Disabling *Cdc42* disrupts bristle patterning.

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Cell rearrangements are known to be involved in sex-comb rotation (Atallah *et al.*, 2009), and they have been implicated in (1) aligning bristles in transverse rows (t-rows) on the 1st-leg tibia (Held, 2002) and (2) ensuring uniform spaces between bristles in longitudinal rows (l-rows) on the 2nd-leg basitarsus (Held *et al.*, 1986). We investigated the role of cell movements in leg development by studying the effects of a dominant-negative allele of *Cdc42*.

Cdc42 encodes a Ras-related GTPase in the Rho subfamily. Other members of this group include *Rac* and *Rho* (Machacek *et al.*, 2009). Genes in the Rho subfamily have been shown to regulate cell polarity, cell shape, and cell movements by modifying the actin cytoskeleton in various ways that are characteristic of the specific genes (Etienne-Manneville and Hall, 2002).

Consistent with the presumption that *Cdc42* mediates cell movements and the hypothesis that cell movements drive sex-comb rotation, t-row fine-tuning, and l-row bristle spacing, we did indeed find disruptions in sex-comb orientation, t-row alignment, and l-row bristle intervals, depending upon the time when we inactivated the *Cdc42* gene.

Materials and Methods

Flies were raised on Ward's *Drosophila* Instant Medium augmented with Fleischmann's live baker's yeast. Culture vials were monitored to prevent larval overcrowding, which can cause developmental delays. Adults were preserved in 70% ethanol. Legs were mounted in Faure's fluid (Lee and Gerhart, 1973) between cover glasses and examined at 400× magnification in a Nikon compound microscope.

One limitation of this mounting technique is that the rigor mortis of the legs constrains their orientation when sandwiched between cover slips. Roughly half of the mounted forelegs have their sex comb suitably positioned (facing up or down versus sideways) so as to permit an assessment of whether the comb has an abnormal "S" shape (see Results). Our minimum sample size was 6 legs per time point (1st-leg pairs of 3 flies), but we examined more flies when needed. Mutant individuals in the earliest cohorts died before eclosion, so those that developed to the pharate adult stage had to be removed from the pupal case before mounting their legs.

Cdc42 was artificially switched OFF at different times by a standard method that involves the transgenic (yeast-derived) components *Gal80ts*, *Gal4*, and *UAS* (Leung and Waddell, 2004), plus incubators at different temperatures (McGuire *et al.*, 2003). The temperature-sensitive (*ts*) mutation *Gal80ts* prevents Gal80 from inhibiting the transcription factor Gal4 at the restrictive temperature of 29°C (Elsaesser *et al.*, 2010) but not at the permissive temperature of 18°C (McGuire *et al.*, 2004). When Gal4 is active, it binds (in *trans*) to an "upstream activating sequence" (*UAS*) and stimulates transcription of any gene attached (in *cis*) to the *UAS* element.

Experimental individuals were obtained as *Dll-Gal4/UAS-Cdc42^{N17}; tub-Gal80ts/+* F_1 progeny of a cross between *w$^+$*; *UAS-Cdc42^{N17}* males and *Dll-Gal4/CyO; tub-Gal80ts* virgin females. Eggs were harvested in one-day laying periods at 22°C (to circumvent paternal sterility at 29°C). In these offspring the expression of the *Gal4* gene is restricted (by its enhancer-trap locus) to the distal tibia and tarsus where its host gene *Distal-less* (*Dll*) is expressed (Wu and Cohen, 1999). *N17* is a dominant-negative allele of *Cdc42* (Luo *et al.*, 1994), which can suppress all *Cdc42* function even though it is only present in heterozygous condition.

At 18°C, *Cdc42* should function normally because *Gal80ts* blocks *Gal4* from activating *UAS* so that there is no transcription of the dominant-negative *Cdc42^{N17}* allele. At 29°C, *Cdc42* should be disabled because *Gal80ts* cannot prevent *Gal4* from activating *UAS* and allowing transcription of *Cdc42^{N17}*. When *Dll-Gal4/UAS-Cdc42^{N17}; tub-Gal80ts/+* larvae or pupae are shifted from 18°C to 29°C, therefore, their *Cdc42* gene

should stop functioning. However, there could be an appreciable time lag until the residual Cdc42 GTPase activity dwindles to a null state—the phenomenon of "perdurance" (Garcia-Bellido and Merriam, 1971; Szabad, 1998).

In order to establish an independent control for assessing delays or other epiphenomena, we conducted a parallel series of temperature shifts using the *Hox* gene *Scr*, whose temporal profile of sensitive periods is known from previous pulse experiments (Held, 2010). For that purpose, we shifted *Dll-Gal4/+*; *UAS-Scr-dsRNAi/tub-Gal80ts* offspring (from a cross of y^1 sc^* v^1; *UAS-Scr-dsRNAi* males with *Dll-Gal4/CyO; tub-Gal80ts* virgin females) from 18°C to 29°C at different times. *Scr* functions at 18°C (*Gal80ts* blocks *Gal4* from activating *UAS*, thus aborting the *Scr RNAi*), whereas *Scr* is blocked at 29°C (*Gal80ts* allows *Gal4* to activate *UAS*, thus expressing *Scr RNAi*)—hence causing 1st legs to look like 2nd legs, which lack sex combs and t-rows.

We also tested whether the loss-of-function effects of *Rac1* and *Rho1* match those of *Cdc42*. For that purpose we performed crosses like those described above, using *UAS-*$Rac1^{N17}$ (on Chromosome 3) or *UAS-*$Rho1^{N19}$ (on Chromosome 1), instead of *UAS-*Cdc^{N17}. We found that these dominant-negative mutations had stronger effects than Cdc^{N17}: $Rac1^{N17}$ individuals that are shifted at or before puparium formation had necrotic, unevenly narrowed legs with clumped, disoriented bristles, while $Rho1^{N19}$ individuals died as pre-pharate pupae, with a few (N = 2) developing to a late enough stage for us to assess the size of their (miniscule) wings. Bristle clumping has been reported for $Cdc42^{N17}$, and tiny wings have been seen with both $Cdc42^{N17}$ and $Cdc42^{L89}$ (Baron *et al.*, 2000). No further data on *Rac1* or *Rho1* are presented here.

Elapsed times at 18°C were converted to equivalent times at 25°C, which is the standard temperature for staging *Drosophila* (Ashburner, 1989), by dividing these numbers by 2.0, which is the ratio of 18°C/25°C rates (Held, 1990). Normalized times computed in this way are reported as ages "@ 25°C." Other abbreviations: h (hours), PF (puparium formation), APF (after PF, with minus signs denoting times before PF), BPF (before PF), ta1-ta5 (tarsal segments 1-5), WPP (white prepupae), and pers. comm. (personal communication).

The WPP stage (0 h APF) marks the start of the pupal period and lasts ~1 h @ 25°C, after which the pupal case quickly turns brown, so it is useful for the precise staging of cohorts. For shifts APF, WPP were collected from 18°C culture bottles and put in moistened test tubes. These tubes were then either placed in a covered 29°C water bath immediately (for a shift time of 0 h APF) or returned to the 18°C incubator for varying durations before transfer to the 29°C bath at a later time. Hence, the ages in our APF cohorts (0, 12, or 24 h APF @ 25°C) varied by $^+$/- 0.5 h @ 25°C.

For shifts before PF, culture bottles were transferred from 18°C to a covered 29°C water bath (for faster equilibration than afforded by air) for ~3 h and then placed on a dry shelf at 29°C. (Leaving bottles at 100% humidity causes delays because larvae seek a dry surface on which to pupariate and will wander for hours if no suitable location is available.) Batches of pupae (WPP and older stages) were then collected from these bottles at either 2-h or 12-h intervals. Hence, the ages in our BPF cohorts (0 to -2, -2 to -4, -4 to -6, -6 to -8, -8 to -10, -10 to -12, -12 to -24, and -24 to -36 h APF @ 25°C) varied by either $^+$/- 1 h or $^+$/- 6 h at 29°C, which is roughly equivalent to developmental times at the standard temperature of 25°C (Held, 2010).

Results and Discussion

When *Dll-Gal4/UAS-*$Cdc42^{N17}$*; tub-Gal80ts/+* flies were raised at 18°C (the "*Cdc42*-ON" state), they eclosed and looked wild-type. However, no such individuals survived to the pharate adult stage when they were raised at 29°C (the "*Cdc42*-OFF" state), following one-day egg-laying periods that were conducted at 22°C (instead of 29°C) in order to circumvent paternal sterility; only their curly-winged (*Dll-Gal4/CyO; tub-Gal80ts/+*) siblings eclosed. This mortality might be due to a subset of *Dll* enhancers that are driving *Gal4* (and hence $Cdc42^{N17}$) in vital organs (of unknown identity) in addition to the legs (Galindo *et al.*, 2011) because null *Dll* mutants die as embryos (Cohen and Jürgens, 1989).

When *Dll-Gal4/UAS-*$Cdc42^{N17}$*; tub-Gal80ts/+* males were shifted from 18°C to 29°C (switching *Cdc42* from ON to OFF) at 12 h APF @ 25°C or earlier, we were surprised to find that they typically displayed S-shaped sex combs (Figure 1, upper panel). This shape bears a striking resemblance to a normal

phase of sex-comb rotation. In wild-type *D. melanogaster* the sex comb begins as an ordinary (horizontal) t-row. It starts rotating toward a longitudinal (vertical) orientation at 16 h APF (Held *et al.*, 2004). By 23 h APF, the comb's midsection has completed about half of its 90° turn, but the termini lag behind, thus giving the comb a sinusoidal shape overall (Atallah *et al.*, 2009). This "S" phase lasts until ~28 h APF, when the midsection has attained an angle of ~65°. Evidently, *Cdc42* function is needed in order for sex combs to proceed beyond this "S" phase.

Figure 1. Effects of disabling *Cdc42* (upper panel) or *Scr* (lower panel) on the male foreleg basitarsus. Numbers along the top denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C. Disabling *Cdc42* at any time from -36 to +12 h APF @ 25°C causes sinusoidally shaped ("S") sex combs. Disabling *Scr* at any time from -4 to +12 h APF @ 25°C causes the top end of the comb to curve ventrally like an inverted "J" (≈ upper half of an "S"), while shifts between -12 and -4 h APF reduce comb size and inhibit comb rotation, and shifts between -36 and -12 h APF eliminate the sex comb and all t-rows (basitarsal and tibial) as part of a homeotic transformation of the 1st leg into a 2nd-leg identity (Held, 2010). Basitarsal length stays constant in the *Scr* series, but it decreases by ~50% in the *Cdc42* series for shifts before -8 h APF. Another distinctive (diagnostic) feature of the *Cdc42* series was necrotic (melanotic) tissue at various leg joints (*e.g.*, the tibia-ta1 joint in the "-2 to -4" leg). The intersegmental membranes at such sites were often puffed out like balloons as well (omitted here because they obscure bristle clarity). These effects on joints might be due to a combination of (1) *Cdc42*'s regulation of

(Figure 1, continued). *Rho1* (Machacek *et al*., 2009) and (2) the roles of *RhoGEFs* and *RhoGAPs* in joint development (Greenberg and Hatini, 2011). See Materials and Methods for procedures and genotypes. In all photos the anterior face of the segment is shown, with proximal at the top, distal at the bottom, dorsal to the left, and ventral to the right. All photos are at the same magnification. Scale bar (lower left) = 100 microns. A minimum of six legs was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted for the sake of conciseness. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs. Combs that are shaped like an inverted-J or "cane" have been described for a variety of other mutants and artificially selected lines, and readers should consult Malagón *et al*. (2014) for incisive mechanistic explanations.

Figure 2. Effects of disabling *Cdc42* on the foreleg tibia. Numbers along the white banner denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C.

(Figure 2, continued) The distal half of the foreleg tibia in wild-type flies has ~6 transverse ("t-") rows, numbered 1 to 6 from distal to proximal, in a triangular area. These rows are used as brushes to clean the eyes (Szebenyi, 1969). When *Cdc42* is disabled at 12 h APF, t-rows 3-6 are slightly disorganized. Shifts at 0 or -2 to -4 h APF disrupt t-row 2 as well, and earlier shifts disrupt the entire t-row area. In the youngest cohorts (-12 to -24 and -24 to -36 h APF) bristle density declines severely on the tibia—a phenotype previously reported for the wing, where the sparseness stems from an inhibitory effect of *Cdc42* on the *Notch* pathway that regulates bristle spacing (Baron *et al*., 2000). This link could explain why we occasionally saw duplicated bristle shafts and missing sockets, since the *Notch* pathway also mediates bristle differentiation (Held, 2002). Disorderly t-rows have also been reported for loss-of-function mutations in *Scr* (Held, 2010) and *Egfr* (Held, 2002), and in both cases the sensitive period is after PF. See Materials and Methods for procedures and genotypes. In all photos the anterior face of the tibia is shown, with dorsal to the left, and ventral to the right. All photos are at the same magnification. Scale bar (upper left) = 100 microns. A minimum of six legs from male flies was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted to reduce clutter. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs.

Figure 3. Effects of disabling *Cdc42* on the midleg basitarsus. Numbers along the top denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C. See Materials and Methods for procedures and genotypes. In all photos the anterior face of the basitarsus is shown, with proximal at the top, distal at the bottom, dorsal to the left, and ventral to the right. The row along the right edge of each basitarsus is row 8 (Hannah-Alava, 1958). The bristles of this row have the most orderly spacing of any longitudinal row on wild-type legs (Held, 1979). With successively earlier shifts before PF, the spacing regularity declines, reaching its most chaotic state in the -24 to -36 h APF cohort. Note, however, that such basitarsi are about half the length of control segments, and growth reduction alone—as witnessed for *Cdc42-null* alleles in wings and eyes (Baron *et al*., 2000; Genova *et al*., 2000)—can disrupt bristle spacing (Held, 1990). Likewise, the bristles on these legs are misaligned and are often missing sockets and bracts, so it is hard to determine which effects are direct consequences of *Cdc42* dysfunction (filopodia-associated) and which are coincidental side-effects (not filopodia-associated). All photos are at the same magnification. Scale bar (lower left) = 100 microns. See text for further discussion. A minimum of six legs

(Figure 3, continued). from male flies was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted to reduce clutter. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs.

Based on Cdc42's role in actin dynamics (Kozma *et al.*, 1995; Nobes and Hall, 1995; Tapon and Hall, 1997; Genova *et al.*, 2000), we propose that Cdc42 is enabling bristle cells to form filopodia, and that these filopodia are needed to drive the sex comb past its "S" phase of rotation, possibly via intercellular signaling (Fairchild and Barna, 2014). Filopodia have been seen on *Drosophila* bristle cells, where they mediate lateral inhibition (de Joussineau *et al.*, 2003). Filopodia are also thought to help align scale cells in moths (Nardi and Magee-Adams, 1986) and pigment cells in zebrafish (Mahalwar *et al.*, 2014), so they might be playing a fine-tuning role here, too. However, the sex comb's bristle cells do not appear to require Cdc42 for alignment *per se* (*i.e.*, chain formation), since most S-shaped combs had single files of bristles. We did see gaps, clumps, and displaced bristles, but they were rare except in younger cohorts.

The *Scr* series also yielded contiguous sex combs, but the comb shapes were different. When *Dll-Gal4/+*; *UAS-Scr-dsRNAi/tub-Gal80ts* males were shifted from 18°C to 29°C (switching *Scr* from ON to OFF) between -4 and 12 h APF @ 25°C, the proximal ends of their sex combs typically curved ventrally. In Figure 1 (lower panel) we denote this shape by an inverted "J" because it resembles the top half of the "S" shape in the *Cdc42* series (Figure 1, upper panel). A similar J-shaped bend occurs when *Scr* is turned OFF by pulses (instead of shifts) between 0 and 18 h APF @ 25°C (Held, 2010). (*N.B.:* The bar labeled "bent" in Figure 2b of that paper was plotted incorrectly; it should range from 6 to 12 h APF.) This "J" distortion disappears with shifts before shifts at -4 h APF, presumably because sex comb size and rotation diminish with successively earlier shifts, as the 1st leg adopts a 2nd-leg identity due to the loss of *Scr* function.

Evidently, *Cdc42* and *Scr* are both permissive agents for rotation, but their roles differ. Both genes appear to enable the proximal end of the comb to overcome a "bottleneck" for cell movements that occurs during the "S" phase (Atallah *et al.*, 2009). The shorter combs from earlier *Scr-RNAi* shifts may avoid this bottleneck by pivoting past the distalmost t-row, instead of colliding with it (Atallah *et al.*, 2009)—a trend that is seen with combs which have been artificially selected for fewer bristles (Malagón *et al.*, 2014).

If so, then why do the shortest combs (7-8 bristles) of our *Cdc42* series (-24 to -36 h APF cohort; N = 8 legs) remain S-shaped? One idea (E. Larsen, pers. comm.) is that these combs lose bristle cells during or after rotation; another possibility (J. Atallah, pers. comm.) is that they get snagged on the tip of the t-row somehow. We don't know why *Cdc42* (but not *Scr*) is needed for the distal end of the "S" to straighten (= a side-effect of $Cdc42^{N17}$'s impact on joints?; *cf.*, necrosis at "-2 to -4" joints in Figures 1, 2, and 3), nor why *Scr* (but not *Cdc42*) is needed for earlier stages of rotation.

The t-rows tell a different story from the sex comb. *Cdc42* is evidently required there in order for bristle cells to form contiguous chains, rather than just for straightening the chains after they arise. Shifts at 24 h APF @ 25°C have no effect, but those at 12 h APF disrupt tibial t-rows 3-6 (9 of 14 legs), where the rows are numbered from distal to proximal (Figure 2). Disruptions include gaps, kinks, zigzags, clumps, and triradii. Shifts between -2 and 0 h APF affect t-rows 2-6 (21 of 22 legs), and earlier shifts (between -4 and -36 h APF) affect all tibial t-rows. A similar wave of disruptions occurs when Epidermal Growth Factor Receptor is disabled (Held, 2002).

Basitarsal t-rows are less regular than tibial t-rows in wild-type flies, so they are less reliable as indicators of misalignment. Nevertheless, they did prove to be informative. They were relatively normal in cohorts shifted at ≥ 12 h APF but were disrupted (like those on the tibia) by shifts ≤ 0 h APF.

Why should $Cdc42^{N17}$ block the concatenation ("self-assembly") of bristles in t-rows (tibial and basitarsal) but not in the sex comb? Conceivably, t-row bristles need more Cdc42 activity than do sex comb bristles, or maybe the *Dll-Gal4* driver is expressed more strongly there, in which case the difference would merely be an artifact (J. Atallah, pers. comm.).

Based on peculiar correlations between bristle spacing and bristle polarity, one of us long ago proposed that bristle cells use filopodia to space themselves at regular intervals in the rows on fly legs (Held *et*

al., 1986). Thus, we wondered whether disabling a putative regulator of filopodia (*Cdc42*) might disrupt bristle spacing. The most orderly longitudinal row on the legs is row 8 on the 2nd-leg basitarsus. Its bristles exhibit a military precision in their intervals.

As shown in Figure 3, we did indeed find spacing irregularities in Row 8 in the earlier cohorts. However, the affected legs also display other anomalies (*e.g*., stunted growth and/or evagination, zigzag bristles, and missing sockets) that confound the analysis. A cleaner test of this hypothesis would be to use a bristle-specific driver (*e.g*., *scabrous*- or *neuralized-Gal4*) with *UAS-Cdc42*^{N17} instead of *Dll-Gal4* (N. Malagon, pers. comm.)—an approach which is now under way.

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Adult sex ratio in *Drosophila melanogaster* developed in different nutritive conditions.

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In most of the animal species, there is approximately equal proportion of females and males (Hamilton, 1967). Sometimes, when one sex is in excess, sex ratio is disturbed. Biased sex ratio is well known for many *Drosophila* species (James and Jaenike, 1990; Montchamp-Moreau and Joly, 1997; Jaenike,