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Proving an old prediction: The sex comb rotates at 16 to 24 hours after pupariation.

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The sex comb is a row of thick, dark bristles (resembling a hair comb) on the forelegs of adult *Drosophila melanogaster* males. Forty-two years ago, the renowned geneticist Chiyoko Tokunaga reported indirect evidence that the sex comb must rotate at some time during development (Tokunaga, 1962). We have now directly determined when this rotation occurs.

We used a *scabrous-Gal4* driver to express green fluorescent protein (GFP) in nascent bristle cells (a.k.a. sensory organ precursors or SOPs) via *UAS-GFP*. Virgin *y w; sca-Gal4/CyO* females were crossed with males carrying an α-tubulin *UAS-GFP* construct (Bloomington Stock Center #7374). F_1 white prepupae were collected and humidified at 25˚C until they reached the desired age. We tried other UAS constructs (nuclear, cytoplasmic, and cell-surface GFP), but none was as effective in delineating SOPs as the α-tubulin marker.

The opaque pupal case precludes observation of the leg epidermis. For pupae older than 12 hAP (hours after pupariation) the case can be entirely removed without any bleeding since by this stage a transparent pupal cuticle has been secreted that covers the fragile epidermis (Poodry, 1980). Wild-type pupae treated thusly survive to eclosion (Held, 1992). To our surprise, however, >95% of the *sca-Gal4; UAS-GFP* pupae died within hours after removal of the case. Death was not due to the insertion site of the #7374 P-element because it also occurred with #1521, nor was it due to fragility since the cuticle remained intact, nor was it rescuable by (1) asepsis, (2) fungicides, or (3) application of a thin coat of oil. Its cause remains unknown.

This lethality prevented us from monitoring cell movements in individual living pupae with time-lapse microscopy. Instead, we pieced together the sequence of events using cohorts of pupae aged for varying lengths of time after collection as white prepupae—namely 16, 17, 18, 19, 21, 22, 24, 26, and 28 hAP. We enriched the frequency of males to 90% by selecting the thinnest 20% of pupae. (Male pupae tend to be thinner than females, though distributions overlap.) Half the F_1 expressed GFP (those inheriting *sca-Gal4*); half did not (those with *CyO*).

Pupae were dissected by the method of Held (1992), placed ventral-side down, and aligned side-by-side (8 per row) in petri dishes whose bottom was replaced by a glass coverslip. Each pupa was rolled ~30˚ so that the inner (anteroventral) surface of its right foreleg could be seen using an inverted (Olympus IX70 epifluorescence) microscope. Forelegs of fluorescent pupae were examined (within a half hour of dissection) at 250× and 500× magnification. A film of water intervened between the coverslip and the pupae, and dishes were kept humidified. We also tried using immersion oil instead of water at the interface, but the resolution was poorer.

Each leg had to be photographed at both magnifications because the higher magnification afforded better acuity, but its field was too narrow to discern the orientation of the tarsus, which was essential to assess the angle of the sex comb. For each leg, we rotated the low-power picture in Adobe Photoshop™ so that the tarsus was vertical and then rotated the high-power picture to the same extent. Angular assessments were aided by using the "central bristle" (Hannah-Alava, 1958) as

a reference point. On the forelegs of adult males, the central bristle is located in the middle of a field of trichomes (hence its name). Tokunaga concluded that this SOP must initially be part of the sex comb. The deviations of the marked cell clones in genetic mosaics convinced her that the sex comb

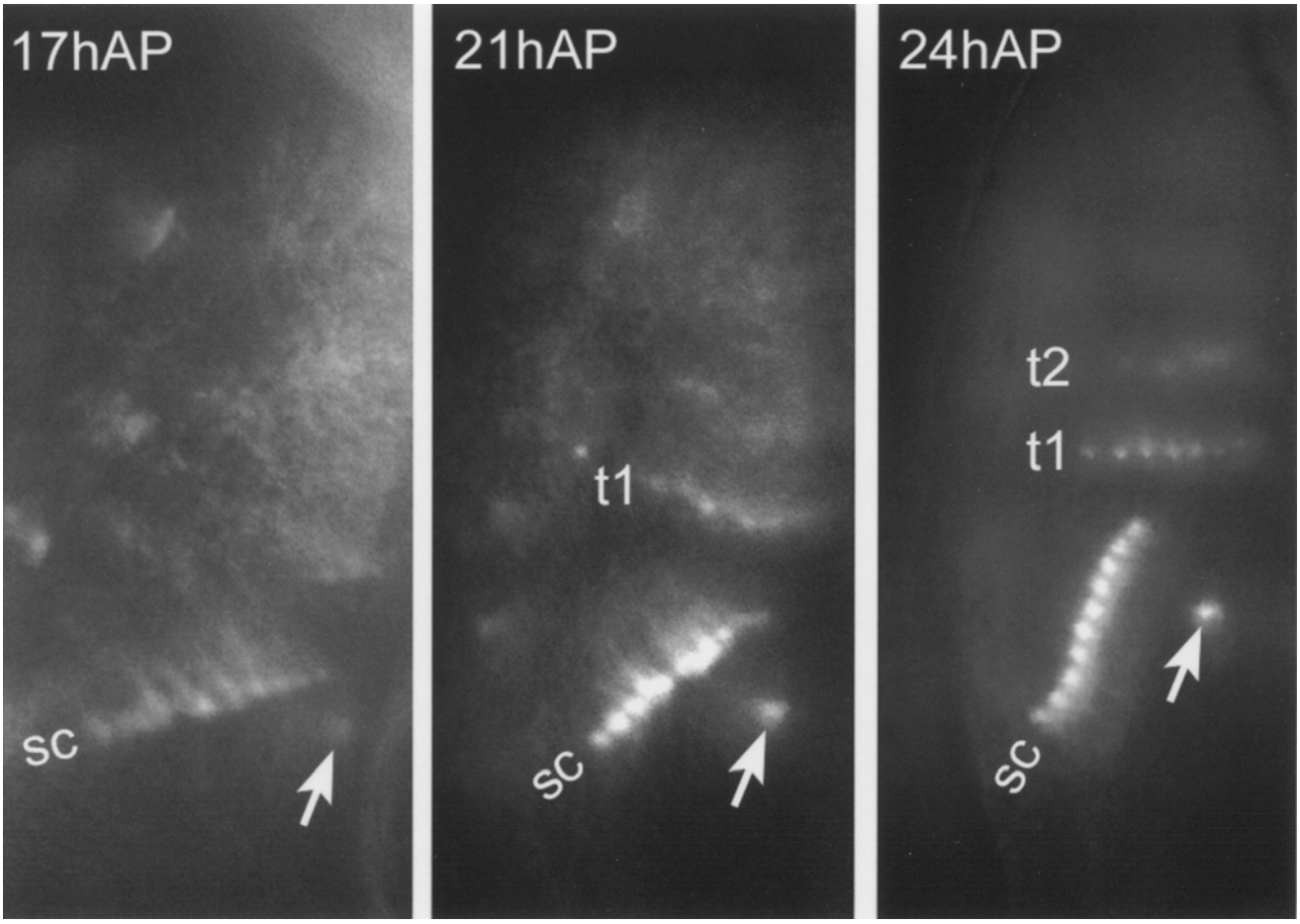

Figure 1. Anteroventral surfaces of right foreleg basitarsi of *sca-Gal4; UAS-GFP* male pupae at 17, 21, and 24 hAP. Basitarsi are oriented with proximal at the top and distal at the bottom, dorsal to the left and ventral to the right. White spots are SOPs. During this period the sex comb (*sc*) appears to pivot from a transverse (~3 o'clock) to a longitudinal (~1 o'clock) orientation. All pictures are at the same magnification. (The mature sex comb is ~60 microns long.) The arrow in each panel points to the SOP that will become the central bristle. The GFP signal intensifies in a distal-to-proximal pattern—with the sex comb shining before the next most distal transverse row (*t1*), and that row shining in turn before its neighbor (*t2*). SOPs tend to be in straighter rows on older legs, and sporadic gaps (*e.g.*, between the 4th & 5th SOP in the 17 hAP pupa and between the 5th & 6th SOP in the 21 hAP pupa) become less frequent. The S-shape of the sex comb in the 24 hAP specimen is common but not universal since other pupae at the same age have their SOPs in a straight line. The number of sex comb SOPs in these legs appears to be 10 (17 hAP pupa), 9 (21 hAP pupa), and 11 (24 hAP pupa). In a control sample of thin pupae that were allowed to complete development, the number of sex comb bristles averaged 10.0, with a range from 8 to 12 bristles (N = 60 adult forelegs). The number of sex comb SOPs is known to become fixed at 14 hAP (Belote and Baker, 1982) when normalized to the 25˚C rate.

must arise as a transverse row that then pivots to become longitudinal, leaving behind its ventralmost SOP, which becomes the central bristle (Tokunaga, 1962).

Consistent with Tokunaga's predictions, we found that the sex comb does indeed arise as a transverse row. This row has clearly pivoted away from the central bristle SOP by 17 hAP (Figure 1), and some rotation is evident in pupae as young as 16 hAP. (Males cannot be discerned from females in the tarsus unless the sex comb has separated from the central bristle SOP.) Attempts to examine pupae before 16 hAP were hampered by faintness of the GFP marker, which only begins to be expressed (via *scabrous*) in sex comb SOPs around this time.

In older cohorts the sex comb has a steeper angle and greater distance from the central bristle SOP, reaching its final (~longitudinal) orientation by ~24 hAP. By this stage, the GFP marker is not only apparent in the sex comb but also in two distal rows of SOPs that remain transverse ("t1" and "t2" in Figure 1). This distal-to-proximal sequence of differentiation has been documented before (Graves and Schubiger, 1981; Held and Bryant, 1984; Held, 1990). Interestingly, the central bristle SOP itself evidently migrates proximally as the comb turns.

Remaining mysteries concern the cellular basis of the movements (Held, 2002). To what extent do mitoses behind the row push it forward? How do cells interchange partners? Do any junctions remain intact as they do so? How do sex comb SOPs stay aligned as the row pivots?

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CpC methylation is present in *Drosophila melanogaster* and undergoes changes during its life cycle.

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Summary

DNA methylation plays an important role in regulation of gene expression in eukaryotes. 5-methyl-cytosine (5mC) is the most commonly found methylated base in the genome of eukaryotes. Due to the low level of 5mC in *Drosophila melanogaster*, the presence of 5mC in the *Drosophila* genome has been controversial. However, there have been conclusive reports confirming presence of 5mC in *Drosophila melanogaster*. Our results show that 5mC is present in the genome and that its pattern varies during different stages in the life cycle.