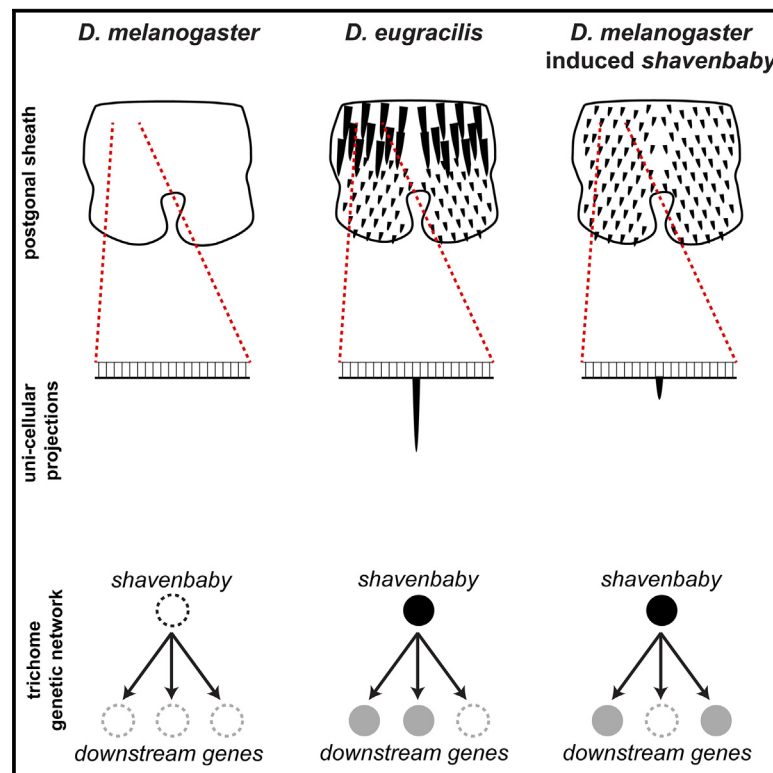


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Co-option of the trichome-forming network initiated the evolution of a morphological novelty in *Drosophila eugracilis*

Graphical abstract



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In brief

Rice et al. show that novel unicellular projections found in the postgonal sheath of *Drosophila eugracilis* express the key trichome transcription factor *shavenbaby* and 14 genes of the known larval trichome genetic network. Expression of *shavenbaby* in the projection-lacking species *Drosophila melanogaster* is sufficient to induce this novel trait.

Highlights

- Unicellular projections of the *D. eugracilis* postgonal sheath express *shavenbaby*
- The *D. eugracilis* projections co-opted a portion of the larval trichome GRN
- *shavenbaby* misexpression recapitulates short projections in *D. melanogaster*
- Upon co-option, the GRN was specialized to produce diverse projection morphologies

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Article

Co-option of the trichome-forming network initiated the evolution of a morphological novelty in *Drosophila eugracilis*

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SUMMARY

Identifying the molecular origins by which new morphological structures evolve is one of the long-standing problems in evolutionary biology. To date, vanishingly few examples provide a compelling account of how new morphologies were initially formed, thereby limiting our understanding of how diverse forms of life derived their complex features. Here, we provide evidence that the large projections on the *Drosophila eugracilis* phallus that are implicated in sexual conflict have evolved through the partial co-option of the trichome genetic network. These unicellular apical projections on the phallus postgonal sheath are reminiscent of trichomes that cover the *Drosophila* body but are up to 20-fold larger in size. During their development, they express the transcription factor Shavenbaby, the master regulator of the trichome network. Consistent with the co-option of the Shavenbaby network during the evolution of the *D. eugracilis* projections, somatic mosaic CRISPR-Cas9 mutagenesis shows that *shavenbaby* is necessary for their proper length. Moreover, misexpression of Shavenbaby in the sheath of *D. melanogaster*, a naive species that lacks these projections, is sufficient to induce small trichomes. These induced projections rely on a genetic network that is shared to a large extent with the *D. eugracilis* projections, indicating its partial co-option but also some genetic rewiring. Thus, by leveraging a genetically tractable evolutionary novelty, our work shows that the trichome-forming network is flexible enough that it can be partially co-opted in a new context and subsequently refined to produce unique apical projections that are barely recognizable compared with their simpler ancestral beginnings.

INTRODUCTION

The mystery surrounding morphological novelties lies in the inherent difficulty of explaining their evolutionary origins. One proposed mechanism for initiating a novel morphological structure is the redeployment of an established gene regulatory network (GRN) to a new developmental context.^{1–4} Such GRN co-option is thought to result from the recruitment of one or a few top-tier regulators within a network to a tissue that previously lacked expression.^{5,6} The mechanism of GRN redeployment has been extensively studied in the field of evolutionary development. It has been implicated across many species^{7–9} in many different contexts^{10–14} and is often linked by shared gene co-expression patterns, pleiotropic enhancer elements, and pleiotropic effects of gene perturbations.^{15–19} While perturbations of many genes of a GRN may disrupt a phenotype, a very small number of genes will be sufficient to induce the phenotype in naive tissues.^{5,6} These novelty-inducing factors have therefore remained elusive because of their relative scarcity within GRNs and the lack of genetic tools in many species that bear novel traits of interest. Inducing a novelty

genetically would allow us to probe the nascent stages of genetic network co-option and examine how subsequent rounds of evolutionary refinement may have proceeded.^{1,20}

Across animals, reproductive structures are some of the most diverse and rapidly evolving morphological traits.^{21–23} For this reason, they are excellent candidates for genetic and developmental investigations of novel traits. As an example, diverse genital morphologies are present in species of *Drosophila*, and the rapid pace of genital evolution has often been attributed to conflict between the sexes.^{21,22,24} *Drosophila eugracilis*, which is a member of the sister group to the *melanogaster* subgroup,^{25–27} shows a unique set of outgrowths covering the surface of the phallus.²³ The adult postgonal sheath (also known as the aedeagal sheath²⁸) of *D. eugracilis* is covered with over 150 differently sized projections, which have been implicated in copulatory wounding to facilitate the entry of male seminal proteins into the female circulatory system, increasing ovulation and reducing remating rates^{23,29} (Figure 1). The recent origin of these projections makes them an ideal model for investigating the genetics behind complex morphological novelties.



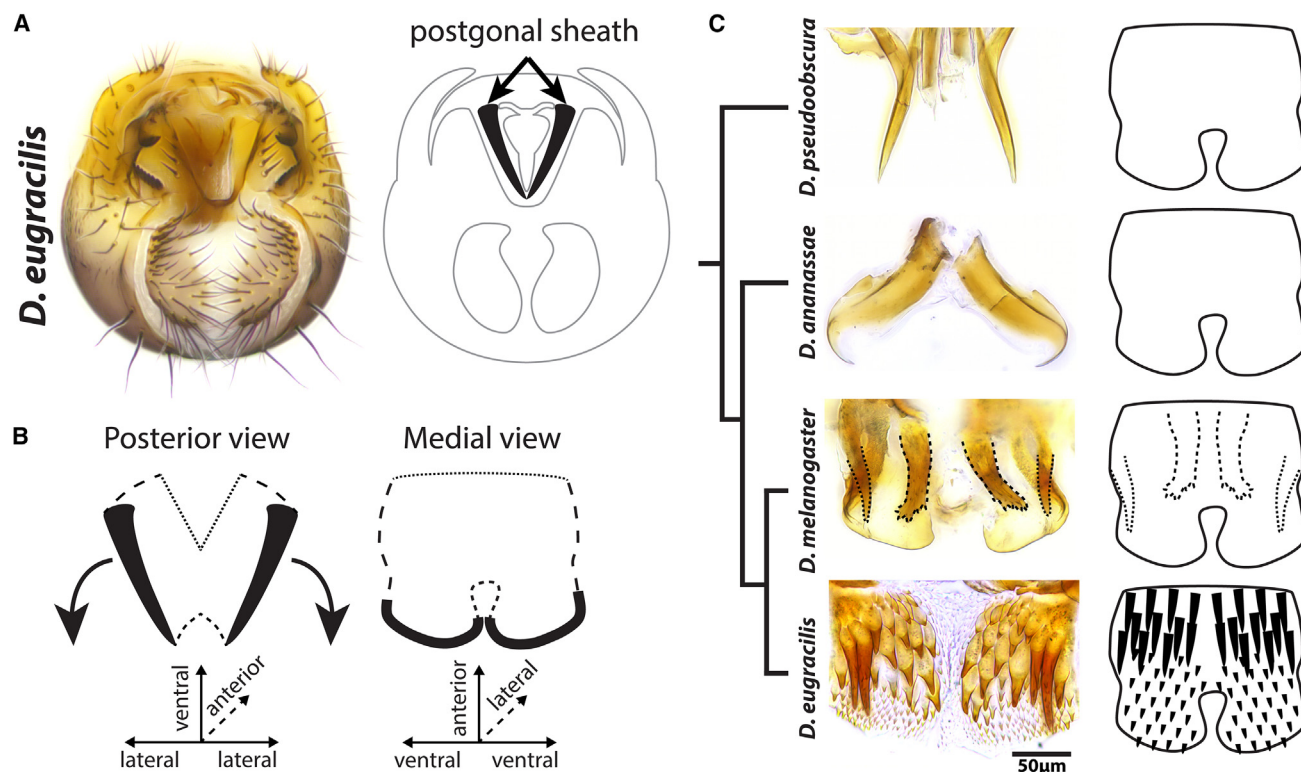


Figure 1. The postgonal sheath of *D. eugracilis* is covered with differently sized projections

(A) Left: light microscopy images of the *D. eugracilis* adult male terminalia (genitalia and analia). Right: a schematic representation of the *D. eugracilis* adult terminalia, highlighting the position of the postgonal sheath.

(B) A schematic representation of the adult postgonal sheath. Left: posterior view of the postgonal sheath, orientation seen while attached to the whole terminalia. Right: medial view of the postgonal sheath, orientation seen once dissected and flattened for imaging.

(C) Medial view of the adult postgonal sheath. Left: light microscopy images of *D. pseudoobscura* (top), *D. ananassae*, *D. melanogaster*, and *D. eugracilis* (bottom) postgonal sheaths. The *D. melanogaster* postgonal sheath contains two pairs of multicellular outgrowths: the ventral postgonal process (small dotted outline) and dorsal postgonal process (large dotted outline). The *D. eugracilis* postgonal sheath is covered with over 150 projections. Right: schematic representations of the *D. melanogaster* and *D. eugracilis* postgonal sheaths. Corresponding images of the *D. eugracilis* female genitalia can be found in [Figure S1](#). Scale bar, 50 μm.

Although the genetic network of the *D. eugracilis* postgonal sheath projections had not been previously studied, the GRN that controls the *D. melanogaster* larval hairs (also known as trichomes) is one of the most thoroughly characterized genetic networks in the literature.^{30–32} The transcription factor genes *shavenbaby* (*svb*) and *SoxNeuro* (*SoxN*) are necessary for trichome development, as evidenced by the loss or large reduction in the height of trichomes in the larvae as well as in the adult wings, legs, and abdomens in *svb* mutants.^{33–36} These two transcription factors bind many of the same genes involved in trichome development but also independently regulate some members of the trichome genetic network.³⁷ Over 150 genes are direct targets of *Svb* in the larval trichomes, including genes that contribute to actin bundling and extracellular matrix (ECM) formation.^{38,39} The loss of *svb* expression has been found to be the key evolutionary change underlying the independent loss of the dorsal larval trichomes in several species of *Sophophora*, indicating that it is a key gene in this network.^{40–43} Furthermore, it has been shown that *svb* and *SoxN* are capable of inducing larval trichomes in cells that do not normally produce them.³⁷ Finally, the trichome types of the larvae (dorsal and ventral) also have unique morphologies, with the ventral

trichomes showing a darkly pigmented saw tooth morphology while the dorsal type 4 trichomes are less pigmented and have a thinner shape.⁴⁴ Despite the creative potential of the trichome network, studies of trichome evolution have necessarily focused on loss, and the genes that cause the unique morphologies of different trichomes have not been well established.

To investigate the genetic network involved in the postgonal sheath projections of *D. eugracilis*, we analyzed their development, both morphologically and molecularly. We found that they are produced by unicellular apical outgrowths similar to larval trichomes. Both *svb* and *SoxN* were expressed in the *D. eugracilis* postgonal sheath, and a large portion of the larval trichome genetic network is species-specifically expressed. Of note, we show that the misexpression of *svb* in the postgonal sheath of *D. melanogaster* is sufficient to recapitulate small projections reminiscent of the *D. eugracilis* novel projections. Comparing the induced trichomes of *D. melanogaster* to the novel trichomes of *D. eugracilis* identifies a core portion of the trichome network shared between larvae and genital contexts. Our work provides a glimpse at the incipient stages of novelty and highlights the challenges of exploring the subsequent steps of elaboration.

RESULTS

Comparative anatomy of *D. eugracilis* and *D. melanogaster* genital traits

To visualize the stereotyped positions and connectivity of the *D. eugracilis* projections, we performed microdissections of the *D. eugracilis* phallus (Figures 1A and 1B). All projections are directly connected to the postgonal sheath (also known as the aedeagal sheath²⁸), an epithelial tissue produced from the genital disc. The anterior portion of the *D. eugracilis* postgonal sheath produces large projections, while the posterior portion produces smaller projections (Figure 1C). Our previous analysis found that the postgonal sheaths of the eight members of the *melanogaster*, *suzukii*, and *ananassae* subgroups have smooth medial surfaces without detectable projections,²⁴ similar to the morphology of the more basal *D. pseudoobscura* (Figure 1C). The *D. melanogaster* postgonal sheath produces four large multicellular spine-like structures,²⁴ known as the postgonal processes (also known as the postgonites²⁸). These large multicellular spines in *D. melanogaster* are similar in size and shape to the largest projections we find in the *D. eugracilis* postgonal sheath. The female external genitalia of *D. eugracilis* are similar in morphology to *D. melanogaster*,⁴⁵ while the internal genitalia (vagina) potentially show a pleated morphology and contain many hair-like structures known as trichomes⁴⁶ (Figure S1).

The *D. eugracilis* projections are unicellular

To investigate possible genetic mechanisms that generated the *D. eugracilis* projections, we first examined how they develop. We initially tested if the *D. eugracilis* projections were produced by multicellular outgrowths similar to the *D. melanogaster* postgonal processes. To visualize the phallic morphology macroscopically, we employed ECAD antibody staining (which highlights apical cellular junctions), revealing that the *D. eugracilis* pupal phallus at 48 h after pupal formation (APF) did not show any multicellular outgrowths (Figures 2E and 2E'). This led us to test if these projections could instead be produced by unicellular outgrowths. Other well-characterized unicellular projections (such as larval trichomes) form actin-rich projections from the cell's apical surface.⁴⁷ Co-staining ECAD and phalloidin (which highlights actin) in the developing postgonal sheath of *D. eugracilis* revealed actin-rich apical projections that each extend from a single cell. These unicellular projections cover the medial postgonal sheath where the projections of the adult are found (Figure 2G'). The unicellular projections begin forming at 44 h APF, similar to when trichomes start forming in the pupal abdominal epidermis⁴⁸ and are close to their adult size at 52 h APF (Figure S2). ECAD/phalloidin co-staining of *D. melanogaster* postgonal sheaths did not show unicellular projections. However, unicellular projections are also found on the aedeagus, medium gonocoxite, and dorsal postgonal process (Figures 2C and S3). The lack of unicellular projections in the postgonal sheath of *D. melanogaster* suggests that the unicellular projections in the *D. eugracilis* postgonal sheath likely represent a novelty.

The *D. eugracilis* postgonal sheath projections are modified trichomes

Since the unicellular projections of the *D. eugracilis* postgonal sheath developed similar to unicellular larval trichomes of

Drosophila, we investigated whether the key transcription factor, *svb*, also known as *ovo* (FBgn0003028), was involved. We established an antibody for *svb* that has binding specificity in both *D. melanogaster* and *D. eugracilis*. As a positive control, we show that it correctly stains the 12 h after egg laying embryonic pattern (Figure S4). Antibody staining and *in situ* hybridization in the developing *D. eugracilis* phallus revealed that *svb* was expressed in the postgonal sheath where the unicellular projections are found and revealed that the anterior regions of the postgonal sheath, which house the largest projections, contain large nuclei (Figures 2I and 2J). Expression of *svb* is first observed at 44 h APF when unicellular projections begin to form and continues until 52 h APF (Figure S2). The same analysis for the phallus of *D. melanogaster* at 48 h APF did not detect *svb* expression in the postgonal sheath (Figures 2G and 2H). Thus, the gain of *svb* expression in the medial postgonal sheath is correlated with the gain of unicellular projections. Expression of *svb* was found in the *D. melanogaster* aedeagus, medium gonocoxite, and dorsal postgonal process (Figure 2J), which corresponds to the unicellular projections we see in these regions. This indicates that the processes of the postgonal sheath are modified trichomes, and we will refer to them as postgonal sheath trichomes throughout the remainder of the text.

We next tested whether the cellular effectors of the larval trichome GRN were expressed in the postgonal sheath of *D. eugracilis*. We examined the expression of 23 known larval trichome GRN cellular effectors^{38,39,49} in the *D. eugracilis* postgonal sheath 48 h APF by *in situ* hybridization (Figure 3). Fourteen out of 23 tested cellular effectors show strong expression in the medial postgonal sheath, while 9 genes did not show strong expression (Figure S5; Table S1). The majority of ECM and actin cellular effectors are expressed in the medial postgonal sheath. Although not all of the larval trichome genetic network showed expression in the postgonal sheath, variation is also seen between larval trichomes.^{38,39,49} Dorsal larval trichomes express 21 out of 23 of the genes we analyzed, while the ventral trichomes express 22 out of 23 (Table S2). Additionally, the *D. eugracilis* medium gonocoxite, which also houses trichomes, expresses 50% of the larval trichome GRN that we analyzed (Figure S5; Table S5). It is also possible that these undetected genes are expressed at a different developmental stage than the one we surveyed. The localized expression of *svb* and 14 cellular effectors in the *D. eugracilis* medial postgonal sheath indicates that a substantial portion of the larval trichome genetic network is present in these novel structures.

svb* is necessary for the largest postgonal sheath trichomes of *D. eugracilis

We next wanted to determine the necessity of *svb* for the *D. eugracilis* postgonal sheath trichome morphology. Null mutations of *svb* have been shown to reduce the height or even lead to the loss of larval, leg, abdominal, and wing trichomes.^{33–37} We tested this by inducing Cas9-mediated mosaic mutants (Figure 4B).⁵⁰ In brief, this technique involves injecting pre-cellularized embryos with a mix of Cas9 protein and short guide RNAs (sgRNAs). This induces clonal regions that are mutant for the target gene throughout the germline and soma. We first injected two sgRNAs targeted for the *white* gene (located on the X chromosome) to calibrate the efficiency of these experiments and

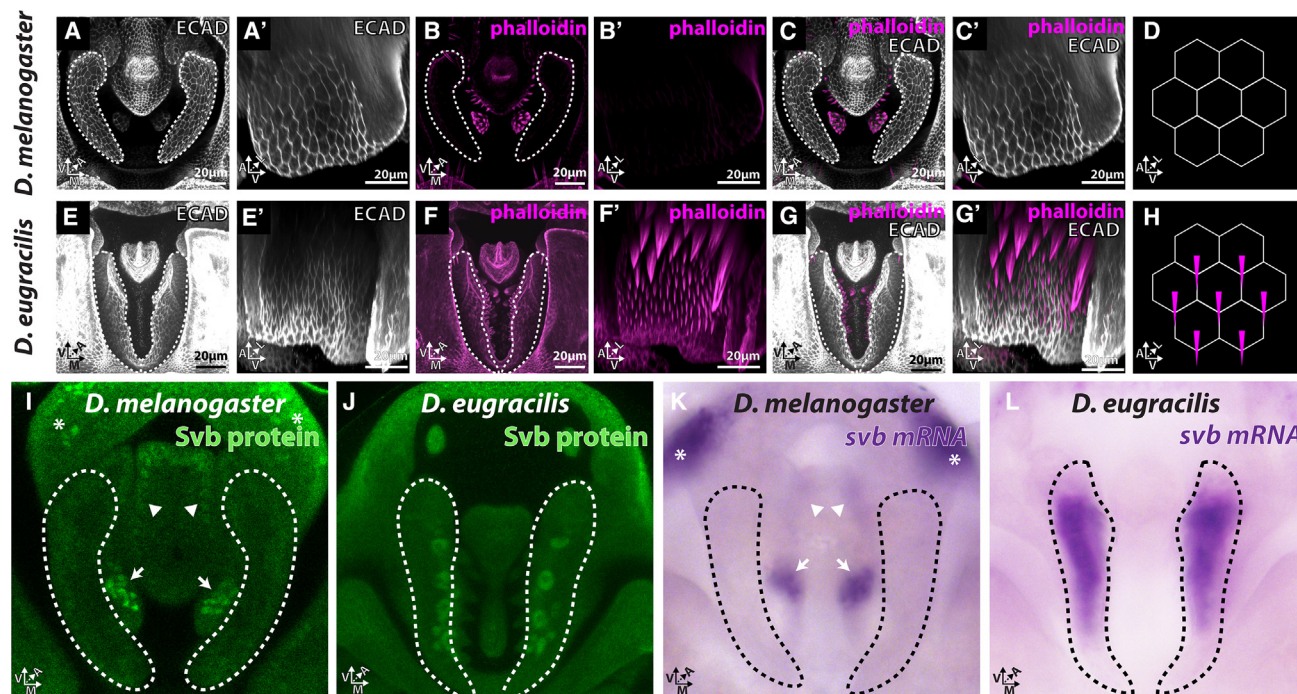


Figure 2. *D. eugracilis* postgonal sheath projections are unicellular trichomes that express *shavenbaby*

(A–H) ECAD staining (white) highlights apical cellular junctions, and phalloidin staining (magenta) visualizes actin in the 48 h APF phallus of *D. melanogaster* and *D. eugracilis*. Scale bar, 20 μ m.

(A–L) Posterior viewpoint. (A'–G') Medial viewpoint.

(A and A') The apical cell junctions of the *D. melanogaster* postgonal sheath (white dotted lines).

(B and B') Phalloidin staining shows strong concentrations of actin-rich apical projections from the aedeagus and dorsal postgonal processes. See also Figure S3.

(C and C') Composite images show that the *D. melanogaster* postgonal sheath lacks actin bundles.

(D) A schematic representation depicting the medial cells of the *D. melanogaster* postgonal sheath.

(E and E') The apical cell junctions of the *D. eugracilis* postgonal sheath (white dotted lines) show that the postgonal sheath is comprised of a continuous flat plate-shaped structure.

(F and F') Phalloidin staining shows that strong concentrations of actin are present in the *D. eugracilis* postgonal sheath. A time course of phalloidin staining can be found in Figure S2.

(G and G') Composite images show that the postgonal sheath is covered with unicellular actin bundles.

(H) A schematic representation depicting the medial cells of the *D. eugracilis* postgonal sheath.

(I) Shavenbaby antibody staining of the *D. melanogaster* phallus. Shavenbaby is not highly expressed in the postgonal sheath but is expressed in the aedeagus (arrowheads), medium gonocoxite (asterisks), and dorsal postgonal process (arrows). Shavenbaby antibody staining of the embryo can be found in Figure S4.

(J) Shavenbaby antibody staining of the *D. eugracilis* phallus. Shavenbaby is expressed in the medial postgonal sheath as well as the medium gonocoxite. The ventral-anterior *svb* expressing nuclei of the postgonal sheath are large in size.

(K) *In situ* hybridization for the long isoform of *shavenbaby* in the *D. melanogaster* phallus. *shavenbaby* RNA is not highly expressed in the postgonal sheath but is expressed in the aedeagus (arrowheads), medium gonocoxite (asterisks), and dorsal postgonal process (arrows).

(L) *In situ* hybridization for *shavenbaby* in the *D. eugracilis* phallus. *shavenbaby* RNA is expressed in the medial postgonal sheath and medium gonocoxite. A time course of *shavenbaby* *in situ* hybridization can be found in Figure S2.

produce a negative control, as *white* has no known genital phenotypes. 43.1% of surviving adult males showed a white mosaic patch in the adult eye (Figure S6B; Table S3).

For our *svb* CRISPR experimental treatment, we used an injection mix that contained Cas9 as well as four sgRNAs (two sgRNAs for *white* and two sgRNAs for *svb*). We performed a pilot study where we injected three different combinations of two sgRNAs targeting *svb*. For most animals, the postgonal sheath trichomes appeared similar to our control injections. However, we noticed that the large trichomes (referred to as major trichomes hereafter) were reduced in size in many of our dissected sheaths (Figure S7A). All three combinations of sgRNAs produced individuals with reduced major trichomes (Figure S8).

Due to a limited number of individuals with mutant phenotypes for each sgRNA combination, we elected to focus on sgRNA pair *svb* sgRNA 1 and 2 to survey a greater number of injected individuals.

For our large-scale *svb* sgRNA 1 and 2 injections (co-injected with *white* sgRNA 3 and 4), 42.7% of our surviving males had mosaic white patches in their eyes, while 16% (9/56) of male adults showed a significant decrease (>3 SD below the *white* control injection mean) in the length of the tallest trichome (Figures 4D, S6B, and S7; Table S3). The lower proportion of identified *svb* major trichome mutants compared with *white* eye mutants is likely due to us only focusing on two major trichome cells for *svb* mutants, while we detected any *white*

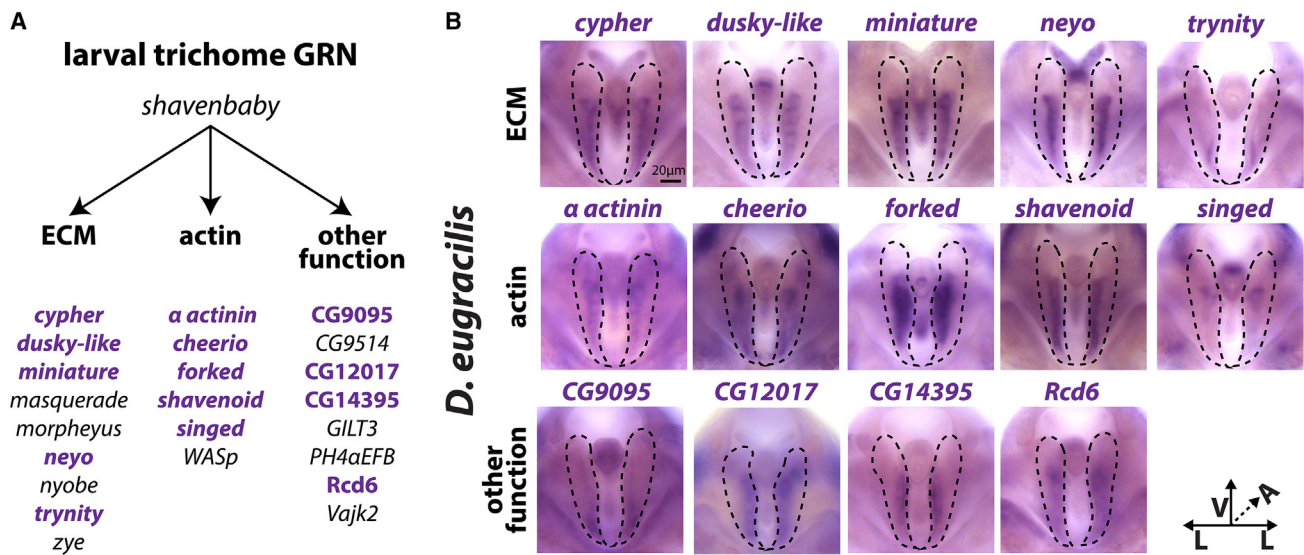


Figure 3. A large portion of the larval trichome GRN is expressed in the *D. eugracilis* postgonal sheath

(A) Genes within the established larval trichome network that were tested. Bolded purple text indicates the genes expressed in the medial postgonal sheath of *D. eugracilis*; see also Table S1.

(B) *In situ* hybridization of the *D. eugracilis* 48 h APF developing phallus. Dashed outlines highlight the postgonal sheath. Images of the genes not expressed in the postgonal sheath are found in Figure S5. Each panel is imaged from the posterior perspective, with the ventral side facing up. The expression of each of these genes in the dorsal and ventral larval trichomes can be found in Table S2 and in the medium gonocoxite in Table S5.

patch across the entirety of the eye for *white* mutants. Our results indicate that *svb* is necessary for the wildtype height of the largest postgonal sheath trichomes but that there is likely a redundant system that induces unicellular trichomes in the postgonal sheath independently of *svb*.

The transcription factor SoxN is known to partially compensate for *svb* loss for larval trichomes in *svb* null lines, leading to the retention of a reduced number of heavily blunted trichomes.³⁷ To test if SoxN may also play a role in the postgonal sheath trichomes of *D. eugracilis*, we performed *in situ* hybridization for SoxN in the developing phallus (48 h APF) of *D. melanogaster* and *D. eugracilis*. We observed strong SoxN expression in the medial region of the *D. eugracilis* postgonal sheath (similar to *svb* expression) and did not find strong expression in the postgonal sheath of *D. melanogaster* (Figure S2).

***svb* is sufficient to induce phallic trichomes and a portion of the larval trichome GRN in the postgonal sheath**

The expression of the *svb* and several of its downstream genes from the larval trichome genetic network in the postgonal sheath of *D. eugracilis* provides strong evidence for the partial co-option of a core trichome network to the novel context of the *D. eugracilis* postgonal sheath. This suggests that the initial deployment of trichomes in this tissue could have been caused by novel ectopic expression of *svb*. To assess the likelihood of this possibility, we expressed the active form of *svb* using the UAS-*ovoB* line,³⁵ in the postgonal sheath of *D. melanogaster* (which naturally lacks postgonal trichomes) driven by the phallic *PoxN*-GAL4 driver (*PoxN*>>*ovoB*).^{13,51} Expressing the active form of *svb* was sufficient to induce small trichomes throughout the adult sheath, transforming the *D. melanogaster* postgonal

sheath to a morphology that partially phenocopies the morphology of *D. eugracilis* (Figure 5A).

We next investigated how the induced postgonal sheath trichomes develop in *D. melanogaster*. Co-staining for actin (phalloidin) and apical cell junctions (ECAD) revealed that the induced projections are indeed unicellular (Figure 5B). Although *PoxN*-GAL4 induces expression on the lateral and medial sides of the postgonal sheath, we only observed trichomes forming on the medial side. Additionally, some cells produced multiple trichomes, which was not observed in the *D. eugracilis* postgonal sheath (Figure 5B).

To determine the composition of the GRN of induced phallic trichomes in *D. melanogaster*, we stained 17 of the downstream members of the larval trichome GRN in these animals. In total, 12 of 17 larval trichome cellular effectors gained expression in the medial postgonal sheath when *svb* was misexpressed (Table S2). We also found that 4 of our 17 genes showed expression in the medial postgonal sheath of control *D. melanogaster*, indicating that a portion of the larval trichome genetic network is activated in this naive context (Figure S10). However, two of the 17 genes (*neyo* and *nyobe*) show decreased expression patterns in the *PoxN*>>*ovoB* background, suggesting that *svb* or other members of this induced network in *D. melanogaster* may repress these genes. Conversely, the other two genes (*cyr*, *Vajk2*) showed weak localized expression in control *D. melanogaster* postgonal sheaths and expanded expression in *PoxN*>>*ovoB* *D. melanogaster*, indicating that *svb* is competent to expand their existing expression. We also found the medium gonocoxite of *D. melanogaster* (which expresses *svb* and displays trichomes) expresses 11 out of 17 of the larval trichome GRN that we analyzed (Figure S10; Table S5). Our findings provide strong evidence that *svb* expression is sufficient to induce a

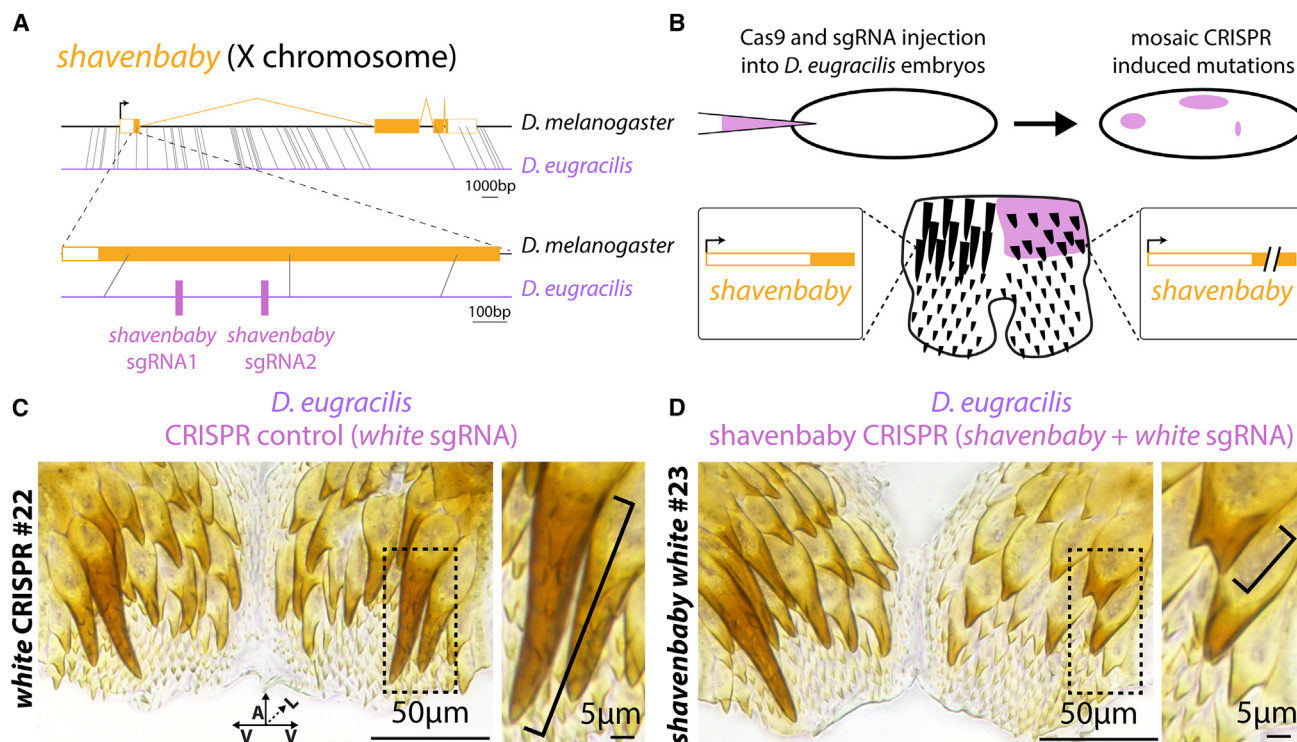


Figure 4. *svb* is necessary for the postgonal sheath major trichome height

(A) Schematic of the gene *shavenbaby* (*svb*). White boxes represent non-coding exons, orange boxes represent coding exons, and lines represent introns. Gray lines represent blocks of homology between *D. melanogaster* (black line) and *D. eugracilis* (purple line), with ≥ 40 bp perfect identity used for the whole gene view (top) and ≥ 30 bp perfect identity used for the zoom-in view (bottom). Target sites for the 2 *svb* sgRNAs (pink bars). Scale bar, 1,000 bp (top) and 100 bp (bottom). (B) Schematic overview of the CRISPR injection procedure. Cas9 and sgRNAs (pink) are injected into <1-h-old embryos, which will induce mutations in a mosaic pattern. Thus, the adult postgonal sheath of injected individuals will have a subset of cells with mutations (slanted lines on the gene schematic). (C) A control postgonal sheath (white sgRNA + Cas9 injection mix). Representative samples of eye phenotypes are in Figure S6. Dashed box represents the area of the zoom-in (right). Brackets represent the measured height of the largest trichome (“major trichome”) on that side. Measurements of major trichome height are found in Table S3. Scale bar, 50 μ m (left) and 5 μ m (right). (D) A representative sample of the postgonal sheath when *shavenbaby* was targeted (white sgRNA + *svb* sgRNA + Cas9 injection mix). Additional postgonal sheath samples are found in Figure S7. Dashed box represents the area of the zoom-in (right). Brackets represent the measured height of the largest trichome on that side. Images of representative postgonal sheath samples using different sgRNAs to target *shavenbaby* are found in Figure S8. Sanger sequencing validation of representative samples is found in Figure S9. Scale bar, 50 μ m (left) and 5 μ m (right).

large portion of the larval trichome GRN in the *D. melanogaster* postgonal sheath.

DISCUSSION

The origins of morphological novelty have long fascinated evolutionary biologists with the conundrum of how a completely new structure might first arise and subsequently adopt its elaborate morphology. Our findings provide evidence that the novel postgonal sheath projections of *D. eugracilis* were generated through the partial co-option and subsequent modification of an ancestral trichome genetic network. The examination of known larval trichome genetic network genes identified a core subset of 14 genes shared between larval and postgonal sheath trichomes, including the key transcription factor-encoding genes *svb* and *SoxN*. Loss-of-function studies demonstrate how *svb* is required for sheath trichome morphology in *D. eugracilis*, consistent with the co-option of this network. Moreover, the induction of small trichomes by *svb* misexpression in *D. melanogaster* illustrates how these trichomes may have first appeared in the

D. eugracilis lineage and highlight the extreme modifications that some of these trichomes underwent as the structures became more specialized. The rewiring of this network that led to this specialized size and shape best encapsulates what makes these trichomes novel rather than a mere repetition of trichome morphologies seen in other parts of the body (Figure 6).

The *D. eugracilis* postgonal sheath trichomes appear to be a novelty as they do not appear in other members of the *melanogaster*, *suzukii*, *ananassae*, and *pseudobscura* subgroups we have previously investigated via microdissection of the adult genitalia.²⁴ Bock and Wheeler previously characterized the overall genital morphology of the *melanogaster* species groups.²³ In their analysis, the phallus of *D. elegans* (*suzukii* subgroup), and *D. ficusphila* (*ficusphila* species group), and *D. orosa* (*montium* species group) have what appear to be bumps near the area of the postgonal sheath. Further characterization of the adult morphology, development, and *svb* expression patterns of these species will be needed to establish if these bumps are trichomes and if there is a deeper origin of the postgonal sheath trichomes.

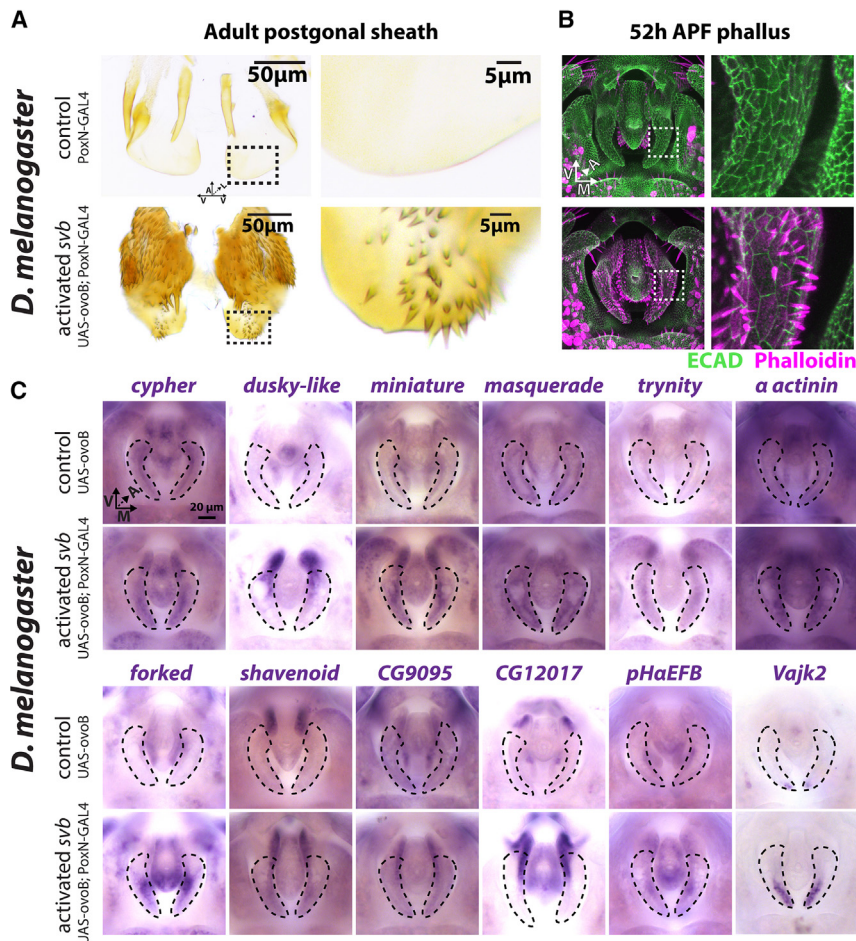


Figure 5. Activated *svb* is sufficient to induce trichomes and a portion of the larval trichome GRN in *D. melanogaster*

(A) Top: the control adult postgonal sheath (UAS-ovoB) has a smooth morphology. Bottom: induction of the activated form of *shavenbaby* (PoxN-GAL4; UAS-ovoB) in the adult postgonal sheath generates small trichomes. Dotted boxes show the closeup view region to the right. Scale bar, 50 μ m (left) and 5 μ m (right).

(B) The developing phallus of the control and *shavenbaby*-induced samples stained with ECAD (green) and phalloidin (magenta). Controls (top) do not produce any unicellular actin outgrowth while *shavenbaby*-induced (PoxN-GAL4; UAS-ovoB) samples gain unicellular actin bundles. There is variation in the induced trichomes, with some cells producing multiple actin projections while others produce one or no actin projections.

(C) *In situ* hybridization for 12 downstream targets of the larval trichome genetic network in control and active *shavenbaby* 48 h APF developing phalluses; see also Figure S10 and Table S1. Expression of these genes in the medium gonocoxite can be found in Figure S10 and Table S5.

The majority of the research on the *svb* genetic network has focused on larval trichomes,^{35,37–39} but trichomes are found across the adult body plan in various shapes and sizes, from the sawtooth-shaped trichomes of the female genitalia to the long, thin trichomes that comprise the arista of the antenna.^{31,35,46} The adult leg trichome genetic network was found to be rewired,¹² with 83% (135/163) of the genes known to be bound by *svb* expressed above background levels in the developing leg transcriptome. This suggests that changes exist in the enhancers and *trans* environments between these two distinct developmental contexts. The medium gonocoxite trichomes of both *D. eugracilis* (Figure S5) and *D. melanogaster* (Figure S10) express only a subset (~50%) of the tested larval trichome GRN genes (Table S5). Even the trichomes of the larvae differ in the expression of downstream targets of *svb*, with 91% and 96% of the genes we analyzed being expressed in the dorsal and ventral trichomes, respectively. The variation in the percentage of the genetic network expressed across trichome type and developmental stages leads us to expect that the co-option of the trichome genetic network will be partial and not wholesale²⁰ in nature even during its initiation.

Indeed, we observe that 71% of the 17 tested larval trichome genes are active when *svb* is ectopically expressed in the *D. melanogaster* postgonal sheath.²⁰ The percentage of larval trichome genes drops to 60% in the postgonal sheath of

D. eugracilis, indicating that the genetic network in the postgonal sheath may have specialized after it first evolved, allowing these trichomes to gain their unique characteristics. Even the trichomes within the *D. eugracilis* postgonal sheath vary in size, with the major trichomes possessing enlarged nuclei that could be established via endoreplication, i.e., localized polyploidy.⁵² Large trichome-like structures have been found in the larvae of botflies and have been suggested as a means to prevent their extraction.^{53,54} This mechanism could explain how a unicellular outgrowth rivals the size of novel multicellular outgrowths like the dorsal and ventral postgonal processes found in *D. melanogaster*.²⁴ That two distinct cellular mechanisms (unicellular and multicellular) are responsible for similarly sized and shaped novelties (Figure 1) provides a clear example of phenotypic convergence. Studying which genes are necessary for different trichome types will allow us to distinguish between the core trichome genetic network and genes that are non-essential but modify cell shape in specific contexts.

In *Drosophila melanogaster* larvae, *svb* mutants lose most of the ventral trichomes and many of the dorsal trichomes. The ventral and dorsal trichomes that remain in *svb* mutants have reduced height,^{33,37} which is similar to what we observe in CRISPR-induced *svb* knockout clones in the postgonal sheath of *D. eugracilis*. This reduction of height is also seen in the trichomes of the wing, leg, and abdomen in *svb* mutants.³⁴ The incomplete loss of larval trichomes is thought to be due to the potential redundancy of the transcription factor *SoxN*, which is sufficient to induce larval trichomes independent of *svb*.³⁷ We found that *SoxN* is expressed in the postgonal sheath of *D. eugracilis*, suggesting that it may be necessary to disrupt both genes to induce a loss of postgonal sheath trichomes.

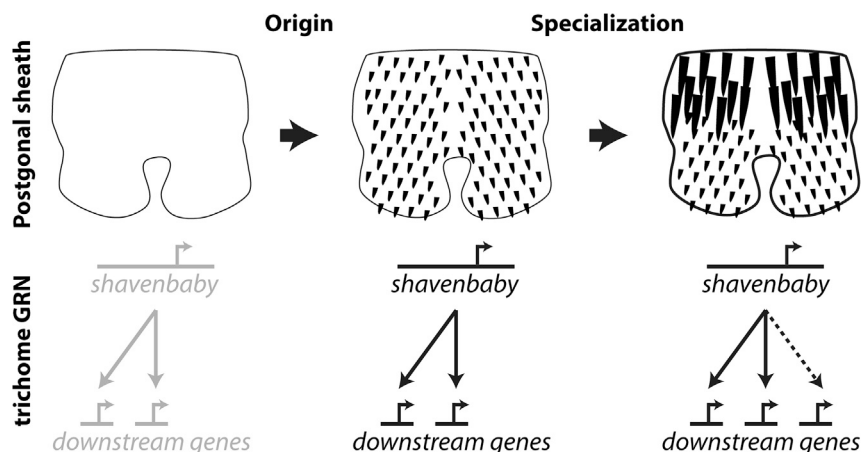


Figure 6. Model of the origin and specialization of the *D. eugracilis* postgonal sheath trichomes

The postgonal sheath of *D. melanogaster* lacks trichomes and the expression of the larval trichome genetic network. The expression of *svb* in *D. melanogaster* (black arrow) induces small postgonal trichomes and much of the larval trichome genetic network. We find that some members of the larval trichome network are expressed in *D. eugracilis*, which naturally houses postgonal trichomes, and not when *svb* is expressed in *D. melanogaster* (dashed line), indicating that the postgonal sheath trichome network may have evolved to incorporate these genes. We predict that specialization (black arrow) allowed *D. eugracilis* to exhibit a unique genetic network leading to a diversity of trichome sizes.

The redundancy of this network begs the question of what genetic changes were sufficient to first establish this novel trichome type.

The theory of the GRN co-option suggests that novel traits can be initiated through the activation of top-tier members of a genetic network in a tissue that did not previously express that genetic network. This would potentially allow hundreds of genes to gain and lose expression in that formerly naive tissue, inducing a large morphological shift. This theory has been implicated in the origin of the beetle horn,¹⁶ echinoderm embryonic skeletons,⁵⁵ treehopper helmets,¹⁷ and many more.⁵⁶ However, to our knowledge, only a single other study has shown that a morphological novelty can be induced through the ectopic expression of an upstream gene.¹⁸ Marcellini and Simpson¹⁸ showed that *Drosophila quadrilineata* possesses an increased number of dorso-central bristles (four pairs) in their thorax compared with *D. melanogaster* (two pairs). The authors further showed that the *D. quadrilineata* enhancer of the proneural *scute* gene was sufficient to induce the two extra pairs of bristles when driving *scute* expression in *D. melanogaster*. The *D. quadrilineata* study shows the ability of a genetic network co-option to induce additional copies of the same trait. On the other hand, the unique size and shape of the *D. eugracilis* postgonal sheath trichomes represents a system where one can test how repeated traits evolve unique features independent of the structure from which it was co-opted.

Although a core GRN may be shared between the co-opted and novel traits, some genes in the bottom tier of the network may become specific to each context and impart specialized characteristics.^{1,20,57,58} This can be seen in novelties such as the tusk of a narwhal. The tusk has been found to be a modified canine tooth, yet this structure is unique in its size and straight spiral shape when compared with other toothed whales, odontocetes, as well as its remaining vestigial teeth.⁵⁹ One could make the case that the tusk is not a true novelty, as it is clearly recognized as a modified tooth. Yet it is the unusual characteristics, not the presence of a clear homology, that pull our focus to these specific structures. If the tusk was simply another tooth with the same repeated morphology in a new position of the body plan, it may not have garnered the same interest or fascination. Future work focusing on

how genetic networks add, remove, and modify genes to evolve a specialized morphology in new contexts of the body plan will provide a much-needed perspective of what makes a trait novel.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark Rebeiz (rebeiz@pitt.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at <https://www.stowers.org/research/publications/libpb-2489> and are publicly available as of the date of publication. Links are listed in the [key resources table](#).
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

The project was conceived by G.R. and M.R. Experiments were carried out by G.R., T.G.-E., and K.C.-O. Data were analyzed by all authors. G.R. and M.R. wrote the paper, assisted by J.Z., T.G.-E., and K.C.-O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
 - *Drosophila* strains
- METHOD DETAILS
 - Sample collection, dissection, and fixation
 - Probe design and synthesis
 - Antibody generation
 - CRISPR
 - Imaging
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal ECAD	DSHB	Cat# DCAD2; RRID: AB_528120
Rabbit monoclonal shavenbaby	GenScript	N/A
Rhodamine Phalloidin	Invitrogen	Cat# R415
Chemicals, peptides, and recombinant proteins		
paraformaldehyde 16% aqueous solution (Methanol free)	Electron Microscopy Sciences	Cat# 15710
Formamide, 500mL	Fisher Scientific	BP227500
Glycerol, 4L	EMD Millipore Corporation	EMD Millipore Corporation
Heptane, 500mL	Fisher Scientific	BP1115-500
Phusion Flash High-Fidelity PCR Master Mix	Fisher Thermo Scientific	Cat# F-548L
salmon sperm DNA sodium salt	Fisher	50-591-966
Dig RNA labeling mix 10X	Dig RNA labeling mix 10X	Cat# 17109821
Halocarbon Oil 27	Sigma-Aldrich	H8773
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	H4784
Tween-20	Fisher Scientific	BP337-500
Triton X-100 electrophoresis grade	Fisher Scientific	BP151-500
RNase inhibitor, Murine	New England Bio Labs	Cat# M0314S
Critical commercial assays		
In-Fusion HD Cloning Plus	Clontech (EV) TAKARA	Cat# 638909
QIAquick Gel Extraction Kit	QIAGEN GmbH	Cat# 28706
DNeasy kit	QIAGEN GmbH	Cat# 69504
T7 RNA Polymerase	Promega / Life Technologies	Cat# P2075
Anti-digoxigenin AP-conjugate	Roche	Cat# 11093274910
BCIP/NBT	Promega	S3771
EnGen sgRNA Synthesis Kit	NEB	Cat # NC1194374
EnGen Spy Cas9 NLS	NEB	Catalog # M0646M
MEGACLEAR Transcription Clean-Up KIT	Invitrogen	Cat # AM1908
Deposited data		
images used for this analysis	Stowers Original Data Repository	Stowers Original Data Repository: http://www.stowers.org/research/publications/libpb-2489
Experimental models: Organisms/strains		
yellow white: yw;+;+	Bloomington Stock Center	#1495
<i>D. eugracilis</i>	National Drosophila Species Stock Center at Cornell	14026-0451.02
<i>D. biarmipes</i>	National Drosophila Species Stock Center at Cornell	14023-0361.09
<i>D. ananassae</i>	National Drosophila Species Stock Center at Cornell	14024-0371.13
<i>D. pseudoobscura</i>	National Drosophila Species Stock Center at Cornell	14011-0121.87
UAS-ovoB	Bloomington Stock Center	#38430
PoxN-Gal4 #13	N/A	N/A
Oligonucleotides		
Primers see Table S4	This paper	NA
Software and algorithms		
InsituPro VIS liquid handling system (insitu robot)	Intavis Bioanalytical Instruments	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drosophila strains

Stocks were obtained from both the National Drosophila Species Stock Center at Cornell (*D. eugracilis* 14026-0451.02), *D. biarmipes* (14023-0361.09), *D. ananassae* (14024-0371.13), *D. pseudoobscura* (14011-0121.87) the Bloomington Drosophila Stock Center: *D. melanogaster yellow white* (y^1w^1 , Bloomington Stock Center #1495), UAS-ovoB on the chromosome II (Bloomington Stock Center #38430). Additionally, PoxN-Gal4 (Poxn-Gal4 construct #13 from Boll and Noll⁵¹) was a gift from the Noll lab.

METHOD DETAILS

Sample collection, dissection, and fixation

Male white pre-pupae were collected at room temperature and incubated in a petri dish containing a moistened Kimwipe at 25°C before dissection. After incubation, pupae were impaled in their anterior region and immobilized with forceps and placed in a glass dissecting well containing phosphate-buffered saline (PBS). The posterior tip of the pupa (20%–40% of pupal length) was separated and washed with a P200 pipette to flush the pupal terminalia into solution. Samples were then collected in PBS with 0.1% Triton-X-100 (PBT) and 4% paraformaldehyde (PFA, E.M.S. Scientific) on ice, and multiple samples were collected in the same tube. Samples were then fixed in PBT + 4% PFA at room temperature for 30 min, washed three times in PBT at room temperature, and stored at 4°C. At least 4 samples were imaged for each *in situ* hybridization probe and are available on Stowers Original Data Repository: <http://www.stowers.org/research/publications/libpb-2489>.

Probe design and synthesis

Templates for 200–300 base pair RNA probes were designed from a large exon present in all annotated isoforms of each examined gene. Exons were chosen by retrieving the decorated FASTA from flybase.org,³⁴ and annotated isoforms were examined using the UCSC genome browser.⁶⁰ After exon selection, Primer3Plus⁶¹ was used to design PCR primers that would amplify a 200–300 base pair region, and 5–10 candidate primer pairs were screened using the UCSC In Silico PCR tool to identify sets that would amplify the region of interest from both *D. melanogaster* and *D. eugracilis*. This screening process was implemented to maximize the utility of any particular primer set for other species. Reverse primers were designed beginning with a T7 RNA polymerase binding sequence (TAATACGACTCACTATAG), and template DNA was PCR amplified from adult fly genomic DNA extracted using the DNeasy kit (–QIAGEN). Digoxigenin-labeled probes were then synthesized using *in vitro* transcription (T7 RNA Polymerase, Promega / Life Technologies), ethanol precipitated, and resuspended in water for Nanodrop analysis. Probes were stored at –20°C in 50% formamide prior to *in situ* hybridization.

Antibody generation

The rabbit antibody for Shavenbaby (Svb) was produced by GenScript against the following amino acid sequence:

MHHHHHGGQSSMMGHFPYGGNPSAYGIILKDEPDIEYDEAKIDIGTFAQNIQATMGSSGQFNASAYEDAIMSDLASSGQCPNGAVDPLQFTATLMLSSQTDHLLQLSDAVIDLSSFLQRSCVDDEESTSPRQDFELVSTPSLTPDSVTPVEQHNANTSQLDALHENLLTQLTHNMARNSSNQQQHHHQHNV.

DNA sequence

CATATGCATCACCACCACCACCACCGAGGGCAATCAAGTATGATGGGACACCCGTTCTACGGTGGCAACCCGAGCGCGTATGGCATCATCTCGAAGGACGAGCCGGATATCGAGTACGACGAAGCGAAAATCGATATTGGTACCTTCGCGCAGAACATCATTCAAGCGACCATGGGTAGCAGCGGCCAATTTAACGCGAGCGCGTATGAAGACGCGATTATGAGCGATCTGGCGAGCAGCGGCCAGTGCCCGAACGGTGCGGTGACCCGCTGCAATTTACCGCGACCCCTGATGCTGAGCAGCCAGACCGATCACCTGCTGGAGCAACTGAGCGACGCGGTGGATCTGAGCAGCTTCCTGCAGCGTAGCTGCGTTGACGATGAGGAAAGCACCAGCCCGCTCAAGACTTTGAACTGGTTAGCACCCCGAGCCTGACCCGGATAGCGTGACCCCGGTTGAGCAGCACAAACGCGAACACCAGCCAACTGGACGCGCTGCACGAAAACCTGCTGACCCAGCTGACCCACAACATGGCGCGTAACAGCAGCAACCAGCAACAGCAACACCAGCAACACAACGTGTAATGAAGCTT

Bold indicates the start codon and His tag.

Italicized indicates the stop codon.

This sequence was cloned into the pET-30a (+) with His tag vector and transformed into *E. coli* strain BL21 Star™ (DE3). Transformants were induced with IPTG. The induced protein was purified via Ni column.

CRISPR

To induce mosaic knockouts⁵⁰ of *white* and *shavenbaby*, we targeted the first or second coding exon and ran the *D. eugracilis* and *D. melanogaster* sequences through the CRISPR Optimal Target Finder (<http://targetfinder.flycrispr.neuro.brown.edu/>)⁶² to avoid off-target effects. We generated our sgRNAs following the protocol outlined in Méndez-González et al.⁶³ 20 bp target-specific primers included the T7 promoter sequence (upstream) in the 5' end and overlapped with the sgRNA scaffold (see Table S4). Each target-specific primer was added with three primers for an overlap extension PCR generating a 130 bp DNA template. The PCR reaction was then used as a template for *in vitro* transcription using EnGen sgRNA Synthesis Kit (NEB), and the MEGACLEAR Transcription

Clean-Up KIT (Invitrogen) was used to purify the sgRNA product. CRISPR-Cas9 injections were performed in-house following standard protocols (<http://gompel.org/methods>). We used an injection mix containing two (*white* control) or four (*white shavenbaby* experimental) sgRNAs targeting the first or second exon (100 ng/ μ l) and CAS9 protein (EnGen Spy Cas9 NLS from NEB, Catalog # M0646M). PCR amplification and sanger sequencing of the regions around the predicted sgRNA targets for 2 individuals were used to confirm that our injections induced the desired mutations. Individuals *shavenbaby white* #36 and #51, which were injected with *shavenbaby* sgRNAs 1,2 and *white* sgRNAs 3,4 (see Figure S9), both show sequence variation around the predicted cut sites in DNA extracted from whole bodies minus the genitalia.

Imaging

After fixation, the developing pupal genitalia of *D. melanogaster* and *D. eugracilis* were stained with rat anti-E-cadherin, 1:100 in PBT (DSHB Cat# DCAD2, RRID:AB_528120) overnight at 4°C, followed by an overnight at 4°C incubation with anti-rat 488, 1:200 (Invitrogen) to visualize apical cell junctions while, Rho-phalloidin (Invitrogen, Catalog # R415), 1:100, was used to visualize actin, *shavenbaby* 1:10 with anti-rabbit 488, 1:200 (Invitrogen) in the genitalia and 1:50 with anti-rabbit 568, 1:500 (Invitrogen) for the embryo.

Fluorescently labeled samples were mounted in glycerol mounting solution (80% glycerol, 0.1 M Tris, pH 8.0) on microscope slides coated with poly-L-lysine (Thermo Fisher Scientific #86010). Samples were imaged at $\times 40$ on a Leica TCS SP8 confocal microscope. We used the MorphoGraphX software package⁶⁴ to render images in three dimensions. This allowed us to rotate the samples to better present the most informative perspectives of the various phallic structures.

We used an InsituPro VSi robot to perform *in situ* hybridization following the protocol of Vincent et al.⁶⁵ Briefly, dissected terminalia were rehydrated in PBT, fixed in PBT with 4% PFA, and prehybridized in hybridization buffer for 1 hr at 60°C. Samples were then incubated with probes for 16h at 60°C before being washed with hybridization buffer followed by PBT. Samples were incubated in PBT block (1% bovine serum albumin) for 2 hr. Samples were then incubated with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) diluted 1:6000 in PBT+BSA. After additional washes, color reactions were performed by incubating samples with NBT and BCIP (Promega) until a purple stain could be detected under a dissecting microscope. Samples were mounted in glycerol on microscope slides coated with poly-L-lysine and imaged at 20X or 40X magnification on a Leica DM 2000 with a Leica DFC450C camera using the ImageBuilder module of the Leica Application Suite.

For light microscopy of adult phallic microdissections, samples were mounted in PVA Mounting Medium (BioQuip) until fully cleared or with glycerol mount. They were then imaged at $\times 20$ magnification on a Leica DM 2000 with a Leica DFC450C camera using the ImageBuilder module of the Leica Application Suite.

QUANTIFICATION AND STATISTICAL ANALYSIS

The distribution of the major trichome height for the *white* control was normal (Shapiro-Wilk normality test $W = 0.9637$, p -value = 0.1788), allowing us to infer outlier major trichome height in the *white shavenbaby* experimental data. We used -3SD below control white major trichome height as our significance cutoff, as values below this number should occur at a rate of 0.15% with a normal distribution. For our 54 samples of *white shavenbaby* experimental data, we would expect to see 0.081 data points below this value if the CRISPR sgRNAs do not affect the major trichome height.