orthologous intron from D. pse. based on the conservation of the flanking exons. The D. pse. intronic sequence is slightly shorter (3.0 kb) with 22% of the intron from D. mel. aligning to the corresponding sequences in D. pse. (Figure 3A). Nonetheless, when inserted at the same position in D. mel. genome, both D. mel. and D. pse. introns were...
Figure 2. Developmental Origin of Eye Size Variation in D. mel. and D. pse

(A) Schematics of the distinct fields of the EAD giving rise to the adult sensory and non-sensory head structures (Ant, antenna; MxP, maxillary palps; Oc, ocelli). Anterior is at the left.

Developmental Cell 50, 780–792, September 23, 2019
able to drive GFP expression in the EAD throughout eye development, revealing global functional conservation (data not shown).

We tested whether, despite their overall functional conservation, subtle changes in ey regulation exist between D. mel. and D. pse. introns (Figures 3C–3D). In early EADs (late embryos and in L1), both D. mel. and D. pse. enhancers drove GFP expression across the entire disk (Figure S4). At the L2 stage, we noted that GFP expression driven by the melanogaster enhancer (D.m.ey3.5) (Figures 3C and 3C) extended further anteriorly into the antennal compartment as compared to the pseudoobscura enhancer (D.p.ey3.3) (Figures 3D and 3D). This means that the posterior retraction of expression driven by the two ey enhancers occurs at different velocities. To quantify this effect, we generated two lines of transgenic D. mel. flies. The first line carries two transgenes driving the expression of the red fluorescent protein mCherry and the green fluorescent protein (GFP), respectively, both under the control of D. mel. ey enhancer. In this control line, any difference in the expression of mCherry and GFP driven by the same enhancer must only be caused by different dynamics of the two fluorescent proteins. In the second line, mCherry was driven by the D. mel. ey enhancer, while GFP was driven by D. pse. ey enhancer. In this case, the differences in expression between the GFP and mCherry is caused both by different dynamics of the fluorescent proteins as well as differences in their transcriptional regulation. Thus, to detect differences in the activity of D. mel. and D. pse. ey enhancers, we performed pairwise comparisons of the difference between GFP and mCherry expression in line 1 versus

Figure 3. Different Temporal Regulation of EAD Subdivision Governs the Trade-Off between eye and Non-eye Fields

(A) Structure of the ey locus showing the location of the intronic ey3.5 eye enhancer (Hauck et al., 1999). E2: exon 2; E3: exon 3.

(B) Alignment of the ey eye enhancer between D. mel. and D. pse. Dark gray boxes represent the fragments of the D.mel. intron sequence that aligned with the orthologous region of D. pse. Using BLAST.

(C–D) GFP expression driven by D. mel. ey3.5 and D. pse.ey3.3 eye enhancers in mid-L2 EADs counterstained with anti-Cut. Green: GFP; magenta: anti-Cut. Yellow arrows indicate the anterior limit of GFP expression. Scale bars: 20 µm. Anterior is at the bottom.

(E and F) Schematics and immunostainings showing ongoing (E, early/mid-L2 stage) and full (F, early L3 stage) posterior retraction of ey enhancer activity during EAD development. (E and F) Pairwise comparison of the difference in expression between GFP and mCherry, measured as the proportion of mCherry that was not colocalized with GFP, when driven by distinct combinations of ey eye enhancers during (E′, early/mid-L2 stage) and after (F′, early L3 stage) posterior retraction of ey enhancer activity. (D.m.ey3.5, D. mel. ey enhancer; D.p.ey3.3, D. pse. Enhancer). (E) Early/mid-L2 stage. Sample sizes (n = 8, n = 12). Two-tailed unpaired t test ***p = 0.0001.

(D) Early-L3 stage. Sample sizes (n = 10, n = 3). Mann-Whitney test n.s. p = 0.6643.
Figure 4. Developmental and Regulatory Origin of Intraspecific Eye Size Variation
(A) Eye versus face width ratio in Canton-S (CS) and Hikone-AS (Hik). Sample sizes: CS: n = 15, Hik: n = 16. Ordinary one-way ANOVA ****p < 0.0001 followed by Tukey’s multiple comparisons; ****p < 0.0001. See full data set in supplement (Figure S6); see also Figure S1.
(B) Ommatidia number. Sample sizes: CS: n = 15, Hik: n = 16. Two-tailed unpaired t-test: ****p < 0.0001.
(C) Third antennal segment (A3) width. Sample sizes: n = 11. Ordinary one-way ANOVA **p = 0.0035 followed by Tukey’s multiple comparisons. **adjusted p = 0.0043. See full figure in supplement (Figure S6).
(D) Frequency distribution of ommatidia numbers in CS (n = 42), Hik (n = 39), and their F1 progeny (n = 54).
(E) Schematics of the \textit{D. mel}. ey eye enhancer (ey3.5) showing the localization of the G>A substitution (chr4: 710326) and of three published Ct binding-sites (C, blue lines) (Wang and Sun, 2012).
(F) Electrophoretic mobility shift assay. The Cut-FLAG expressing nuclear extract elicits a bandshift when incubated with the G-Probe (black arrow). Excess unlabeled G-probe, but not A-probe effectively competes with the binding, suggesting a higher affinity of Ct for the former. Incubation with an anti-FLAG induces a supershift (empty arrowhead). Gray arrow: non-specific binding.
(G) Ommatidia number variation following RNAi-mediated knockdown of ct. Sample sizes from left to right (n = 13; n = 23; n = 10; and n = 8). Ordinary one-way ANOVA ****p < 0.0001 followed by Sidak’s multiple comparisons; ****p < 0.0001, *p = 0.0126. See also Figure S5.
(H–I) Pairwise comparison of the expression of the four \textit{D. mel}. ey enhancer alleles: ey3.5 (Hikone-AS); ey3.5A (Canton-S); ey3.5CC (ConsensusCt); ey3.5NC (NoCt).
(G and H) Schematics and immunostainings of EADs with ongoing (F, early/mid-L2 stage) and full (G, early L3 stage) posterior retraction of \textit{ey} enhancer activity.
A Conserved Mechanism of Sensory Trade-Offs

To understand the genetic basis of sensory trade-off in Drosophila, we exploited the fact that such trade-offs have also been observed within single fly species (Cowley and Atchley, 1990; Posnien et al., 2012; Arif et al., 2013; Norry and Goetz, 2017; Gaspar et al., 2019). We found that two wild-type D. mel laboratory strains, Canton-S and Hikone-AS, show different eye-to-face ratios. This is associated with changes in ommatidia number (12, 5% more facets in Canton-S) and diameter (Figures 4A, 4B, and S1A–S1D, Table 1) as well as variation of antennal width (Figure 4C). P1 progeny of Canton-S and Hikone-AS parents presented intermediate ommatidia numbers relative to their parents, demonstrating the heritable nature of this trait (Figure 4D). We asked if facet number variation between Canton-S and Hikone-AS also originate from changes in the subdivision of the EAD into eye versus non-eye territories. We compared the subdivision of early L3 EADs between the two strains. While the size of the entire EAD was unchanged, the eye field was proportionally larger in Canton-S than in Hikone-AS (Figures 2E–2E’). These data suggest that despite 17–30 million years of separated evolution between the two species groups (Obbard et al., 2012), ommatidia number variation between D. mel. and D. pse. and between two D. mel. strains shares a common developmental logic.

A Single Nucleotide in a Ct Binding Site Distinguishes the Canton-S and Hikone-AS ey Regulatory Sequences

Does the difference in EAD subdivision between the Canton-S and Hikone-AS also result from a differential temporal regulation of ey? To answer this question, we cloned and aligned the ey cis-regulatory sequence from the Canton-S and Hikone-AS strains (Figure 4D). In contrast to the significant divergence observed between D. mel. and D. pse, Hikone-AS and Canton-S intron sequences were nearly identical and differed only by a single nucleotide over the entire 3.2 kb intronic region, a G>A substitution at position chr4: 710326. In silico analyses revealed that this single nucleotide variant is located in a Ct binding site, distinct from the three sites previously described in the ey cis-regulatory sequence (Figure 4E) (Wang and Sun, 2012). Interestingly, the two variants are predicted to display different affinities for the repressor, in a manner that anti-correlates with the number of ommatidia: the A-allele presents a lower affinity score (4.56) and is associated with larger eyes (Canton-S) as compared to the G-allele (Hikone-AS; predicted affinity score 5.22) (Figures 4E and S4). We thus performed electrophoretic mobility shift assays using a tagged recombinant Ct protein (Figure 4F), and found that Ct has the ability to bind to this sequence. Next, we tested the affinities of the two sequences by performing a competition assay in which unlabeled A-probes or unlabeled G-probes competed with the labeled G-probe. While unlabeled G-probes effectively suppressed the shift, the unlabeled A-probes did not, providing biochemical evidence that the two variants have different affinities for Ct. Put together, these results suggest that the strength of ey repression by Ct, a selector TF for antennal fate and a repressor of ey expression, influence eye size (Wang and Sun, 2012; Weasner and Kumar, 2013). Consistent with this, RNAi knockdown of ct expression during EAD development was sufficient to increase facet number in the adult eye (Figures 4G and S5). We note, however, that this did not consistently result in antagonistic trends in face and antennal width, possibly due to pleiotropic effects of ct loss of function on head development (Figure S5).

These findings raise two questions: first, is the G to A substitution in the ey cis-regulatory sequence sufficient to cause temporal changes in its activity; and second, if so, might such

<table>
<thead>
<tr>
<th>Table 1. Ommatidia Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td>Canton-S</td>
</tr>
<tr>
<td>Hikone-AS</td>
</tr>
<tr>
<td>D. pse.</td>
</tr>
<tr>
<td>F1 CS/Hik</td>
</tr>
<tr>
<td>ct5687</td>
</tr>
<tr>
<td>ct5687/TM3,Sb</td>
</tr>
<tr>
<td>Canton S BH</td>
</tr>
<tr>
<td>Canton S TP</td>
</tr>
<tr>
<td>Canton S RD</td>
</tr>
<tr>
<td>ey3.5G</td>
</tr>
<tr>
<td>ey3.5G/A</td>
</tr>
<tr>
<td>ey3.5G/D fri</td>
</tr>
<tr>
<td>ey3.5G/A/D fri</td>
</tr>
</tbody>
</table>

Ommatidia numbers are counted on SEM images (count) or estimated from light-microscopy images using an ellipse-based method (estimated, see STAR Methods and Figure S2).

*Flies reared at 21°C.

(G’ and H’) Pairwise comparison of the difference in expression between GFP and mCherry, measured as the proportion of mCherry that was not colocalized with GFP, when driven by distinct combinations of ey enhancers during (F’) early/mid-L2 stage and after (G’, early L3 stage) posterior retraction of ey enhancer activity. ey enhancer variants: ey3.5G, G-variant (Hikone-AS); ey3.5G’, A-variant (Canton-S); ey3.5G<sup>C</sup>, ConsensusCt-variant; ey3.5G<sup>C</sup>, NoCt-variant.

(G) Mid-L2 stage. Sample sizes: (n = 16, n = 24, n = 20, and n = 12). Ordinary one-way ANOVA ***p < 0.0001 followed by Dunnet’s multiple comparisons versus ey3.5G<sup>C</sup>.” **Supplemental Experimental Procedures.****adjusted p = 0.0001, n.s. adjusted p = 0.9828; “*adjusted p = 0.0167.

(H) Early-L3 stage. Sample sizes from left to right (n = 9; n = 10; n = 6; and n = 9). Ordinary one-way ANOVA n.s. p = 0.9652.
changes be caused by alterations in the regulation of the ey enhancer by Ct? To tackle these two questions, we used the same strategy described above for comparing the D. pse and D. mel enhancers using GFP and mCherry reporters. We first compared the activities of the Canton-S (A-allele) and the Hikone-AS (G-allele) of the ey 3.5 cis-regulatory sequences. At early/mid L2, during EAD subdivision, mCherry and GFP co-expression differed between the alleles such that the A-carrying variant (Canton-S; larger eyes) showed further posterior retraction of GFP expression than the G-carrying variant (Hikone-AS; smaller eyes) (Figures 4H and 4I). In contrast, at early L3, after EAD subdivision is completed, the two alleles drove similar expression of GFP and mCherry (Figures 4J and 4K).

Could this differential temporal retraction of the ey enhancer be caused by changes in ey repression by Ct? To test this, we created two new synthetic ey enhancers, based on in silico predictions (Figure 4E). The first, which we call the NoCt variant, is predicted to abolish Ct binding to the site harboring the G/A SNP (predicted affinity score <3). The second, which we call ConsensusCt, creates a Ct consensus-binding motif at that position (predicted affinity score 6.62). Remarkably, the ConsensusCt variant behaved similarly to the G-allele, while the NoCt variant mimicked the A-allele in that it caused faster posterior retraction of ey enhancer activity (Figures 4H–4I). This further suggests that the Canton-S A-allele may constitute a lower affinity site for the Ct repressor as compared to the Hikone-AS G-allele.

Thus far, we showed that the changes in EAD subdivision between Hikone-AS and Canton-S and between D. mel and D. pse, are both driven by differential temporal dynamics of the posterior retraction of ey expression. Between Hikone-AS and Canton-S, this is associated with a single nucleotide variant in ey enhancer, which introduces subtle changes in ey regulation, by affecting its repression by the antennal selector TF Ct.

A Common SNP in D. mel. Natural Populations Is Associated with Facet Number Variation

Because Hikone-AS and Canton-S flies have been in artificial lab culture conditions for decades, we asked if either of these two alleles is found in natural fruit fly populations. By investigating allele frequency patterns in whole-genome data of worldwide population samples, we found that most natural populations from Europe, North America, Asia, and Australia are polymorphic at this position. Thus, neither of the two alleles corresponds to a de novo mutation and variation at this position corresponds to a relatively frequent SNP (Figure 5A and 5A'; Table S2). Populations from sub-Saharan Africa are mostly fixed for the G-allele suggesting that the A-allele is a derived variant that appeared after D. mel left Africa and colonized the rest of the world. In line with this hypothesis, we found statistical evidence (FET test, p = 0.02) that the few African populations carrying the A-variant are more likely to be admixed with the European genetic variation than the ones with the putatively ancestral G-allele (Table S3). Moreover, the frequency of the A-variant decreased from West to East in European populations (Figures 5A' and 5B). The slope of the longitudinal frequency cline of the ey SNP deviated significantly from that of 21,008 genome-wide SNPs in short introns that presumably evolved neutrally (Figure 5C), suggesting that the clinal pattern is not solely the result of neutral evolution or demography (see also Figure S7).

Causal Effect of the SNP on Eye Size

We further noted that natural populations from North-East America, where the Canton-S strain originated, are highly polymorphic for the ey SNP (Figure 5A; Table S2). By comparing Canton-S flies from three laboratories, we discovered that, while our Canton-S lab isolate (henceforth Canton-SBH) carries the A-allele, two other strains from two different laboratories in Paris, France (T. Pre´at) and Florida, USA (R. Davis) were homozygous for the G-allele, similar to Hikone-AS. This strongly suggests that the original Canton-S population was polymorphic and that the two alleles were eventually segregated during the separate maintenance of different laboratory stocks (Colomb and Brems, 2014). This provided a unique opportunity to quantify the contribution of the G/A SNP to eye size in a relatively homogeneous background. By comparing ommatidia numbers between the three stocks, we observed that Canton SBH flies have significantly more facets than its two siblings (Figure 6A; Table 1), a difference that anti-correlates with their face and antennal width (Figure S6). These data suggest that the A-variant may be sufficient to drive larger facet numbers, possibly at the expenses of other head structures. To test this idea directly, we used CRISPR/Cas9 to introduce the A-allele in a G-homozygous stock. We recovered one transformant male carrying the A-allele and controlled that it bore no other mutation in the ey regulatory intron. Comparing ommatidia numbers between engineered G>A flies and the G-carrying control revealed an increase in eye size associated with the G>A substitution (Figure 6B; Table 1). It recapitulated up to 49% of the difference between Hikone-AS and Canton-SBH and up to 86% of the variation observed between Canton-SBH and Canton-SBD and Canton-STP, respectively. Three other A-homozygous lines deriving from the same transformant male were established to account for subtle differences in the genetic background of the flies. The three stocks followed the same trend, i.e., an increased facet number when compared to the G-homozygous controls, reaching statistical significance in two out of the three (Figure S6). This result suggests that the ey cis-regulatory SNP is causal to facet number variation. Next, we combined the two alleles with Dr(4) J2, a large deficiency covering the entire ey locus. These flies recapitulated the phenotypes of the corresponding homozygous alleles, indicating that an undetected lesion outside of the ey locus did not cause the effect on eye size attributed to the A-allele (Figure 6C). In addition, in both cases, the antennae show a trend toward decreasing in size, but this trend did not reach statistical significance (Figure S6), likely because of the small size of the difference.

DISCUSSION

In 1987, Montgomery Slatkin proposed a mathematical model (Slatkin, 1987), which he referred to as “unrealistically simple,” predicting that mutations modifying the time at which “traits developing from the same tissue” begin to grow independently changes the relative size of the traits. Size trade-offs between head sensory organs represent precisely the types of traits referred to in Slatkin’s model. However, whether visual-olfactory sensory trade-offs follow a “Slatkin model” and if so, what the genetic basis of such a model are, remained unexplored.
In this study, we find that differential subdivision of the head primordium into eye and non-eye progenitor fields constitutes a developmental mechanism for creating different proportions of head structures in fruit flies, including trade-offs between the olfactory antennae and the eyes. We further demonstrate that this is associated with differential temporal regulation of the expression of the conserved eye selector transcription factor, Ey/Pax6. We propose a model (Figures 6D–6F) whereby early in development, the homogenous expression of ey, which promotes its proliferation (Zhu et al., 2017), causes homogenous growth throughout the entire EAD. Later, the progressive retraction of ey expression from the anterior antennal compartment creates an asymmetry in growth rate. Modulating the velocity of ey retraction through mutations affecting the bistable switch between GRNs governing antennal versus eye identity, changes the relative time during which the anterior and posterior compartments grow at different rates resulting in their different proportions. This provides direct biological evidence for mathematical models linking heterochrony in development to changes in adult traits (Riska, 1986; Slatkin, 1987; Cowley and Atchley, 1990). Our observation of similar changes in ey temporal regulation between and within species further suggests that the temporal mechanism we identify may represent a “preferred route” to relative variation in sensory organ size because it results in no dramatic “pleiotropic” effects associated with changes to growth, patterning, or specification. We speculate that such variation of the temporal regulation of EAD partitioning could be caused by a variety of molecular changes acting either in cis or in trans on the GRNs governing eye and antennal compartment identities.

In this work, we uncovered an example of such a molecular change to the regulation of these GRNs. Specifically, within D. mel., a naturally occurring SNP in the eye-enhancer of ey is sufficient to modulate the velocity of the posterior retraction of the enhancer activity and to vary facet numbers in the adult eye. The SNP, a G/A substitution, is located in a binding site for ey repressor Ct. In-silico predictions and EMSA experiments indicate that the G/A substitution modifies the affinity of Ct for the binding site. This suggests that different levels of ey repression by Ct are responsible for changing the dynamics of ey regulation.
Figure 6. The Non-coding G>A SNP in ey Enhancer Causes Facet Number Variation

(A) Ommatidia numbers in three Canton-S strains with different ey SNP alleles (in red). Sample sizes: Canton-S<sup>BH</sup> (CS-BH, n = 18), Canton-S<sup>TP</sup> (CS-TP, n = 16), Canton-S<sup>G</sup> (CS-RD, n = 19); Kruskal Wallis test: ***p < 0.0001 followed by Dunn’s multiple comparisons: *adjusted p = 0.0041; **** adjusted p < 0.0001; n.s. adjusted p = 0.1180. See also Figure S6.

(B) Ommatidia numbers in CRISPR A-variant and control G-variant homozygous fly eyes imaged by scanning electron microscopy. Sample sizes: ey<sup>3.5G</sup>; n = 13; ey<sup>3.52G/+</sup>; n = 12. Two-tailed unpaired t test: *p = 0.0356. See also Figure S6.

(C) Ommatidia numbers in CRISPR A-variant and control G-variant over a deficiency covering the entire ey locus. Sample sizes: n = 18. Two-tailed unpaired t test with Welch’s correction: *p = 0.0138. See also Figure S6.

(4) Boxes indicate interquartile ranges, lines medians, and whiskers data ranges.

(F-C) Model of the developmental origin of the trade-off between EAD derived structures.

(F) The subdivision of the EAD into an anterior (“antennal”) and posterior (“eye”) compartments involves a bistable switch by which GRNs promoting eye (GRNe, in red) and antennal (GRNa, in blue) identity act antagonistically by activating their own and repressing the alternative GRN’s activity.

(G) The bistable switch between the GRNs and GRNe results in the progressive segregation of the expression domains of TFs promoting eye (posterior, in red) versus antennal (anterior, in blue) fates during successive stages of EAD development. Anterior is on the left.

and ultimately for causing morphological variation, a lower affinity of the binding site for Ct resulting into faster enhancer retraction and larger eyes and vice versa. This view is further supported by our findings that (1) synthetic mutations predicted to support or reduce Ct binding mimic the effect of the SNP on the velocity of the enhancer retraction; and that (2) knocking down Ct expression increases compound eye size. Interestingly, the highly divergent ey enhancers from <i>D. mel</i> and <i>D. pse</i>. display similar differences in regulation, i.e., a faster retraction of the enhancer of the “larger eye” species. However, what feature of the two enhancer sequences causes their different temporal regulation is not known. A recent study identified a single nucleotide insertion in <i>Drosophila</i> by modulating the affinity for a TF named Klumpfuss (Anderson et al., 2017). Together with our work, this suggests a general role for variation in suboptimal TF binding sites in sensory evolution (Crocker et al., 2016).

In vertebrates, antagonistic relationships between GRNs and signaling pathways that promote different sensory identities also regulate the subdivision of the multipotent sensory placode (Grocott et al., 2012; Singh and Groves, 2016). The anterior placodal region, which gives rise to the lens and olfactory placodes, expresses pax6. In the absence of pax6, both lens and olfactory placodes fail to thicken and to develop properly (Quinn et al., 1996; Ashery-Padan et al., 2000; Collinson et al., 2000). Interestingly, the temporal regulation of the pax6 placode enhancer is altered by manipulating suboptimal binding sites for one of its activators (Rowan et al., 2010). In addition, the pax6 ectodermal enhancer shows evidence of accelerated evolution in subterranean mammalian species (Partha et al., 2017). This is consistent with our model whereby naturally occurring mutations that alter pax6 regulation, either in cis or in trans, constitute a common genetic origin of the trade-offs between visual and olfactory organ size.

In The Origin of Species Charles Darwin referred to the evolution of the eye as a challenge to his theory (Darwin, 1872). He also discussed the importance of correlation between body parts concluding that it was “most imperfectly understood.” During the last decades, the common origin of animal eyes and their evolution over long evolutionary distances has been abundantly documented (Gehring, 2014). However, the developmental mechanisms by which small-scale variation in eye size or shape can take place without disrupting its organization and function remain largely elusive (Dyer et al., 2009). We have demonstrated that a single nucleotide change in a core regulator of eye development is sufficient to generate reciprocal sensory organ size variation, potentially providing a quick route to behavioral changes and perhaps adaptation. As predicted by Darwin,
adaptive variation in head derived structures, including the eye, can be produced by the accumulation of modest morphological changes, which our data suggest may be caused by a small number of genetic variants affecting the temporal regulation of core regulatory networks.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Species
  - Developmental Stages
  - Density-Controlled Culture Conditions
- **METHOD DETAILS**
  - Generation of Reagents
  - Imaging and Image Quantification
  - Immunostainings
  - In Silico Analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND CODE AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.devcel.2019.07.027.

**ACKNOWLEDGMENTS**

This work was supported by the program “Investissements d’avenir” ANR-10-IAPU-06, ICN, VIB, the WiBrain Interuniversity Attraction Pole network (Bel-spo), the Paul G. Allen Frontiers Group, FLiACT Marie Curie ITN (FP7, EU) and Fonds Wetenschappelijk Onderzoek (FWO) grant G.0503.12 (B.A.H.). M.K. and T.F. were supported by a Swiss National Science Foundation and Fonds Wetenschappelijke Onderzoeks (FWO) grant G.0503.12 (B.A.H.).

**REFERENCES**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.


environment reveals temporally heterogeneous trajectories of selected alleles. Mol. Ecol. 27, 4931–4941.


<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Anti-Drosophila Cut protein product Monoclonal Antibody, Unconjugated (1:10)</td>
<td>DHSB</td>
<td>DSHB Cat# 2b10; RRID:AB_528186</td>
</tr>
<tr>
<td>Mouse anti-Eyes Absent (Eya) protein antibody (1:75)</td>
<td>DHSB</td>
<td>DSHB Cat# eya10h6; RRID:AB_528232</td>
</tr>
<tr>
<td>Rat-Elav-7E8A10 anti-elav monoclonal antibody (1:100)</td>
<td>DHSB</td>
<td>Cat# 7E8A10; RRID: AB_528218</td>
</tr>
<tr>
<td>Rat anti-Eyless (Ey) polyclonal antibody (1:300)</td>
<td>Laboratory of P. Callaerts</td>
<td>Halder et al. (1998)</td>
</tr>
<tr>
<td>Rabbit PhosphoDetect Anti-Histone H3 (pSer10) (7-20) Rabbit pAb antibody (1:1000)</td>
<td>Millipore</td>
<td>Cat# 382159-50UG; RRID:AB_565299</td>
</tr>
<tr>
<td>Sheep anti-Atonal (Ato) polyclonal antibody (1:250)</td>
<td>Laboratory of A. Jarman</td>
<td>Jarman AP; Development. 1995 Cat# ato; RRID:AB_2568143</td>
</tr>
<tr>
<td>Mouse Anti-Green Fluorescent Protein (GFP) Monoclonal Antibody, Unconjugated, Clone 3E6 (1:1000)</td>
<td>Molecular Probes</td>
<td>Molecular Probes Cat# A-11120; RRID:AB_221568</td>
</tr>
<tr>
<td>Rabbit Living Colors® DsRed Polyclonal Antibody (1:10000)</td>
<td>Takara Bio</td>
<td>Takara Bio Cat# 632496; RRID: AB_10013483</td>
</tr>
<tr>
<td>Donkey Anti-Rat IgG (H+L) Antibody, Alexa Fluor 488 Conjugated (1:200)</td>
<td>Thermo Fisher Scientific</td>
<td>Thermo Fisher Scientific Cat# A-21208; RRID:AB_141709</td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG (H+L) Highly Cross-adsorbed Antibody, Alexa Fluor 488 Conjugated (1:200)</td>
<td>Thermo Fisher Scientific</td>
<td>Thermo Fisher Scientific Cat# A-11029; RRID:AB_138404</td>
</tr>
<tr>
<td>Donkey Anti-Mouse IgG (H+L) Antibody, Alexa Fluor 488 Conjugated (1:200)</td>
<td>Thermo Fisher Scientific</td>
<td>Thermo Fisher Scientific Cat# A-21202; RRID:AB_141507</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated (1:200)</td>
<td>Thermo Fisher Scientific</td>
<td>Thermo Fisher Scientific Cat# A-11008; RRID:AB_143165</td>
</tr>
<tr>
<td>Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (1:200)</td>
<td>Thermo Fisher Scientific</td>
<td>Thermo Fisher Scientific Cat# A-31572; RRID:AB_162543</td>
</tr>
<tr>
<td>Monoclonal ANTI-FLAG® M2 antibody (1:200)</td>
<td>Sigma-Aldrich</td>
<td>Sigma-Aldrich Cat# F3165; RRID: AB_259529</td>
</tr>
<tr>
<td>Chemicals, Peptides, and Recombinant Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qiagen Effecten Transfection reagent</td>
<td>Qiagen</td>
<td>Qiagen Cat# 301425</td>
</tr>
<tr>
<td>Vectashield</td>
<td>Vector Laboratories</td>
<td>Vector Laboratories Cat#H-1000</td>
</tr>
<tr>
<td>Critical Commercial Assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qiagen DNeasy Blood and Tissue Kit</td>
<td>Qiagen</td>
<td>Qiagen Cat#69504</td>
</tr>
<tr>
<td>pENTR-D-TOPO kit</td>
<td>ThermoFischer Scientific</td>
<td>ThermoFischer Scientific Cat#K2400-20</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES-C212-BLASTICIDIN SUPPORT Kit</td>
<td>ThermoFischer Scientific</td>
<td>ThermoFischer Scientific Cat#K515001</td>
</tr>
<tr>
<td>LightShift™ Chemiluminescent EMSA Kit</td>
<td>ThermoFischer Scientific</td>
<td>ThermoFischer Scientific Cat# 20148</td>
</tr>
</tbody>
</table>

**Deposited Data**

- **D. melanogaster** Reference Genome BDGP release 6
  - Berkeley Drosophila Genome Project
  - GCA_000001215.4

- **D. pseudoobscura** release Dpse_3.0
  - Baylor College of Medicine
  - GCA_000001765.2

- **Drosophila** Genome Nexus
  - [http://www.johnpool.net/genomes.html](http://www.johnpool.net/genomes.html)
  - N/A

- **D. melanogaster** European genomes
  - European Drosophila Population Genomics Consortium (DROSEU)
  - PRJNA388788

**Experimental Models: Cell Lines**

- **D. melanogaster**: Cell line S2: S2-DRSC
  - Laboratory of Norbert Perrimon
  - FlyBase: FBtc0000181

**Experimental Models: Organisms/Strains**

- **D. melanogaster**: Canton-S^BR^.
  - Laboratory of B. Hassan
  - N/A

- **D. melanogaster**: Canton-S^TP^.
  - Laboratory of P. Callaerts
  - N/A

- **D. melanogaster**: Canton-S^RD^.
  - Laboratory of P. Callaerts
  - N/A

- **D. melanogaster**: Hikone-AS
  - Kyoto Stock Center DGGR
  - RRID:DGGR_105668

- **D. melanogaster**: DGRP-208
  - Bloomington Drosophila Stock Center
  - RRID:BDSC_25174

  - Bloomington Drosophila Stock Center
  - RRID:BDSC_45603

- **D. melanogaster**: w^{1118}; P[y^{y+7.7} w^mC=GMR33F07-GAL4]attP2/TM3, Sb^1^.
  - Kyoto Stock Center DGGR
  - RRID:DGGR_104957

- **D. melanogaster**: UAS-RNAi^rt^.
  - Vienna Drosophila Resource Center
  - VDRC Cat#v5687

- **D. melanogaster**: UAS-RNAi^rt^.
  - Vienna Drosophila Resource Center
  - VDRC Cat#v5687

- **D. melanogaster**: y^1^ v^1^.
  - Vienna Drosophila Resource Center
  - RRID:BDSC_31603

- **D. melanogaster**: D.m.ey3.5^GFP^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: D.m.ey3.5^GFP^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: D.m.ey3.5^NocGFP^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: D.m.ey3.5^ConsensusGFP^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: D.m.ey3.5^mcherry^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: D.m.ey3.5^mcherry^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: ey3.5^G^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: ey3.5^G^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: ey3.5^G^.
  - Laboratory of B. Hassan
  - this study

- **D. melanogaster**: ey3.5^G^.
  - Laboratory of B. Hassan
  - this study

- **D. ananassae; isofemale WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14024-0371.30

- **D. ananassae; isofemale WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14024-0371.31

- **D. yakuba; WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14021-0261.00

- **D. yakuba; WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14021-0261.01

- **D. pseudoobscura; isofemale WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14011-0121.121

- **D. pseudoobscura; isofemale WT**
  - Drosophila Species Stock Center
  - DSSC Cat#14011-0121.118

- **D. virilis; isofemale WT**
  - Drosophila Species Stock Center
  - DSSC Cat#15010-1051.118

**Oligonucleotides**

- Primers, see Table S5
  - Laboratory of B. Hassan
  - this study

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bassem Hassan (bassem.hassan@icm-institute.org). Reagents generated during this study are available upon request from the authors.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Species

*Drosophila Melanogaster*

*D. melanogaster* stocks were cultured on standard cornmeal diet food at 25°C except mentioned otherwise in the corresponding figure legend. Developmental stages used in each experiment are indicated in the corresponding Method Details section. Morphological measures on adult flies were performed on females only. The fly strains used in this study were: Canton-S^H^ (Hassan lab); Canton-S^T^ (Préat lab, provided by P. Callaerts); Canton-S^D^ (Davis lab, provided by P. Callaerts); Hikone-AS (Kyoto DGRC 103421); DGRP-208 (D.M. 3^D^, Bloomington #25174); Act5C-Cas9 (Port et al., 2014) (Bloomington #54590); ct^5^Gal4 (Pfeiffer et al., 2008) (Bloomington #45603); hth^NP5332-Gal4 (DGRC Kyoto #104957); UAS-RNAi ct (VDRC #v5687); UAS-RNAi ct (VDRC #v4138); UAS-RNAi luc (Bloomington #31603); D.m.ey3.5GGFP (this study); D.m.ey3.5AGFP (this study); D.m.ey3.5GmCherry (this study); D.m.ey3.5AmCherry (this study); D.m.ey3.5NoCtGFP (this study); D.m.ey3.5ConsensusCtGFP (this study); ey3.5G (this study); ey3.5G>A-1 (this study); ey3.5G>A-2 (this study); ey3.5G>A-3 (this study); ey3.5G>A-4 (this study).

*Drosophila Ananassae*

Isofemale *D. ananassae* stocks were cultured on standard cornmeal diet food at 21°C. Morphological measures on adult flies were performed on females only. Stock origin: Kisangani, Congo (DSSc 14024–0371.30) and Mumbai, India (DSSc 14024–0371.31).

## Tables

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gateway^R^ pDONR^®^/221 Vector</td>
<td>ThermoFischer Scientific</td>
<td>ThermoFischer Scientific Cat#12536017</td>
</tr>
<tr>
<td>pAWF vector</td>
<td><a href="https://emb.carnegiescience.edu/Drosophila-gateway-vector-collection">https://emb.carnegiescience.edu/Drosophila-gateway-vector-collection</a></td>
<td>DGRC Cat#1111</td>
</tr>
<tr>
<td>pStingerGFP vector</td>
<td>Aerts et al. 2010; Quan et al. 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>pStingermCherry vector</td>
<td>Aerts et al. 2010; Quan et al. 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>pU6-BBS1-chiRNA vector</td>
<td>Gratz et al. 2013</td>
<td>Addgene #Cat45946</td>
</tr>
<tr>
<td>pU6gRNA^ey</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pAWF-Cut</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pEntry-ey^3.3Pse</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pEntry-ey^3.5CSBH</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pEntry-ey^3.5ShIk</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pEntry-ey^3.5NoCi</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pEntry-ey^3.5ConsensusCt</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingerGFP-ey^3.3Pse</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingerGFP-3.5CSBH</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingerGFP-3.5ShIk</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingerGFP-3.5NoCi</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingerGFP-3.5ConsensusCt</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingermCherry-3.5CSBH</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>pStingermCherry-3.5ShIk</td>
<td>Laboratory of B. Hassan</td>
<td>this study</td>
</tr>
<tr>
<td>Software and Algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prism 8</td>
<td>Graphpad Software Inc.</td>
<td><a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a></td>
</tr>
<tr>
<td>FIJI</td>
<td>Schindelin et al. 2012</td>
<td><a href="http://fiji.sc/">http://fiji.sc/</a></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Code for mcherry/GFP colocalization macro</td>
<td>Oliva et al. 2016</td>
<td><a href="https://github.com/rejsmont/FijiScripts/blob/master/mColoc3D.py">https://github.com/rejsmont/FijiScripts/blob/master/mColoc3D.py</a></td>
</tr>
</tbody>
</table>
**Drosophila Yakuba**

*D. yakuba* stocks were cultured on standard cornmeal diet food at 21°C. Morphological measures on adult flies were performed on females only. Stock origin: Ivory Coast (DSSc 14021–0269.0261.00) and Liberia (Reference Genome strain DSSc 14021–0261.01).

**Drosophila Pseudoobscura**

Isofemale *D. pseudoobscura* stocks were cultured on standard cornmeal diet food at 25°C except when mentioned otherwise in the corresponding figure legend. Morphological measures on adult flies were performed on females only. Stock origin: Catalina Island, California, USA (Cat; DSSc 14011–0121.121) and Chiracahua Mountains, Arizona, USA (DSSc 14011–0121.118).

**Drosophila Virilis**

The isofemale *D. virilis* stock was cultured on standard cornmeal diet food at 21°C. Morphological measures on adult flies were performed on females only. Stock origin: Gikongoro, Rwanda (DSSc 15010–1051.118).

**Developmental Stages**

For selecting specific developmental stages, embryos were collected on grape fruit plates complemented with yeast paste changed every 2 hours. Freshly hatched L1 larvae were collected every two hours and transferred to corn meal food vials in a density-controlled fashion (20 larvae/vial). Staging was performed at 25°C. Correspondence of developmental stages between *D. mel.* and *D. pse.* was determined based on developmental transitions – larval hatching, L2 to L3 molt, pupa formation - and morphological features – embryo morphology, rows of differentiated photoreceptors in the EAD, size of the EAD.

**Density-Controlled Culture Conditions**

Morphological measurements were performed on flies raised in density-controlled conditions: batches of 20 young females and males (2-5 days old) were put together and cultured at 25°C. They were transferred in fresh vials every 24 hrs. For each vial, the individuals eclosing during the first two days of eclosion only were used for measurements.

**METHOD DETAILS**

**Generation of Reagents**

**Constructs**

Enhancer reporter constructs were generated using the Gateway Recombination Cloning Technology (ThermoFischer Scientific). *D. pse. ey3.3* and *D. mel. ey3.5* regulatory sequences were amplified respectively from *D. pse.* (from stock Cat; DSSc 14011–0121.121), Hikone-AS (for the G-variant) and Canton-S\(^{BH}\) (for the A-variant) genomic DNA (extracted using Qiagen DNeasy Blood and Tissue Kit #69504) and cloned into the Gateway pDONR221 entry vector (ThermoFischer Scientific #12536017) following the provider specifications. Primers for the enhancer amplifications are:

- pEntry-ey3.3Pse: forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGTGGTAGTGGACTAGG and reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGAATTTTGCTAACGC;
- pEntry-ey3.5CSBH and pEntry-ey3.5Hik: forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTGGACTAGGCGGTATTGCT and reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTGCTCACACATTTG.

The entry vectors with mutated forms of *ey3.5* enhancer, pEntry-ey3.5NoCt and pEntry-ey3.5consensusCt were generated by modifying the pEntry-ey3.5\(^{BH}\) using primers carrying the corresponding mutations. These primers were (mutated nucleotides are in capital letters):

- pEntry-ey3.5NoCt: forward: caataaaatggttgg C\(\text{a}\)aG\(\text{a}\)tttttcgaactttcg and reverse: cgaaagttcgaaaaa C\(\text{t}\)G\(\text{c}\)caaccattttattg;
- pEntry-ey3.5consensusCt: forward: taaaatggtt T\(\text{a}\)gaactttttcgaactttcg and reverse: gaaaaagttcA\(\text{a}\)aaccattttattttt.

Enhancer inserts were next transferred using Gateway recombination into mCherry- and GFP-expressing enhancer reporter vectors amenable to phiC31 integration –mediated transgenesis (Aerts et al., 2010; Quan et al., 2016).

**pAWF-Cut**: ct cDNA was kindly provided by I. Lohmann (U. Heidelberg). The full length cDNA was cloned, without its ATG, into a Gateway pEntry vector using the pEntr-D-TOPO kit (ThermoFischer Scientific K2400-20) following provider specifications. It was transferred using Gateway recombination into the pAFW vector (DGRC#1111), resulting in the addition of 3 x Flag tag coding sequence upstream ct cDNA.

**pU6gRNA\(^{ey}\)**: the following complementary phospho-oligomers were used to generate a double strand DNA sequence encoding the ey eye-enhancer guide RNA (gRNA): forward: phospho-CTTCGTCGAAAACAATAAAATGGT; reverse: phospho-AAACACCATTTATTGTATTG. After hybridization, the resulting double-strand DNA was cloned into the pU6-BBS1-chiRNA plasmid (Addgene #45946) (Gratz et al., 2013).

**Enhancer-Reporter Lines**

Transformant flies carrying enhancer reporter constructs were generated by BestGeneInc. All constructs were integrated at the Attp2 landing site using phiC31 recombination.
**CRISPR/Cas9 Engineering**

For editing the ey eye enhancer, we injected SNP\(^G\) homozygous *D. mel.* Act5-Cas9 embryos (Port et al., 2014) with two constructs respectively encoding the guide RNA (pU6gRNA\(^p\)) and the SNP\(^A\)-carrying ey eye enhancer sequence (pEntry-ey\(^A\), 5'CSBBH), each of them at a concentration of 500 ng/µl (Port et al., 2015). Candidates were screened using allele-specific PCR. We isolated one CRISPR modified male from which we established four CRISPR SNP\(^A\) lines. In parallel, a control line was established by mating non-injected Act-Cas9 flies following the same scheme their injected siblings. Sequencing the ey eye enhancer from the transformed SNP\(^A\) and of the non-injected SNP\(^A\) control stocks confirmed that they were differing only by this single nucleotide.

**Allele-Specific PCR**

SNP\(^A\) and SNP\(^G\) alleles were detected by allele-specific PCR using a common reverse primer (Ey-R3: AGAAATATCACATGGCCGAG) and one of two specific forward primers differing by the 3' most nucleotide (either A or G) and including a mismatch (underlined) to increase binding specificity (Ey-SNP\(^G\)-F: GGAATCGAAAACAATAAAATGGCTGG; Ey-SNP\(^A\)-F: GGAATCGAAAACAATAAAATGCTGA).

**Cut-FLAG Fusion Protein Expressing S2 Cells**

S2 cells cultivated at 25°C in Schneider’s medium supplemented with 10% fetal calf serum were co-transfected with 2 µg of pAWF-Cut and 0.2 µg of pcOBlast vector (Thermo Fisher Scientific) using Effecten transfection reagent (Qiagen) according to the manufacturer’s instructions (1/10 DNA-Effecten ratio). Blasticidin selection (10 µg/µl) was applied three days after transfection. After one week of selection, cells were harvested, and expression of Cut-FLAG fusion protein was checked by western-blot using an anti-FLAG antibody (Sigma-Aldrich Cat# F3165). For long-term culture, cells were maintained in 2 µg/µl blasticidin.

**Imaging and Image Quantification**

**Image Processing**

Except mentioned otherwise, all image processing was performed using ImageJ (versions 1.45 to 1.48) (Schneider et al., 2012).

**Scanning Electron Microscopy**

Whole flies were fixed overnight at 4°C in a 1:1 mix of 4% formaldehyde in phosphate buffer pH 7.2 and 100% ethanol and dehydrated successively in graded ethanol series, hexamethyldisilazane (HMDS) and in a dessicator. Fly heads were mounted on specimen studs using silver paint in two distinct orientations: dorsal head up (for whole head imaging) and lateral (for ommatidia counts and measures). Samples were subsequently coated with platinum and images acquired in LEI mode with a JEOL JSM 7401F microscope at magnifications ranging from 120 (heads overview) to 1900 times (ommatidia width) (Schneider et al., 2012).

**Transmitted Light and Confocal Microscopy**

Preparations of adult heads for the acquisition of light microscopy images were acquired from non-fixed, freshly cut adult heads glued laterally on glass slides. Images were acquired using a camera DFC295 (Leica) mounted on a DMRXA (Leica) microscope, operated via the open-source software Micro-Manager (Edelstein et al., 2014). Fluorescent preparations of embryos and imaginal discs were acquired using a Nikon A1R Eclipse Ti, a Leica TCS SPS II or a Leica SP8 confocal microscope operated by the accompanying company software.

**Measuring Adult Eye, Face and Antennae**

All head and eye measurements were performed on female flies using ImageJ (Schneider et al., 2012). Adult Eye:Face ratio was expressed as E (Figure 1). Ommatidia width was measured on high magnification SEM images as the distance between one interommatidial bristle and the opposite angle of the facet. For each sample, measures of six adjacent ommatidia localized at the center of the eye were taken. To limit underestimation of the ommatidia width due to perspective projection distortion, samples were carefully oriented prior to image acquisition.

**Ommatidia Numbers**

Ommatidia numbers were manually counted on SEM images using the ImageJ plugin “Cell counter”. We also developed an alternative method based on the approximation of the compound eye to an ellipse. With this method, the ommatidia number is calculated as the surface of an ellipse whose large and small axes correspond to the numbers of ommatidia along the compound eye anterior-posterior and dorso-ventral axes (Area = π·a·b/4 with a and b as the lengths of the large and small ellipse axes; Figure S4). This method accommodates lower resolution images and does not require the use of SEM. Bland-Altman method (Bland and Altman, 1986) was used to compare the outcome of the two methods applied on a common set of SEM images. The ellipse method results in an overestimation of approximately 20 ommatidia as compared to the manual counting (bias mean = 20.29; SD = 11.70). Importantly, this difference is independent of ommatidia number (Figure S2). Facet number estimation of Cut RNAi and CRISPR flies (Figure S5) was performed blind regarding to the genotype.

**Measuring Embryonic and Larval EADs**

Numbers of Ey-positive embryonic eye-antennal disc cells were counted manually. To measure the surface of the larval eye-antennal disc and eye progenitor field, regions of interest were selected manually using the ImageJ freehand selection tool. The number of mitotic pH3-positive cells was automatically counted using the Dead-Easy Mito–Glia ImageJ Plugin (Forero et al., 2010). The mitotic index was calculated as the number of mitotic cells per surface of the Eya-positive eye progenitor field.

**MCherry and GFP Colocalization**

Protocol for pixel-based quantifications of mCherry and GFP colocalization was adapted from (Oliva et al., 2016). We used Fiji/ ImageJ2/ImgLib2 (Pietzsch et al., 2012; Rueden et al., 2017; Schindelin et al., 2012) macro implemented in Jython. Raw images were imported using BioFormats library (Linkert et al., 2010). EADs were manually segmented in each stack by the user. Stack
threshold levels for each channel were calculated using preselected auto-thresholding algorithms available in Fiji (Huang for both channels). Determined threshold levels were used to calculate Mander’s overlap coefficient using Fiji implementation of the colocalization algorithm. Code for the macro is available on GitHub, https://github.com/rejsmont/FijiScripts/blob/master/mColoc3D.py (Oliva et al., 2016). The proportion of pixels expressing solely mCherry is used as a measure for GFP retraction.

**Behavioral Measures of Visual Acuity.** The experimental set-up is modified from (Buchner, 1976). It exploits the spontaneous tendency of fruit flies to adjust their trajectory to the surrounding landscape. Presented with rotating vertical black stripes, tethered flies spontaneously follow their movement. Narrowing the angular distance between the stripes beyond its resolving capacities makes the fly move in the opposite direction, due to an interference phenomenon similar to what we perceive when looking at the wheels of a starting train. It consists of two tracking balls and of two computer screens on which moving vertical black and white stripes are displayed in a window of 90° horizontal and 74° vertical extensions. The width of the stripes (spatial wavelength λ) as they move on the flat screen are adjusted such that they subtend a constant angle as seen from the fly 40 mm away of the screen. Pattern speed w is adapted to maintain the "contrast frequency" at 1 Hz. A positive optomotor response indicates the tendency of the flies to follow the direction of the movement of the stripes. Reduction of λ below the resolving power of the eye causes an inversion of the apparent direction of the movement of the stripes due to the geometrical interference between the fly’s vertical columns of ommatidia and the vertical stripes and is accompanied by the inversion of the fly response towards negative values. The λ value at which the response of the fly is inverted (zero-crossing angle, 2Δφ) provides a measure of spatial resolution (or visual acuity). Recordings of female D. mel. (Canton-S; n=9) and D. pse. (Cat; n=9) were performed simultaneously, with alternating assignments. We calculated the zero-crossing and its variance from the two average responses surrounding the zero-crossing (one positive, one negative) using linear interpolation and error propagation followed by t-test for differences between 2 means.

**Immunostainings**

**Antibodies**

We used the following primary antibodies: mouse anti-Ct (1:10, DHSB hybridoma supernatant 2B10, deposited by Rubin G. M.), mouse anti-Eya (1:75, DHSB hybridoma supernatant eya10H6, deposited by Benzer, S. and Bonini, N.M.), rat anti-elav (1:100, DHSB hybridoma supernatant Rat-Elav-7E8A10, deposited by Rubin G. M.), mouse anti-futsch (1:100; DHSB hybridoma supernatant 22C10 deposited by Benzer, S. and Colley, N.); rat anti-ey (1:300, received from P. Callaerts (Halder et al., 1998)); anti-phosphorylated histone 3 (1:1000, pSer10; Merck Millipore #382159); sheep anti-atonal (1:1000, received from A. Jarman and P. zur Lage (Jarman et al., 1995)); mouse anti-GFP 3E6 (1:1000, Invitrogen, #A11120); rabbit anti-DesRed (1:1000, Clonetech, #632496). Secondary antibodies conjugated with Alexa 488, Alexa 555 and Alexa 647 were used at 1:200 (Molecular Probes). Nuclei were counterstained using Draq-5 (1:1000 in PBS, Abcam #ab108410). All samples were mounted in Vectashield (Vector laboratories #H-1000).

**Procedure**

Fixation and immunostainings were performed following standard procedures as described in (Patel, 1994) (embryos) and in (Blair, 2000) (EADs). Briefly, embryos were collected from grape juice agar plates and dechorionated with bleach (sodium hypochlorite 3%). They were fixed in a 1/1 mixture of n-heptane and 3.7% formaldehyde in PBS and devitellinized with methanol. Larval and pupal mouth complexes including the EADs, brain and pharynx were dissected in cold PBS and fixed in 3.7% formaldehyde in PBS for 20 minutes, then washed in PBT (embryos: PBS tween 0,1%; L1 and L2 EADs: PBS Triton-X 0,1%; L3 EADs: PBS Triton-X 0,3%) for 2 hours. Blocking was performed 1 hour at room temperature in blocking solution (5% normal goat serum in PBT) and incubation with primary antibodies diluted in blocking solution overnight at 4°C. After 2 hours of washes, tissues were incubated 2 hours at room temperature with the secondary antibodies diluted in blocking solution. After two hours of washes in PBT, tissues were rinsed in PBS, incubated with Draq5 (1/500 in PBS) 1 hour at room temperature or overnight at 4°C and mounted in Vectashield (Vector laboratories cat#H-1000). DAPI stainings were performed by adding DAPI in the mounting medium (final concentration 1.5 μm).

**In Silico Analysis**

**Genome Assemblies**

All D. mel. Genome positions refer to BDGP release 6 assembly (GCA_000001215.4) and D. pse. Genome positions refer to genome release Dpse_3.0, Baylor College of Medicine (GCA_000001765.2).

**Alignment of D.mel. D. pse. ey enhancers**

Pairwise alignment of D. pse. and D. mel. intronic ey enhancers was performed with BLASTn (McGinnis and Madden, 2004) as described in (Swanson et al., 2011). Namely, the following parameters were selected: “somewhat similar” sequences and the lower complexity filter was off. The sequences used for the alignment were: for D. pse. chrU:2,848,595-2,851,564 and for D. mel. chr4:707,672-710,917. The 22% of alignment corresponds to “query coverage” in BLASTn output and indicates the amount of D. mel. intron sequence included in blocks aligned to the D. pse ortholog by BLASTn.

**Ct Binding Site Predictions**

To predict possible Ct binding sites, the SNP surrounding region (500bp up and down) was scored with Cluster-Buster (Frith et al., 2003) (https://github.com/weng-lab/cluster-buster version, options -c0 -m3 -f5 -G0) using the 3 available Drosophila Ct PWMs (MA0128.1 from JASPAR (Mathelier et al., 2014), Ct_Cell_FBgn0004198 and Ct_SOLEXA_FBgn0004198 from FlyFactorSurvey (Zhu et al., 2011) for the 5 different alleles (Reference, Hikone-AS, Canton-S, NoCt, ConsensusCt). Predicted Binding sites overlapping our SNP are shown with either their corresponding score in Figure 4 for the best scoring PWM (Ct_SOLEXA_FBgn0004198), and in Figure S5 for all PWMs. Predictions for the entire region are shown in Table S2.
Population Genetics

We compiled whole genome sequencing data from multiple geographic samples collected in Africa, Europe, North America, Asia and Australia to investigate worldwide allele frequency patterns of the eyeless SNP at position Chr 4: 710326 in natural populations. This data set consisted of single individual sequencing data (Campo et al., 2013; Grenier et al., 2015; Lack et al., 2015; Langley et al., 2012; Pool et al., 2012) and Pool-Seq data from various sources (Bastide et al., 2013; Bergland et al., 2014; Kapun et al., 2016; Orozco-terWengel et al., 2012; Reinhardt et al., 2014) (Table S2). For single individuals, we obtained genotypes of the focal SNP from the Drosophila Genome Nexus website (DGN; http://www.johnpool.net/genomes.html) and estimated allele frequencies based on the number of chromosomes carrying the A-variant for populations with at least ten sequenced individuals. For Pool-Seq datasets, we re-mapped quality-filtered raw data as described in (Kapun et al., 2016) and estimated allele frequencies based on read counts of the A-variant relative to the total coverage. To increase sequence coverage in Queensland and Tasmania, we merged libraries of multiple collections at the corresponding locations (Reinhardt et al., 2014). We further used a collection of Pool-Seq data from 48 population samples collected across Europe by the DrosEU consortium (Kapun et al., 2018) (accession number: PRJNA388788) for an in-depth analysis of spatial distribution of the A-variant. Specifically, we tested for clinal distribution along the latitudinal and longitudinal axes by means of generalized linear models (GLMs) in R based on allele counts to account for the biallelic nature of the focal SNP. We further contrasted the clinality of 4: 710,326 to 21,008 putatively neutral genome-wide SNPs located in short introns (<60bp) and in distance to chromosomal inversions (Clemente and Vogl, 2012; Parsch et al., 2010). To test if the observed p-value from a GLM at the focal SNP deviates from neutral expectation we empirically assessed significance. We therefore generated empirical cumulative density functions (ECDF) based on the –log10 transformed p-values of all neutral SNPs and calculated the area under the ECDF confined by the –log10 p-value of the focal SNP and the upper tail of the distribution by integration. This area corresponds to the percentile of neutral SNPs with p-values equal or smaller than the focal SNP and thus summarizes the significance of clinality for 4:710,326 relative to genome-wide neutral estimates. We further characterized chromosome-wide patterns of genetic variation by estimating the population genetics statistics r and Tajima’s D for all 48 samples from the DrosEU dataset using Pool-Gen (Kapun et al., 2018) with implemented corrections for Pool-Seq data (Futschik and Schlötterer, 2010; Kofler et al., 2011). At last, we tested whether very rare occurrences of the A-variant in Sub-Saharan Africa may be due to admixture with non-African genetic variation. We therefore used admixture probability estimates from (Lack et al., 2015) (see Table S2) to classify African lines as admixed (>10% of the autosomes admixed) or non-admixed (≤10% of the autosomes admixed) and compared genotype counts for admixed and non-admixed lines by means of Fisher exact tests (FET) in R.

Electrophoretic Mobility Shift Assays

Nuclear extracts of stably transfected S2 cells expressing the Cut-FLAG fusion protein were prepared from cells pelleted and resuspended for 15 minutes at 4°C in the membrane lysis buffer (Tris-HCl pH7.4 20mM, NaCl 10mM, MgCl2 3mM, DTT 0.5mM, NP40 1%, protease inhibitors). Cell extracts were centrifuged 5 minutes at 13000 RPM. Pelleted nuclei were then rinsed twice with PBS 1X and lysed for 30 minutes at 4°C in the nuclear envelope lysis buffer (Tris-HCl pH7.4 20mM, NaCl 100mM, MgCl2 3mM, EDTA 1mM, glyc- ecol 10%, DTT 0.5mM, SDS 0.1%, sodium deoxycholate 0.5%, Triton X-100 1%, protease inhibitors). After centrifugation for 5 minutes at 13000 RPM, the supernatant containing the nuclear extract was recovered and stored at -80°C. Cold Electrophoretic Mobility Shift Assays (EMSA) were performed with 8 or 16μg of nuclear extract using the LightShift Chemiluminescent EMSA kit (ThermoFisher Scientific) according to the manufacturer’s instructions, with a modified binding buffer (Tris-HCl pH7.5 10mM, KCl 50mM, DTT 1mM, MgCl2 5mM, EDTA 1mM, Ficoll 400 5%, BSA 0.1μg/μl, NP40 0.05%, poly dI-dC 50ng/μl or Tris- HCl pH7.5 10mM, KCl 100mM, DTT 1mM, MgCl2 5mM, EDTA 1mM, NP40 0.05%, poly dI-dC 50ng/μl).

5’-biotinylated and unlabeled probes corresponding to the G and A-alleles of the ey enhancer were generated. To this goal, labeled or non-labeled complementary oligonucleotides were synthesized and hybridized. Oligonucleotide sequences are: G-allele: 5’-ACAATAAATGTTGGAACATTTCGAAACCTT-3’ A-allele: 5’-ACAATAAATGTTGGAACATTTCGAAACCTT-3’

The binding was performed for 20 minutes at room temperature, followed by electrophoretic migration onto native 5% polyacryl- amide gel in TBE 0.5× buffer. The supershift experiment was performed adding 2μg of the M2 anti-FLAG antibody (Sigma-Aldrich Cat# F3165).

Quantification and Statistical Analysis

In all experiments, sample size was determined a priori. Data were excluded exclusively prior to quantification based on poor image quality or inadequate developmental stage, explaining differences in sample size between groups. Except where stated otherwise, all statistical tests and charts were performed using GraphPad Prism 7 (GraphPad Software Inc.). Normality of the data were systematically assessed and statistical tests selected accordingly. Details on statistical tests, sample sizes and p values are indicated in figure legends except where mentioned otherwise.

Data and Code Availability

The code for the macro used for quantification of GFP/RFP colocalization (Oliva et al., 2016) is available on GitHub at the following link: https://github.com/rejsmont/FijiScripts/blob/master/mColoc3D.py. Raw images are available upon request from the Lead Contact.
Supplemental Information

Altering the Temporal Regulation of One Transcription Factor Drives Evolutionary Trade-Offs between Head Sensory Organs

Ariane Ramaekers, Annelies Claeys, Martin Kapun, Emmanuèle Mouchel-Vielh, Delphine Potier, Simon Weinberger, Nicola Grillenzoni, Delphine Dardalhon-Cuménal, Jiekun Yan, Reinhard Wolf, Thomas Flatt, Erich Buchner, and Bassem A. Hassan
Supplemental Information

Altering the temporal regulation of one transcription factor drives evolutionary trade-offs between head sensory organs


Figure S1, Related to Figure 1
Figure S2, Related to Figures 1, 4, 6
Figure S3, Related to Figure 2
Figure S4, Related to Figures 3, 4
Figure S5, Related to Figure 4
Figure S6, Related to Figures 4, 6, S5
Figure S7, Related to Figure 5
Table S1, Related to Figures 1, S1
Table S2, Related to Figure 4
Tables S3 and S4, Related to Figure 5
Table S5, Related to Star Methods
Figure S1. Natural variation in eye size in *Drosophila*, Related to Figure 1

(A to C’) Eye size comparison between females from five *Drosophila* species: *D. melanogaster* (*D. mel.*), *D. yakuba* (*D. yak.*), *D. ananassae* (*D. ana.*), *D. pseudoobscura* (*D. pse.*), *D. virilis* (*D. vir.*). Different numbers indicate different strains (*see Methods*). Boxes indicate interquartile ranges, lines medians and whiskers data ranges.

(A) Phylogenetic relationship between the five species (tree branches are not scaled).
(B) Eye: Face ratio measured from SEM images.
(C) Ommatidia number counted on SEM images. Ordinary one-way ANOVA **** p<0.0001 followed by Dunnett’s multiple comparisons. See also Table S1.
(D) Ommatidia width. Ordinary one-way ANOVA **** p<0.0001 followed by Dunnett’s multiple comparisons. See also Table S1

For this experiment, flies were raised at 21°C.
Figure S2. Ommatidia number variation: scaling and methods, Related to Figures 1, 4 and 6


(B) Bland-Altman chart plotting the difference in ommatidia number measured by two methods (ellipse-based estimation vs direct counting) over their mean (Bland and Altman, 1986). Comparison of fits indicates that the difference between the two measurements is independent of the mean (null hypothesis, grey line: slope= 0.0; alternative hypothesis blue line: slope unconstrained = -0.02372; \( p=0.6212 \)).

(C) Mesothoracic tibia (T2) length in three wild-type *D. mel.* stocks (Canton-S\textsuperscript{BH}, Canton-S\textsuperscript{TP}, Hikone-AS) and *D. pse*.
Sample sizes from left to right (n=21, n=23, n=29, n=21). Kruskal Wallis test **** \( p<0.0001 \) followed by Dunn’s multiple comparisons: **** \( p<0.0001 \); n.s. \( p>0.9999 \).

(D) Mesothoracic tibia (T2) length in CRISPR/Cas9 engineered and control lines. Sample sizes (n=20). Ordinary One way ANOVA n.s. \( p=0.7600 \).
Figure S3. Developmental origin of eye size variation in *D. mel.* and *D. pse.*, Related to Figure 2

(A) Schematics of the first steps of retinal differentiation showing the singling-out of committed Ato-expressing R8 ommatidia progenitor cells and subsequent steps of ommatidia assembly.

(A’ and A’’) The density of Ato-expressing R8 progenitors (in red in A’ and A’’) is similar in the two species. *Red:* anti-Ato immunostaining; *blue:* DAPI. Anterior is at the left. Scale bars: 5 µm.
Figure S4. Eyeless enhancer activity in early EADs. Related to Figure 3 and Figure 4.

The *D. pse.* and the four *D. mel.* alleles of the *ey* eye enhancer drive GFP expression in the entire EAD in stage 16 embryos (arrows in upper panel) and in 1st instar larvae (L1; yellow dashed line in lower panel). Green: GFP; Blue: DAPI; Red: anti-Futsch (22C10). Scale bars: 20 μm.
Figure S5: Ct TF binds ey enhancer and regulates eye size, Related to Figure 4.
(A) Visualization of Cluster-Buster Ct predicted binding sites for natural and synthetic ey enhancer alleles at the SNP location. Scores are represented by a grey scale. PWMs corresponding sequence logos plotted by seqLogo (https://rdrr.io/bioc/seqLogo/) are shown on the left.

(B) Electrophoretic mobility shift assay. The Cut-FLAG nuclear extract induces a band shift (black arrow) with oligonucleotide probes corresponding to both G and A-enhancer alleles. Both shifts are eliminated when corresponding non-labeled competitors are added.


(D) Overexpression of two UAS-ctRNAi and one UAS-luciferaseRNAi constructs under the control of ctGAL4. Sample sizes from left to right (n=23, n=13, n=8, n=10, n=8, n=7, n=6). Ordinary one-way ANOVA **** p<0.0001 followed by Sidak’s multiple comparisons: ctRNAi5687/ctGal4 vs ctRNAi5687/TM3, Sb **** p<0.0001; ctRNAi5687/ctGal4 vs ctGAL4/+ **** p<0.0001; ctRNAi4138/ctGal4 vs ctRNAi4138/TM3, Sb * p=0.0126; ctRNAi4138/ctGal4 vs ctGAL4/+ **** p<0.0001; lucRNAi/ctGal4 vs lucRNAi/TM3, Sb n. s. p>0.9999; lucRNAi/ctGal4 vs ctGAL4/+ ** p=0.0036.

(E) Overexpression of two UAS-ctRNAi and one UAS-luciferaseRNAi constructs under the control of hthGAL4. Sample sizes, from left to right (n=2, n=5, n=15, n=17, n=10, n=7). Sample size for ctRNAi5687/hthGAL4 was low due to the lethality or gross morphological defects caused by this allelic combination. Ordinary one-way ANOVA ** p=0.0089 followed by Sidak’s multiple comparisons: ctRNAi4138/hthGal4 vs ctRNAi4138/TM3, Sb ** p=0.0047; lucRNAi/hthGal4 vs lucRNAi/TM3, Sb n. s. p=0.2152.

(D and E) Scatter dot plots. Line indicates the mean.
Figure S6. Eye: Face ratio, absolute A3 width and absolute face width. Related to Figure 4, Figure 6 and Figure S5.

Boxes indicate interquartile ranges, lines medians and whiskers data ranges.

(A) Sample sizes (n=12, n=14, n=10). Ordinary one-way ANOVA **** $p<0.0001$ followed by Tukey’s multiple comparisons: **** adjusted $p<0.0001$.

(A’) Sample sizes (n=11, n=11, n=13). Ordinary one-way ANOVA ** $p=0.0035$ followed by Tukey’s multiple comparisons: ** adjusted $p=0.0043$; * adjusted $p = 0.0184$; n.s adjusted $p=0.7600$.

(A’’) Sample sizes (n=12, n=14, n=10). Ordinary one-way ANOVA **** $p<0.0001$ followed by Tukey’s multiple comparisons: **** adjusted $p<0.0001$.

(B) Sample sizes (n=19, n=19, n=12, n=12). Unpaired t-tests: **** $p<0.0001$; n.s. $p=0.37831$.

(B’) Sample size (n=12). Unpaired t-tests: * $p=0.0288$; * $p=0.0444$.

(B’’) Sample sizes (n=13, n=13, n=12, n=12). Unpaired t-tests: ** $p=0.0042$; * $p=0.0163$.

(C) Sample size (n=42). Unpaired t-tests: ** $p=0.0060$.

(C’) Sample sizes (n=16, n=14). Unpaired t-tests: n.s. $p=0.0553$.

(C’’) Sample size (n=42). Unpaired t-tests: n.s. $p=0.5831$.

(D) Sample sizes (n=9; n=16). Unpaired t-tests: n.s. $p=0.2625$.

(D’) Sample sizes (n=9; n=16). Unpaired t-tests: n.s. $p=0.2220$.

(D’’) Sample sizes (n=9; n=16). Unpaired t-tests: n.s. $p=0.5353$.

(E) Estimated ommatidia numbers in control G-carrying and the four CRISPR engineered A-carrying variants imaged by light microscopy. Sample sizes: from left to right (n=24, n=8, n=32, n=33, n=45); Ordinary one-way ANOVA *** $p=0.0009$ followed by Dunnet’s multiple comparisons between the control and the four CRISPR lines.
Figure S7. Genetic variation of the fourth chromosome in Europe, Related to Figure 5

The distribution of \( \pi \) (top panel) and Tajima’s \( D \) (bottom panel) in 50kb windows with 10kb step-size for 48 population samples from Europe. The vertical dashed black line indicates the approximate genomic position of the focal SNP at position Chr 4: 710326.
### Table S1. Natural variation in *Drosophila* eye size, Related to Figures 1, S1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ommatidia Number</th>
<th>Ommatidia width</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adjusted p value</td>
<td>adjusted p value</td>
</tr>
<tr>
<td><em>D. m. 2</em></td>
<td>n=7 p = 0.0001</td>
<td>n=24 p = 0.0161</td>
</tr>
<tr>
<td><em>D. m. 3</em></td>
<td>n=8 p = 0.0001</td>
<td>n=24 p = 0.0002</td>
</tr>
<tr>
<td><em>D. y. 1</em></td>
<td>n=5 p = 0.9396</td>
<td>n=24 p = 0.0462</td>
</tr>
<tr>
<td><em>D. y. 2</em></td>
<td>n=4 p = 0.2764</td>
<td>n=24 p = 0.9922</td>
</tr>
<tr>
<td><em>D. a. 1</em></td>
<td>n=8 p = 0.9770</td>
<td>n=24 p = 0.0001</td>
</tr>
<tr>
<td><em>D. a. 2</em></td>
<td>n=8 p = 0.0083</td>
<td>n=24 p = 0.0001</td>
</tr>
<tr>
<td><em>D. p. 1</em></td>
<td>n=9 p = 0.0001</td>
<td>n=24 p = 0.9994</td>
</tr>
<tr>
<td><em>D. p. 2</em></td>
<td>n=8 p = 0.0001</td>
<td>n=24 p = 0.9072</td>
</tr>
<tr>
<td><em>D. v.</em></td>
<td>n=4 p = 0.6782</td>
<td>n=24 p = 0.0001</td>
</tr>
</tbody>
</table>

Sample sizes and results of Dunnett’s multiple comparison tests following ordinary one-way ANOVA from Figure S1. Comparisons towards *D. m. 1* (Canton-S\textsuperscript{BH}; ommatidia number sample size n=6; ommatidia width sample sizes n=24).
Table S2. Ct binding site predictions at the SNP location, Related to Figure 4

*Data are presented in a separate Excel document.*

Predictions of Ct binding sites in a 1 kb region surrounding the SNP at position Chr 4: 710326 (500 bp up and down) scored with Cluster-Buster (Frith et al., 2003).
Table S3. Worldwide allele frequency patterns, Related to Figure 5

<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>Data Type</th>
<th>Data Reference</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>Sorell Pool</td>
<td>Reinhardt et al. 2012</td>
<td>0.206</td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>Queensland        Pool</td>
<td>Reinhardt et al. 2012</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>Gross-Enzersdorf Pool</td>
<td>Kapun et al. 2016</td>
<td>0.159</td>
<td></td>
</tr>
<tr>
<td>Cameroon</td>
<td>Oku Single</td>
<td>Pool et al. 2012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>Beijing Single</td>
<td>Grenier et al. 2015</td>
<td>0.070</td>
<td></td>
</tr>
<tr>
<td>Egypt</td>
<td>Cairo Single</td>
<td>Lack et al. 2015</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Gambella Single</td>
<td>Lack et al. 2015</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Lyon Single</td>
<td>Pool et al. 2012</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Gabon</td>
<td>Franceville Single</td>
<td>Pool et al. 2012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Malawi</td>
<td>Mwanza Single</td>
<td>Langley et al. 2012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>Houten Single</td>
<td>Grenier et al. 2015</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Rwanda</td>
<td>Gikongoro Single</td>
<td>Pool et al. 2012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>Barcelona Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Homestead Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Hahira Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.154</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Eutawville Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Raleigh Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Charlottesville Pool</td>
<td>Kapun et al. 2016</td>
<td>0.055</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Winters Single</td>
<td>Campo et al. 2013; Bergland et al. 2014;</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Linvilla Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Ithaca Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Lancaster Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.230</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Cross Plains Pool</td>
<td>Kapun et al. 2016; Bergland et al. 2014;</td>
<td>0.112</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Bowdoinham Pool</td>
<td>Kapun et al. 2016</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td>Siavonga Single</td>
<td>Pool et al. 2012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Cyprus</td>
<td>Nicosia Pool</td>
<td>Kapun et al. 2018</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>City</td>
<td>Pool Type</td>
<td>Reference</td>
<td>Value</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------</td>
<td>-----------</td>
<td>-------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Turkey</td>
<td>Yesiloz</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Turkey</td>
<td>Yesiloz</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.019</td>
</tr>
<tr>
<td>Portugal</td>
<td>Recarei</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.034</td>
</tr>
<tr>
<td>Spain</td>
<td>Lleida</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Spain</td>
<td>Lleida</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.029</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Yalta</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Yalta</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>France</td>
<td>Gotheron</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.128</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Odessa</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Odessa</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Odessa</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Portugal</td>
<td>Recarei</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.034</td>
</tr>
<tr>
<td>Spain</td>
<td>Lleida</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Spain</td>
<td>Lleida</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.029</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Yalta</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Yalta</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Switzerland</td>
<td>ChaletAGobet</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.096</td>
</tr>
<tr>
<td>Switzerland</td>
<td>ChaletAGobet</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.081</td>
</tr>
<tr>
<td>Austria</td>
<td>Seeboden</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.055</td>
</tr>
<tr>
<td>Germany</td>
<td>Munich</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.045</td>
</tr>
<tr>
<td>Germany</td>
<td>Munich</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.021</td>
</tr>
<tr>
<td>Germany</td>
<td>Broggingen</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.052</td>
</tr>
<tr>
<td>Germany</td>
<td>Broggingen</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.090</td>
</tr>
<tr>
<td>Austria</td>
<td>Mauternbach</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.053</td>
</tr>
<tr>
<td>Austria</td>
<td>Mauternbach</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.029</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Uman</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>France</td>
<td>Viltain</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.029</td>
</tr>
<tr>
<td>France</td>
<td>Viltain</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.131</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Drogoibych</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Kharkiv</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Kharkiv</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Piryutatin</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Kyiv</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.071</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Kyiv</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.024</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Varva</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>ChernobylApple</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.020</td>
</tr>
<tr>
<td>Ukraine</td>
<td>ChernobylPolisske</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.015</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Chernobyl</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.000</td>
</tr>
<tr>
<td>Ukraine</td>
<td>ChernobylYaniv</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.036</td>
</tr>
<tr>
<td>UK</td>
<td>Lutterworth</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.078</td>
</tr>
<tr>
<td>UK</td>
<td>MarketHarboroug</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.072</td>
</tr>
<tr>
<td>UK</td>
<td>Sheffield</td>
<td>Pool</td>
<td>Kapun et al. 2018</td>
<td>0.011</td>
</tr>
<tr>
<td>Origin</td>
<td>Data Type</td>
<td>Data Source</td>
<td>Allele Frequency</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>Lund</td>
<td>Pool</td>
<td>0.121</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>Karensminde</td>
<td>Pool</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>Karensminde</td>
<td>Pool</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>SouthQueensferry</td>
<td>Pool</td>
<td>0.173</td>
<td></td>
</tr>
<tr>
<td>Russia</td>
<td>Valday</td>
<td>Pool</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Finland</td>
<td>Akaa</td>
<td>Pool</td>
<td>0.048</td>
<td></td>
</tr>
<tr>
<td>Finland</td>
<td>Akaa</td>
<td>Pool</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Finland</td>
<td>Vesanto</td>
<td>Pool</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Origin, data type, data source and allele frequencies of the A-variant of the focal SNP at position Chr 4: 710326 of world-wide populations with sample sizes ≥ 10 individuals.
Table S4. Isofemale line genotypes, Related to Figure 5

Data are presented in a separate Excel document.

Genotypes and admixture status for isofemale lines from Sub-Saharan Africa.
<table>
<thead>
<tr>
<th>name</th>
<th>sequence</th>
<th>used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>ey3.3Pse_F</td>
<td>GGGGACAAGTTTTGTACAAAAAAGCAGGCTAAGTGTAGTGGACTAGG</td>
<td>cloning of ey enhancer</td>
</tr>
<tr>
<td>ey3.3Pse_R</td>
<td>GGGGACCACTTTGTACAAGAAAGCAGGCTTGGCTAGAATTTTGCTACAAGCG</td>
<td>cloning of ey enhancer</td>
</tr>
<tr>
<td>ey3.5Mel_F</td>
<td>GGGGACAAGTTTTGTACAAAAAAGCAGGCTAGGACTAGGCTACAGGCTATTGCT</td>
<td>cloning of ey enhancer</td>
</tr>
<tr>
<td>ey3.5Mel_F</td>
<td>GGGGACCACGTTTTGTACAAAAAAGCAGGCTAGGACTAGGCTACAGGCTATTGCT</td>
<td>cloning of ey enhancer</td>
</tr>
<tr>
<td>ey3.5NoCt_F</td>
<td>caataaaatgttggCaGttttaactttcg</td>
<td>site directed mutation of ey enhancer</td>
</tr>
<tr>
<td>ey3.5NoCt_R</td>
<td>cgaagttcgaaaaaCtcgaaccattttattg</td>
<td>site directed mutation of ey enhancer</td>
</tr>
<tr>
<td>ey3.5ConsensusCt_F</td>
<td>taaaagtgttTgacatttttcgaacctttcg</td>
<td>site directed mutation of ey enhancer</td>
</tr>
<tr>
<td>ey3.5ConsensusCt_R</td>
<td>gaaaaagttctaaaccatattttttttt</td>
<td>site directed mutation of ey enhancer</td>
</tr>
<tr>
<td>ey3.5gRNA_F</td>
<td>phospho-CTTCTacgaaaaaatatatgtt</td>
<td>guideRNA construct</td>
</tr>
<tr>
<td>ey3.5gRNA_R</td>
<td>phospho-AAACaccatatttttttcgaC</td>
<td>guideRNA construct</td>
</tr>
<tr>
<td>ey_R3</td>
<td>agaataatcacatggcggag</td>
<td>allele-specific PCR</td>
</tr>
<tr>
<td>ey-SNPG-F</td>
<td>ggaatcgaaaaaatatatgttgg</td>
<td>allele-specific PCR</td>
</tr>
<tr>
<td>Ey-SNP^A-F</td>
<td>ggaatcgaaaaaatatatgtgga</td>
<td>allele-specific PCR</td>
</tr>
<tr>
<td>EMSA_G</td>
<td>ACAATAAAAATGGTTGGAACCTTTTCGAACCTTT</td>
<td>EMSA</td>
</tr>
<tr>
<td>EMSA_A</td>
<td>ACAATAAAAATGGTTGGAACCTTTTCGAACCTTT</td>
<td>EMSA</td>
</tr>
</tbody>
</table>