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Cell Dynamics of Sex Comb Morphogenesis in *Drosophila melanogaster*

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Abstract

The sex comb (SC) of *Drosophila melanogaster* is a linear arrangement of bristles found on the basitarsus of male forelegs, and has long been considered an excellent model for studying evolutionary developmental biology. Although many of its developmental regulators have been discovered, how they are translated into cell dynamics and eventually SC morphology still remain unclear. Previous studies have demonstrated the dramatic remodeling of tarsal epithelium proximal and distal to SC during SC development displays a variety of cellular processes characteristic of systems modulated by surface mechanics. To explore the possibility of modulation of surface tension as the mechanism underlying SC ontogeny, we examined the effect of varying SC length on the cell dynamics of epithelial cells in the presumptive SC field. Confocal time series of SC morphogenesis in four lines of flies with increasingly longer SC were obtained and analyzed with ImageJ. Apical cell surface dimensions were measured and cell positions relative to landmark bristles were tracked. Our results demonstrated that changes in epithelial cell size and shape are closely correlated with the size of the SC being rotated. Furthermore, we showed that changes in cell position within the epithelium over time appeared to be random, and that cell intercalation is perhaps not actively contributing to SC rotation in contrast to what was previously believed. In summary, our findings provide evidence of association between epithelial remodeling and the size of SC. This study paves the way for future experiments in investigating the modulation of surface tension as the mechanism underlying SC morphogenesis.

Introduction

Sex comb (SC) is a linear arrangement of modified bristles found on prothoracic legs of a subclade of *Drosophila* species [1]. It is a sexually dimorphic trait that occurs only in males and is crucial for courtship and mating behaviours [1]. Not only does it exhibit incredible morphological diversity among species in its size, orientation, and teeth morphology, it has also been shown that even identical morphologies in closely related species can be developed by different cellular processes [2]. SC has long piqued the interest of evolutionary developmental biologist due to its potential as a model for addressing important questions such as how do developmental processes affect the evolution of traits [3]. It is an excellent model for both independent modifications of shared ancestral states as well as independent ontogeny of similar morphological structures in separate lineages [2]. These in conjunction with its rapid pace of evolution make it a powerful comparative tool for studying the genetic and developmental changes underlying convergent and divergent evolution [3]. However, before this model can be utilized widely to address the questions of evolutionary developmental biology, detailed understanding of its development must be established.

This paper will focus on the cellular processes of SC formation in *Drosophila melanogaster* due to the large amount of knowledge already available on the model organism and the availability of well-established methods [1]. Since novel forms are frequently produced by novel regulations of conserved mechanisms [2], our findings from this study may be widely applicable to SC development in other species as well. SC in *D.melanogaster* originates from the most distal Transverse Bristle Rows and has been shown to develop by male specific morphogenesis [4]. During this process, SC rotates anteriorly from its initial transverse position parallel to the anterior-posterior axis (AP-axis) of the leg to its final longitudinal position parallel to the proximal-distal axis (PD-axis) of the leg [5]. A lot of research has been done in the past to identify key signaling pathways involved in the regulation of this process. It has been shown that a HOX gene, *Sex combs reduced* (*Scr*), and a sex determination gene, *doublesex* (*dsx*), play a central role in coordinating regulatory inputs and specifying the SC morphology, such as position, size, orientation and degree of rotation [6]. Some of the regulatory genes involved in the process include the leg patterning genes such as *dachshund* (*dac*), *bric a brac* (*bab*), *Wingless* (*Wg*), and the leg segmentation regulators such as the Notch signaling pathway [1, 7-9]. However, how this information is translated into changes in cell dynamics in the SC region and eventually leading to the adoption of the longitudinal position of SC remains poorly explored.

One possible mechanism through which this may be done is through the modulation of surface tension. Intercellular surface tension has been shown to influence shape of cells within a tissue, and is dependent on cell adhesion and cortical tension

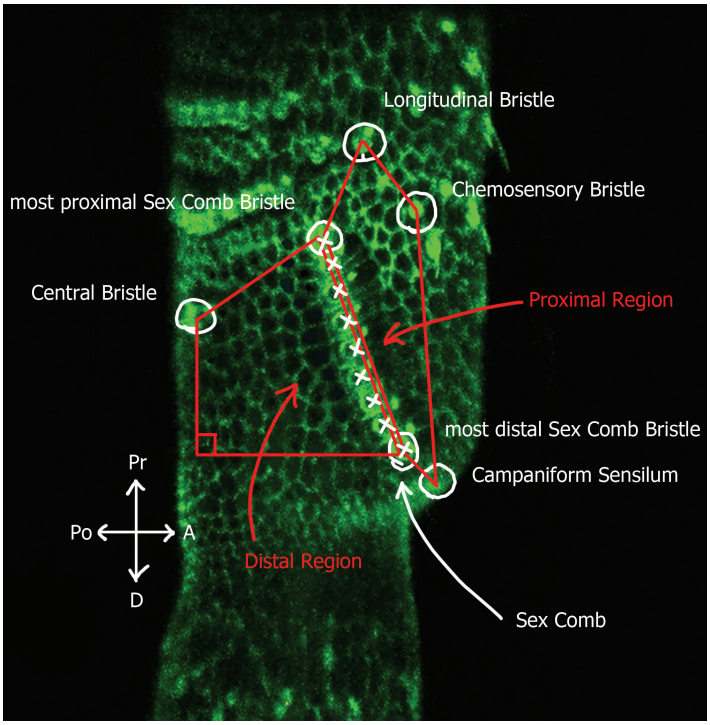

Figure 1: Visual representation of landmark bristles and key regions under study. Shown above is the confocal micrograph of the 1st Tarsal Segment (TS1) of a male *D. melanogaster* expressing ubi*D*Ecad::GFP 36hrs after pupariation (AP). Landmark bristles are represented by white circles; the Sex Comb is represented by white cross-marks; and the key regions are represented by red boxes. The Proximal Region (red box 1) refers to the tarsal epithelium surrounded by five landmark bristles (white circles): Longitudinal Bristle, Chemosensory Bristle, most proximal SC Bristle, most distal SC Bristle and Campaniform Sensilum (for motion detection & balance enhancement) [1]. The Distal Region (red box 2) is the tarsal epithelium surrounded by three landmark bristles (white circles) and a right angle: Central Bristle (the bristle that originated in the same transverse bristle row as the SC but got left behind during SC rotation) [1], most proximal SC Bristle, and most distal SC Bristle. At the beginning of SC morphogenesis when SC originates in its transverse position, the DR is distal to SC and the PR is proximal, hence their names. However at the end of the morphogenetic event, the DR appears posterior and PR appears anterior due to remodeling of the epithelium during the event.

[10]. Tissue surface tension, on the other hand, has been shown to explain global geometry of the tissue, and is dependent on cell adhesion [10]. Both have been used to explain the cell dynamics of many well-known morphogenetic events such as mesoderm invagination, convergent extension, dorsal closure, and retina development in *D.melanogaster* [10]. During the remodeling of the tarsal epithelium through SC rotation, epithelial cells seem to display a variety of behaviours similar to those occurring in systems experiencing complex surface mechanics, suggesting similar mechanisms may be at work. Malagon *et al.* have shown that regions of the presumptive SC field undergo considerable changes in area during SC rotation [11], while Tanaka *et al.* observed notable changes in cell shapes in the same regions [2]. Moreover, Atallah *et al.* have observed intercalation of cells similar to those seen during germ-band extension [4], suggesting a possible functional correlation of the process in both systems.

In order to evaluate association between surface tension and SC rotation, we studied three cell behaviours that are under the modulation of cell surface mechanics [10]: changes in cell size, changes in shape, and changes in position. Under the assumption that greater extent of changes would be required to generate greater motor forces, and eventually rotating a larger SC, we examined correlation between changes in cell size as well as cell shape and the length of SC. We also examined correlation in patterns of cell rearrangement among wild type (WT) individuals. The regions studied were parts of the tarsal epithelium immediately proximal and distal to the SC at the beginning of the rotation, termed the Proximal Region (PR) and the Distal Region (DR). The vertices of these regions were defined by land mark bristles to ensure consistency across all lines of flies studied (Figure 1). The PR is outlined by the Longitudinal Bristle, Chemosensory Bristle, Campaniform Sensilum, the most proximal SC bristle, and the most distal SC bristle; while DR is outlined by the same SC bristles, in addition to Central Bristle, and a right angle. Note that by the end of rotation, the PR becomes anterior to SC while the DR becomes posterior. The PR and DR in females refer to the regions proximal and distal to the most distal transverse bristle row homologous to SC, and are defined by a similar set of landmark bristles. To monitor changes in cell dimensions and positions, confocal time series of the tarsal epithelium during SC development were created. The movies were analyzed with ImageJ to acquire measurements of apical surfaces of cells, and statistical analyses were done on the results using MedCalc, and G*Power.

Our results demonstrated a strong correlation between changes in cell size, as well as cell shape, in the cellular neighbourhood of SC and the number of teeth in SC. Furthermore, the exchange of neighbours in the same regions seemed to vary largely among WT individuals and appeared stochastic. Although it is difficult to predict the role of cell rearrangement in SC rotation at the moment, our analysis do show that cell intercalation is perhaps not actively driving SC rotation in contrast to what was previously believed. Our results suggest possible cause and effect relationship between epithelial remodeling and SC rotation. These also provide support for future investigation of modulation of surface tension as a mechanism behind SC morphogenesis.

Materials and Methods

Fly stocks

Four lines of flies with varying length of SC expressing ubi*D*Ecad::GFP were studied (Table 1): WT-Female, WT-Male, Low Line and High Line. WT-Female was used as a control; the female region homologous to the male SC region as defined by a similar set of landmark bristles was studied. DR and PR in females are proximal and distal to the most distal transverse bristles which are homologous to SC in males. Low and High were lines of flies with abnormally low and high number of SC bristles compared to WT-Male, and were created through divergent artificial selection [12].

Table 1: Lines of flies studied and their respective length of SC. We used four lines of flies with increasingly longer SC to probe relationships between cell behaviours and SC rotation.

Line	Length of SC (# of bristles)	Region of leg imaged
WT-Female	0 [11]	TS1
Low	4-5 [12]	TS1
WT-Male	9-10 [11]	TS1
High	12-13 [12]	TS1

Confocal time-lapsed series

Confocal movies documenting epithelial cell dynamics of the TS1 during SC development between 23-36 hrs AP were obtained from works previously done by others with permission [4, 11]. The time interval between the acquisition of z-stacks were 30 minutes for WT-Female, Low Line, and High Line; and 20 minutes for WT-Male [4, 11].

Measuring changes in cell size and cell shape

The confocal movies were analyzed with ImageJ frame by frame. First, epithelial cell boundaries in the region of interest were manually outlined with white coloured lines using the brush tool. Second, the image was converted into 2-bit using the built-in "Make Binary" function and cropped to remove unnecessary regions. Third, the image was further edited using the brush tool and paint bucket tool to remove noise and blemishes until each cell was represented with a contoured patch of solid black sitting against a solid white background. Lastly, the edited image was processed with the built-in "Analyze Particles" subroutine to measure cell surface area, height and width. At least three samples were studied for each line of flies. To analyze change in cell size, cells were divided into two categories according to surface area: $<9\ \mu m^2$ were considered "Small"; while $\geq 9\ \mu m^2$ were considered "Large". The proportions of cells belonging to each size category (p(cell size)) at 23 hrs and 36 hrs in both DR and PR were calculated for each sample. To analyze change in cell shape, cells were divided into three categories according to height to width ratio: $0.5<H/W<1.5$ was considered square; $H/W<0.5$ was considered horizontal rectangle, $H/W>1.5$ was considered vertical rectangle. The proportion of cells belonging to each shape category (p(cell shape)) at, again, 23-36 hrs in both DR and PR were calculated.

Measuring changes in cell position (cell rearrangement)

The confocal movies were also analyzed with ImageJ frame by frame. Linear arrays of cells parallel to the AP-axis in regions of interest were manually labeled and followed from 23 hrs to 28 hrs AP. Cells were classified into six types of rearrangements based on local behaviours: one line of cells becoming two were considered intercalation along the PD-axis; two lines of cells becoming one were considered intercalation along the AP-axis; a group of minimum three cells rotating in unison clockwise (CW) or counter-clockwise (CCW) were considered CW rotation or CCW rotation respectively; cells not changing position were considered static; and lastly, cells that were disappearing from epithelium were considered as extrusion. Only WT flies were investigated for cell rearrangement. Three samples were studied.

Statistical analysis

All statistical tests were done using MedCalc unless otherwise specified. To assess the correlation between SC length and cell size, Spearman's coefficient of rank correlation (ρ) was calculated for the proportion of cells that got larger (Δp(Large Cell)) between 23-36 hrs AP and the length of SC (# of bristles) in each of the lines studied. The sample size required to yield statistically significant result for each rho was obtained using the Correlation Coefficient Sampling function to assess the significance of correlation. Furthermore, two sample t-tests were performed to analyze statistical significance of the difference inΔp(Large Cell) between all four lines of flies studied. Similar procedure was taken to assess relationship between SC length and cell shape, except that ρ was calculated for the proportion of cells that got more elongated along the PD-axis (Δp(Rectangular Cell)) between 23-36hrs AP. Lastly, for the cell rearrangement data, Chi-Square Test of Independence (χ^2) was done using Excel to assess whether the three samples of WT-Males studied differ from one another. Minimum required sample size was calculated using G*Power's "A priori Power Analysis" function for "Goodness-of-fit tests: Contingency tables".

Results

The tarsal epithelium surrounding the SC displays dramatic remodeling during SC morphogenesis [11]. To explore the possibility of regulation of surface tension being the mechanism underlying SC rotation, we studied several cellular processes often modulated by surface tension, and attempted to determine their relationship with SC rotation.

Changes in area of apical surface of cells strongly correlate with SC length

To determine the relationship between changes in cell size and SC rotation, we quantified the changes in apical area of epithelial cells during SC rotation in four lines of flies with increasingly larger SC. Under the assumption that cell surface area is generally indicative of cell size, our results showed that cells in the DR consistently increased in size while cells in PR consistently reduced in size for males. At 23 hrs the DR began with a large proportion of small cells and almost no large cells (Figure 2A), while at 36 hrs there was a reduction in the proportion of small cells and an increase in the proportion of large cells (Figure 2B), indicating that cells had become larger over time. In DR, the change in the proportion of large cells between 23 hrs to 36 hrs AP positively correlated with the number of SC teeth (ρ= 1.00, $P<0.0001$, α=0.05, β=0.20, Minimum Sample Size = 4). In other words, more cells became larger in the DR when the SC being rotated was longer (Figure 2C). The opposite trend was observed for the PR. At 23 hrs the PR began with more large cells in lines with longer SC, and more small cells in lines with shorter SC. Females did not seem to follow the trend (Figure 2D). At 36 hrs the PR ended with much more small cells and the proportion of small cells was roughly similar in different lines of flies. Females, again, did not seem to follow the trend (Figure 2E). In contrast to DR, the change in the proportion of large cells in PR negatively correlated with the number of SC teeth (ρ= 1.00, $P<0.0001$, α=0.05, β=0.20, Minimum Sample Size = 4). In other words more cells became smaller in the PR when the SC being rotated was longer (Figure 2F). It is also interesting to note the difference between males and females. While all males showed an increase in cell size in the PR, females showed a decrease. The patterns for the two sexes were also opposite in the PR. A comparison was drawn between cell size heat maps of a WT individual at the beginning (Figure 3B) and end (Figure 3C) of the rotation to visually present the extent of change. Notice how the cells in DR dramatically enlarged, while the cells in PR dwindled.

Changes in shape of apical surface of cells strongly correlate with SC length

To determine the relationship between changes in cell shape and SC rotation, we quantified changes in apical shape of epithelial cells during SC rotation in four lines of flies with increasingly larger SC. Our results showed that cells in both DR and PR elongated along the PD-axis of the leg during the course of rotation for males. At 23 hrs, there were a lot of square cells in the DR, the proportion of square cells are higher in lines of flies

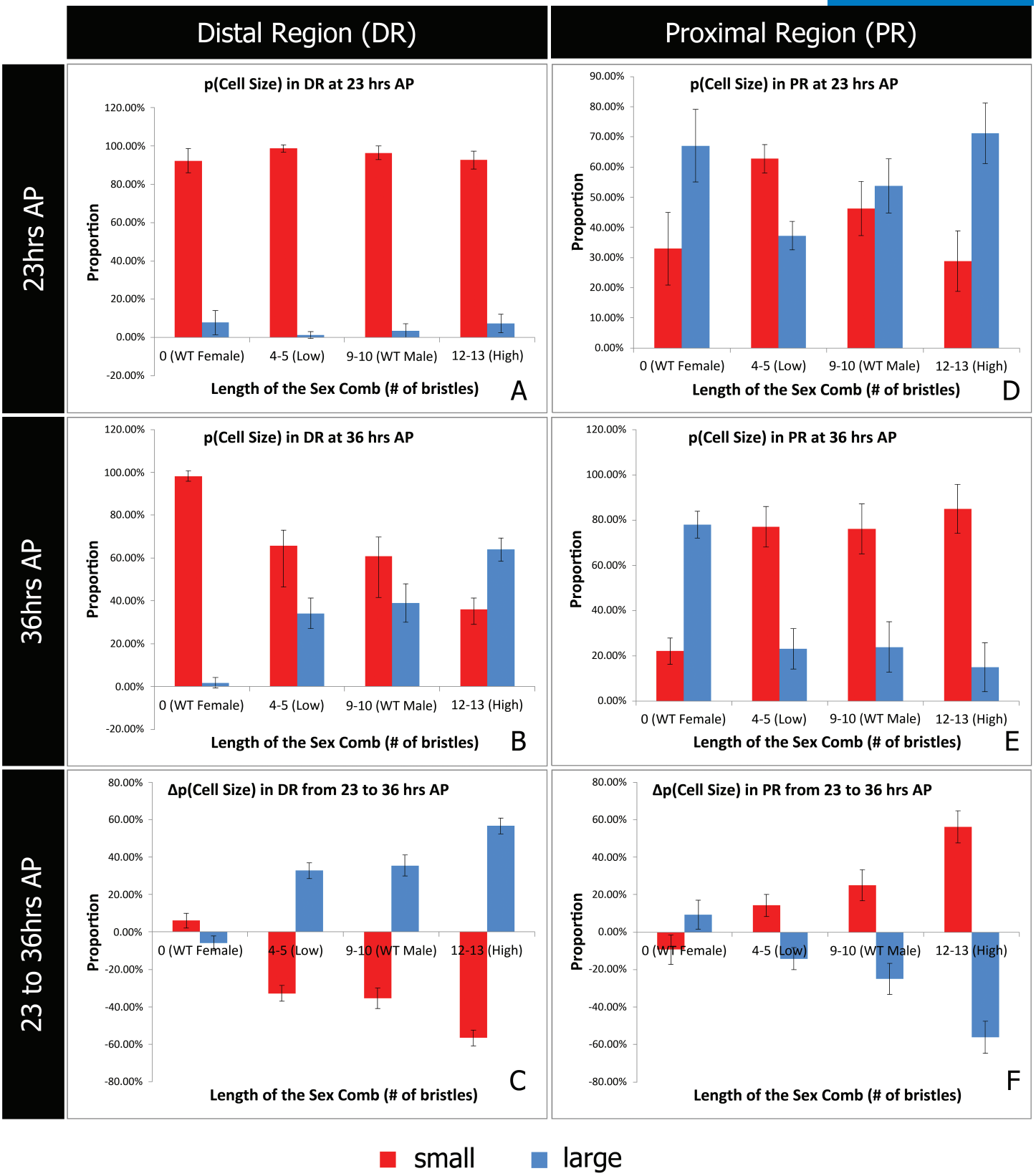

Figure 2: Effect of varying SC length on the apical surface area of epithelial cells in DR and PR [11]. The x-axis depicts the lines of flies arranged by their corresponding number of SC teeth from the smallest to the largest; y-axis represents the proportion of cells belonging to different size categories. **(A,B)** The proportion of "small" (red) and "large" (blue) cells in the DR at 23 hrs and 36 hrs AP respectively. **(C)** Change in the proportion of "small" and "large" cells from 23 hrs to 36 hrs AP. Notice the dramatic increase in cell size. Also notice how the proportion of cells that got larger positively correlates with the length of SC. **(D-F)** Same as above except for PR. The cells dramatically decreased in size, the extent of reduction positively correlates with the length of SC.

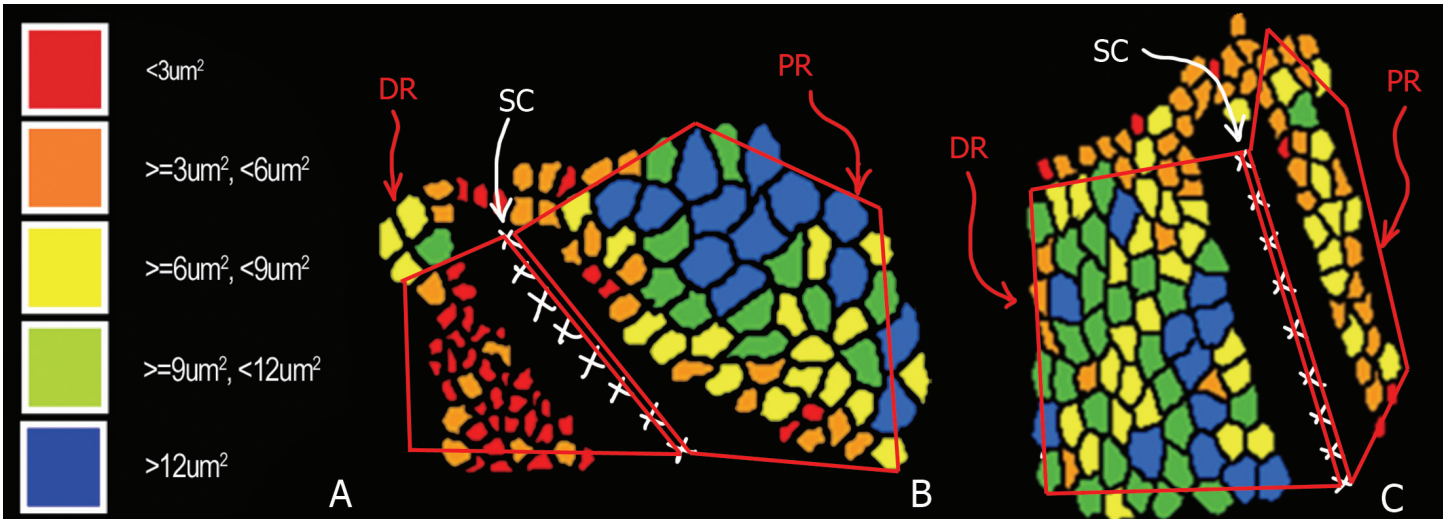

Figure 3: A visual representation of the changes in the apical surface area of epithelial cells in DR and PR [11]. **(A)** Color code for cells belonging to different size categories **(B)** Sample heat map of cell size in the DR and PR of a WT-Male at 23 hrs AP. The array of cross marks represents the position of SC. Red boxes outline the DR and PR. **(C)** Sample heat map of cell size in the DR and PR of the same WT-Male at 36 hrs AP. Both diagrams are shown at the same scale. There is considerable increase in the number of large cells (green, blue) in the DR and smaller cells (orange, yellow) in the PR.

with longer SC (Figure 4A). The female flies, as expected, did not follow the trend. At 36 hrs, a large increase in the proportion of vertical cells was observed and all four lines of flies ended up with similar proportion of both square and vertical cells (Figure 4B). This suggested that a lot of cells in the DR had elongated along the PD-axis during SC rotation. In DR, the change in the proportion of vertical cells between 23hrs to 36hrs AP positively correlated with the number of SC teeth (ρ= 1.00, P<0.0001, α=0.05, β=0.20, Minimum Sample Size = 4). In other words, more cells in DR elongated along the PD-axis when the SC being rotated was longer (Figure 4C). A similar trend was observed for the PR. At 23 hrs, the PR began with a large excess of square cells, the proportion of square cells were higher in lines with longer SC (Figure 4D). At 36 hrs, the proportion of vertical cells dramatically increased, while the proportion of square cells decreased. All four lines of flies ended with a similar proportion of both square and vertical cells (Figure 4E). Similar to DR, the change in the proportion of vertical cells in PR also positively correlated with the number of SC teeth (ρ= 1.00, P<0.0001, α=0.05, β=0.20, Minimum Sample Size = 4). In other words, more cells in PR elongated along the PD-axis in lines of flies with longer SC, similar to DR (Figure 4F). Furthermore, it is also notable that the change in the proportion of vertical cells in the DR was no longer opposite between the two sexes, suggesting that the magnitude of change in the DR is perhaps not as large as in that the PR. Finally, a comparison was drawn between the cell shape heat maps of a WT male at the beginning (Figure 5B) and end (Figure 5C) of the rotation to depict the extent of changes. Notice how a large number of cells elongated along the PD-axis in both the DR and the PR.

Patterns of cell rearrangement vary largely among individuals and are perhaps stochastic

In order to determine the relationship between the exchange of cell neighbours and SC rotation, we manually tracked the movement of individual epithelial cells near SC during SC rotation. Due to time limitations, we only compared the patterns of cell rearrangement among individuals of WT flies and we observed the following. First, cell rearrangement in DR and PR showed significant variations with no associations between individuals. (For DR, χ2=0.86, DF=10, P=0.9999, Effect Size=1.27, α=0.05, β=0.20, Minimum Sample Size=11; For PR,χ2= 0.55, DF=10, P=0.9999, Effect Size=1.40, α=0.05, β=0.20, Minimum Sample Size=9) This was the case in terms of both the proportion of cells undergoing each type of rearrangement as shown in Figure 6, as well as the spatial distribution of those cells as shown in Figure 7. For instance, in the DR, clockwise rotation (Rotation-CW) was completely missing in Pupa 1, but was the dominant type of rearrangement in Pupa 2 and 3 (Figure 6A). Meanwhile in the PR, intercalation along the AP-axis (Intercalation-AP) was very prevalent in Pupa 3 but was present only to a small extent in Pupa 1 and Pupa 2 (Figure 6B). Moreover, when considering the spatial distribution of cells undergoing different rearrangements, no consistent patterns were observed either. For example, CW rotations occupied the entire anterior section in Pupa 1 but was only restricted to the distal region in Pupa 2 and to the proximal section in Pupa 3 (Figure 7B, D, F). Furthermore, the diversity of cell rearrangement in the DR was considerably smaller than in the PR. In the DR, only three types of rearrangements were present and more cells remained static (Figure 6A). While in the PR, all five types of rearrangements were observed and fewer cells remained static (Figure 6B).

Discussion

Association between changes in cell size and SC length suggests the interplay between cell surface area, intercellular surface tension, and tissue surface tension plays an important role in SC rotation

In contrast to liquid where surface tension is constant, cell surface tension has been shown to closely correlate with cell surface area [10]. Therefore, if surface tension is indeed fundamental behind SC morphogenesis, we might be able to observe some kind of association between changes in cell surface area and SC rotation. Our results demonstrated a strong correlation between changes in cell size during SC rotation and SC length. While cells generally increased in size in the DR, and reduced in size in the PR, the extent of change was larger in lines with longer SC, and smaller in lines with shorter SC. Such correlation suggests that changes in cell size may play an important role in SC rotation. Moreover, the

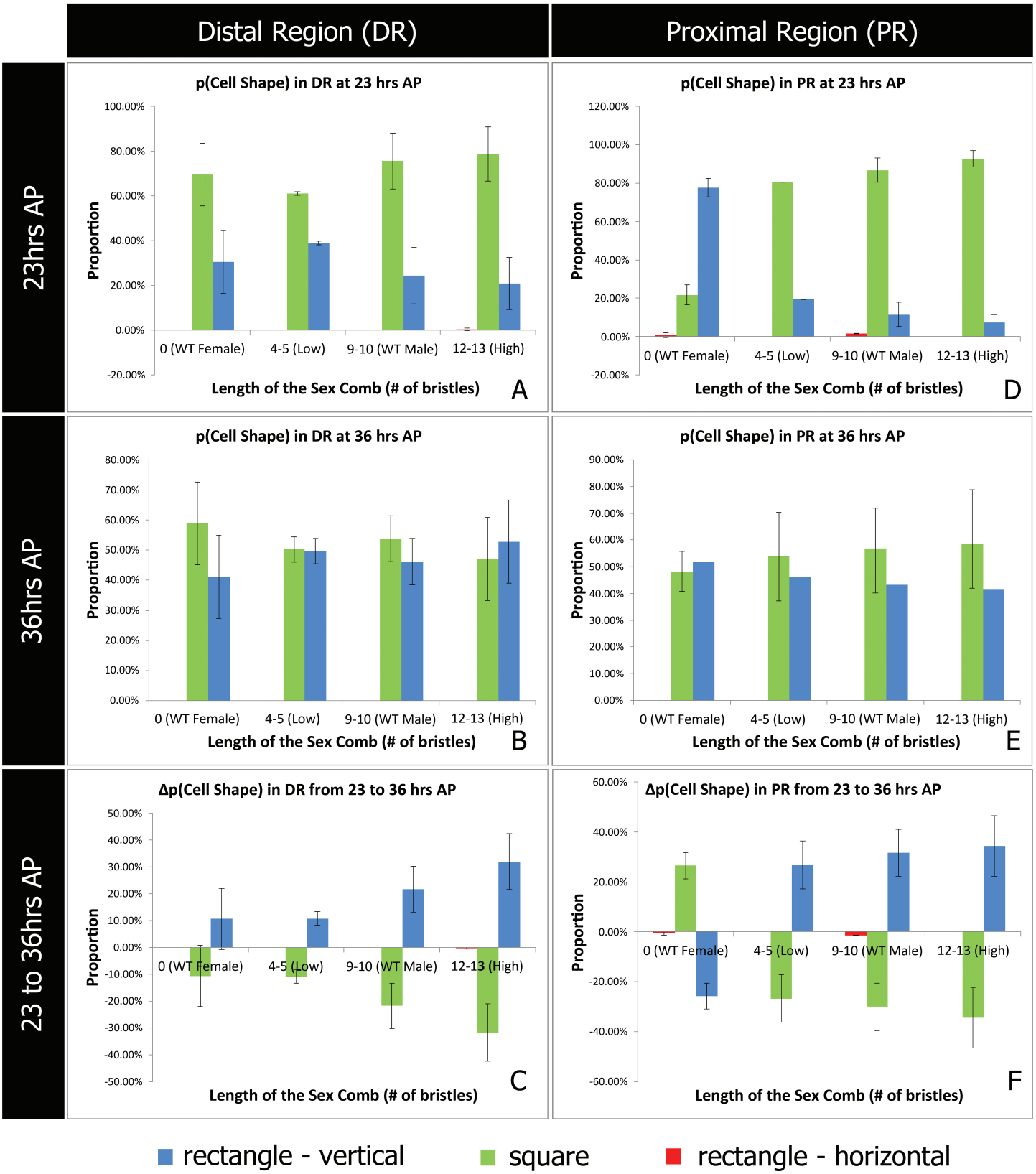

Figure 4: Effect of varying SC length on the apical surface shape of epithelial cells in DR and PR [11]. The x-axis depicts the lines of flies arranged by their number of SC teeth from the smallest to the largest; y-axis represents the proportion of cells belonging to different shape categories. **(A,B)** Proportion of cells belonging to "square" (green), "rectangle - horizontal" (red), and "rectangle - vertical" shape categories at 23 hrs and 36 hrs AP respectively. **(C)** Change in the proportion of cells belonging to the three different shape categories between 23 hrs to 36 hrs AP. Notice the dramatic increase in the proportion of vertical rectangles in DR. Also notice how the increase in the proportion of vertical rectangles positively correlates with the length of SC. **(D-F)** Same as A-C except for PR. Again, cells elongated along the PD-axis, the proportion of cells that did so positively correlate with the length of SC.

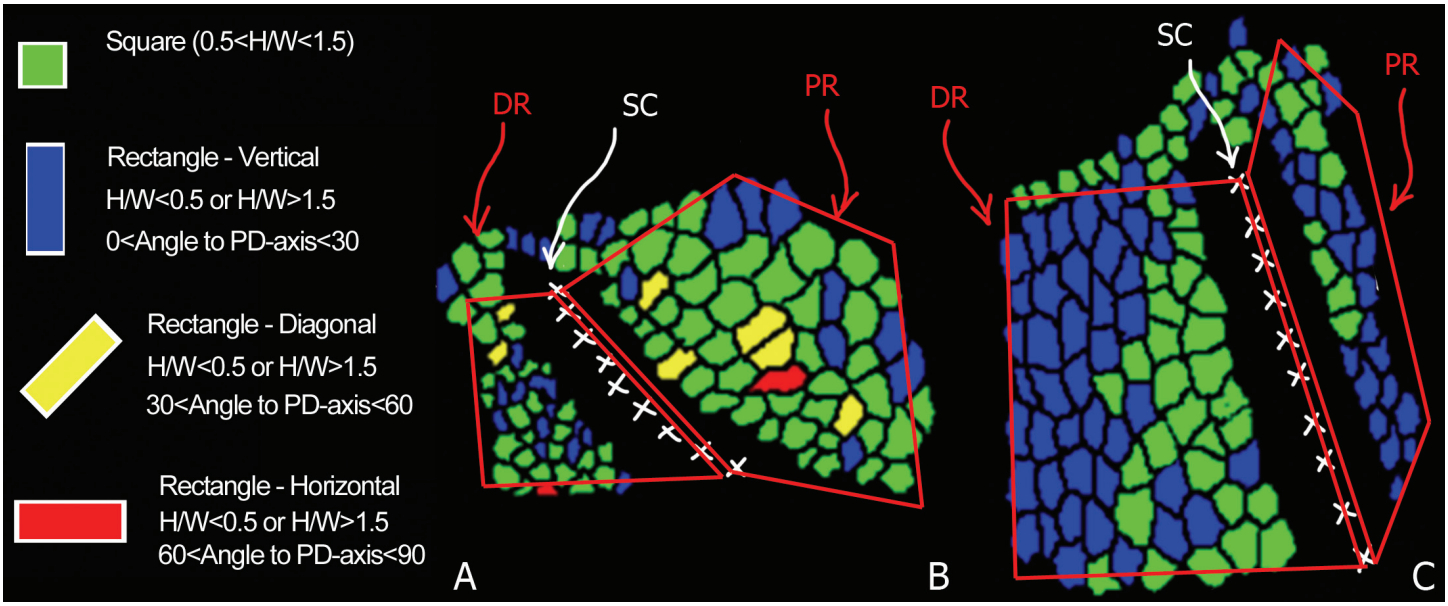

Figure 5: A visual representation of the changes in the apical surface shape of epithelial cells in DR and PR [11]. **(A)** Color code for cells belonging to different shape categories **(B)** Sample heat map of cell shape in the DR and PR of a WT-Male at 23 hrs AP. The array of cross marks represents the position of SC. The red boxes represent the boundaries of DR and PR. **(C)** Sample heat map of cell shape in the DR and PR of the same WT-Male at 36 hrs AP. Both diagrams are shown at the same scale. Notice how both DR and PR start off with a lot of squares (green) and end up with a lot of vertical rectangles (blue), indicating systematic elongation in the regions.

extents of changes were in opposite direction in males and females in both DR and PR, suggesting the changes are sexually dimorphic, providing a further piece of evidence of association between cell size changes and SC rotation. However, it remains unclear as to whether these changes are the cause of the rotation or simply a result of it based on our findings alone.

Changes in apical surface area of cells have been shown to produce the necessary motor force needed to drive morphogenetic event such as mesoderm invagination in *D.melanogaster* [10]. The dramatic expansion and reduction in the DR and PR could allude to a similar role played by cell size changes in the SC system. If cell size changes are indeed the cause, three predictions could be made. First, the correlation could be a result of the differences in the amount of tissue tension necessary for rotating SCs of varying length, based on the assumption that greater changes could produce greater forces. Second, since changes in cell surface area could significantly impact the area of intercellular contact, which in turn influences intercellular and tissue surface tension [10], we speculate surface tension could be acting in synergy on both cellular and tissue level to drive SC rotation. Third, based on the models of SC rotation put forth by Malagon *et al.* [4] and Atallah *et al.* [11], we speculate that the role of PR and DR may alternate during the course of the event. Previous studies have demonstrated that larger contact area between cells as a result of better adhesion strengthens tissue surface tension [10] while smaller contact area as a result of less favorable adhesion weakens tissue tension [10]. Based on this observation, we speculate the constriction in PR may be needed for driving the rotation at the beginning due to initially larger cell sizes in the region producing stronger tissue surface tension. At this stage PR may be exerting a "pull" on the SC. As time progresses the cells in PR get smaller and the force exerted by the region diminishes, while cells in DR get larger and begin to exert stronger forces on the SC instead. At this stage the expansion in DR might be able to produce a "push" and take over the role of driving SC rotation. On the other hand, if cell size changes are the result of SC rotation, they may be explained as passive response to the motor forces applied on the epithelium during SC rotation.

Association between changes in cell shape and SC length suggests cell elongation, likely regulated by intercellular surface tension, plays an important role in SC rotation

Spatial patterns of cell shape are regulated by intercellular surface tension [10]. Cells in tissues have been shown to adopt similar shapes as bubbles in 2D foam during morphogenetic events that span medium to long timescale [13]. This is due to the fact that both cells and bubbles share the similar tendency to optimize packing and minimize surface energy [13]. Similarly, the global shape of cell aggregates has been found to be under the regulation of tissue surface tension[14], evident in the formation of spheres by cell aggregates and the spontaneous sorting of mixed groups of cells [10]. Given the close correlation between cell shape and surface tension, if morphogenesis is indeed achieved by regulation of surface tension, we should be able to observe certain kind of association between changes in cell shape and SC rotation.

Our results demonstrated that cells in both DR and PR elongated along the PD-axis during SC rotation. The extent of change was positively correlated with the length of SC, in both DR and PR, suggesting a possible role played by changes in cell shape during SC morphogenesis. Moreover, the proportion of cells elongating along the PD axis changed in opposite directions in PR of males and females, suggesting cell elongation in the region is male specific and adds a further piece of evidence supporting the association between cell shape changes and SC rotation. In contrast, the pattern of shape change observed in the DR was similar for both sexes – cells in the DR of both males and females elongated along the PD-axis. This is either due to an artifact of our data limited by its small sample size, or could suggest that the elongation of cells in the DR is intrinsic to proper leg development, such as the formation of joint, and is therefore

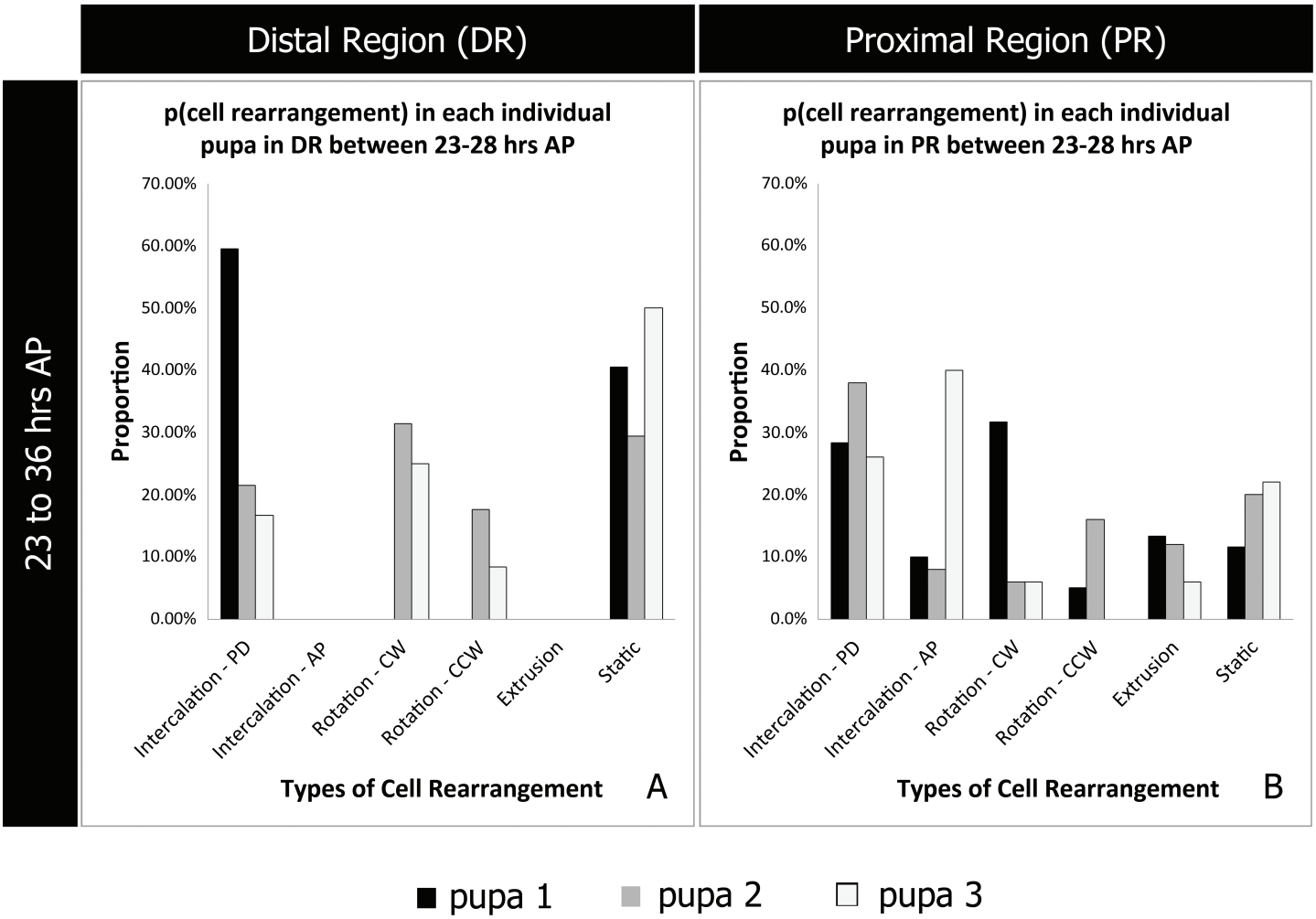

Figure 6: Quantification of epithelial cell rearrangement among three different WT-Males. The x-axis represents different types of cell rearrangement, while the y-axis represents the proportion of cells partaking in each type of rearrangement. **(A)** Proportion of cells undergoing each type of cell rearrangement in the DR of three WT-Males between 23-28 hrs AP. The black bars represent data from pupa 1, grey bars from pupa 2, and white bars from pupa 3. **(B)** Same as (A) except for the PR. The proportion of cells doing each type of rearrangement showed significant variation from individual to individual and lacked any patterns. It should be also noted that cell intercalations along the AP-axis and cell extrusions were only present in the PR.

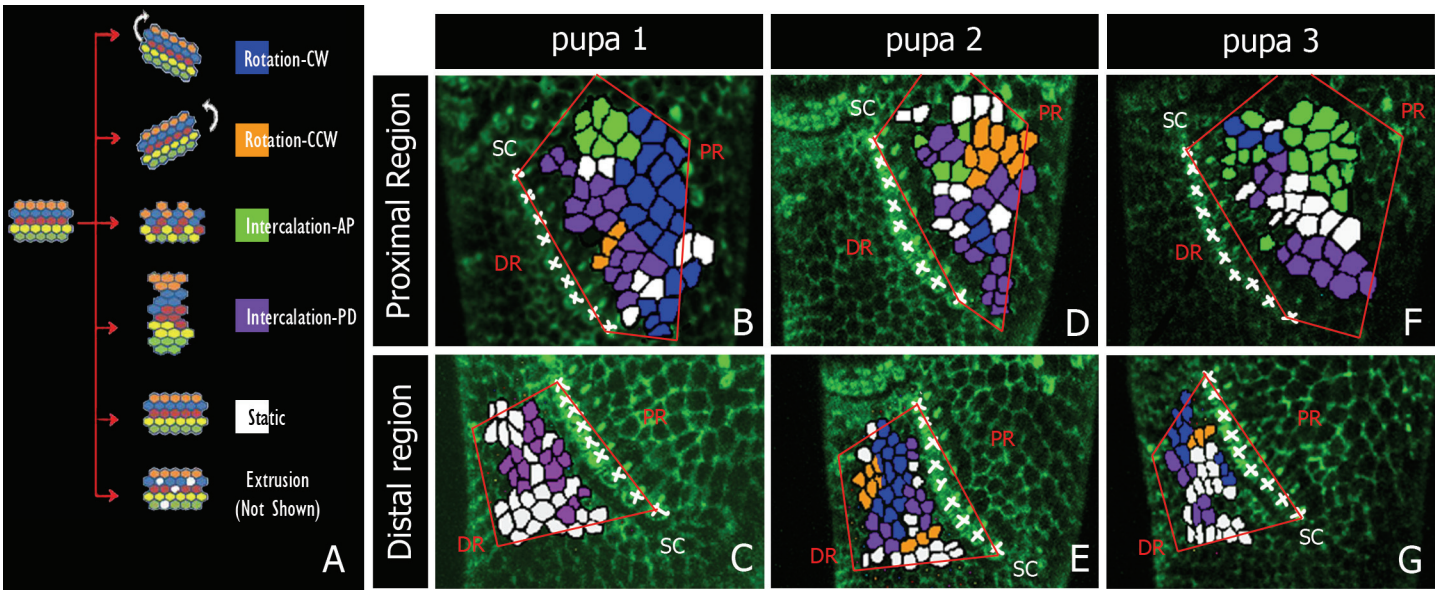

Figure 7: Spatial pattern of epithelial cell rearrangement in 3 different WT-Males [11]. **(A)** Color code of cells belonging to different categories of rearrangement. **(B-G)** Fate maps of cells of three WT-Males (column 1-3) at about 28 hrs AP in the PR (top row) and DR (bottom row). The array of cross-marks represent the position of SC. The red boxes represent the boundaries of DR and PR. Notice the significant variations in the spatial pattern of rearrangement between individuals.

present to a certain degree in both males and females. Although the trend where more cells elongated in lines with longer SC still existed in DR, but it was present to a lesser extent, suggesting DR is probably less involved in SC rotation than PR.

Again, due to the nature of the study, although we were able to demonstrate the correlation between shape changes and SC rotation, we were unable to determine whether cell shape changes are the cause or the effect of SC rotation. We speculate it is perhaps the latter because changes in cell shape regulated by intercellular surface tension have been known to drive morphogenesis and pattern formation in a variety of systems, including the development of retina in *D.melanogaster* [10].

Lastly, to further characterize cell shape changes, further experiments could attempt to identify the polygonal shapes taken by the cells. Epithelium regulated by intercellular surface tension has been known to favour the formation of three-fold vertices where three edges of the neighbouring cells meet at 120 degree, and result in the formation of hexagonal networks [13]. Therefore, observation of similar patterns in the tarsal epithelium could provide further evidence of surface tension at work.

The lack of association between patterns of cell rearrangement among individuals demonstrates that it is perhaps stochastic in nature, and that cell intercalation is likely not driving SC rotation as previously anticipated

Cell rearrangement has been known to cause changes in overall tissue geometry, particularly in systems where tissue mechanics has been used to explain morphogenesis [10]. For instance, cell intercalation has been shown to produce the motor forces needed to drive germ-band extension, and to results from cell shape changes regulated by intercellular surface tension [10, 15]. To investigate the role of cell rearrangement in SC rotation, we followed changes in cell positions during the initial stage of rotation (23 to 28 hrs AP). Due to the time consuming nature of this procedure, instead of examining extent of changes in lines of flies with varying SC length, we observed variations among WT individuals. Consistent patterns of cell intercalation were observed among individuals for germ-band extension. If cell rearrangement is also driving SC rotation, we might be able to observe similar consistency for the SC system as well.

The classification of each type of cell rearrangement is based on changes in cell position relative to their neighbours and landmark bristles. One of the limitations of this method is that the groups of cells assessed for their types of rearrangement are chosen arbitrarily at the discretion of the experimenter and the result is heavily dependent on subjective choices. Another limitation is that manual tracking does not permit analysis of large sample sizes due to the amount of work involved. However, this may be resolved in the future by tagging nuclear envelopes with fluorescent proteins and then tracking and analyzing the position of each nucleus using automated image processing software [11]. Not only would this allow batch processing of large amounts of samples, but this would also eliminate the necessity for arbitrary assignment of cells into groups for more precise assessment. Our interpretation of the results below should be considered in light of these limitations.

The most prominent feature of cell rearrangement in the DR and PR is the large variation among individuals in terms of the number of cells undergoing each type of rearrangement and the spatial distribution of these cells. This is the opposite of what was seen for cell size and cell shape, where overall changes remained largely similar on the level of individual organisms, despite differences on the level of individual cells. However, although this result suggests that cell rearrangement is probably stochastic, it is difficult to say whether rearrangement is actively contributing to SC rotation or not. It has been shown that some degree of disorder could almost always be seen on finer scales during tissue reorganization, reflecting a limit to the extent of tight genetic regulation [10]. The reason that systems could usually produce consistent morphologies is due to the robustness of the developmental process, which enables the systems to tolerate a certain extent of noise [10]. Furthermore, examples of self-organization where apparently random behaviours consistently result in similar outcomes have been found in both the development of mouse optic cup [16] and zebra fish heart [17]. In these systems, a set of genes specify parameters for the system, then simply allow spontaneous interactions between components of the system to achieve the desired phenotype [18]. While the behaviour of each component may seem random, the overall outcome is always consistent because the components act within the confinement of parameters [18]. On the other hand, instead of actively contributing to SC rotation, cell rearrangement may also be a passive response to the other processes at work, such as the changes in cell size and cell shape mentioned previously, or to the rotation of SC, which applies mechanical force on the tarsal epithelium. Similar haphazard behaviours have been found in fly notum where apparently random delamination occurs in response to mechanical stress caused by over-crowding to ensure proper packing of epithelial cells [19].

Another interesting observation from our results is the higher diversity in the types of rearrangement in PR when compared to DR. In the PR, fewer cells remained static and all five types of rearrangement were observed. While in the DR, more cells remained static and only three types of rearrangement were present. Under the assumption that cell rearrangement is stochastic, one possible cause of this disparity in diversity is that shrinking cells in PR reduces contact area at cell boundaries and therefore weakens cell-cell adhesion. This would in turn allow for easier exchange of neighbours and lead to more varied patterns of rearrangement. Furthermore, since PR reduces in area during SC rotation it is likely that it will experience more mechanical pressure from the SC and thus require more reposition of cells to accommodate the constriction in space.

Finally, previous studies done by Atallah *et al.* [1, 4] have observed cell intercalations in the PR, and suggested that it might play a similar role as the intercalations during germ-band extension. In order to evaluate the functional similarity of cell intercalation in both systems, we compared the formation and resolution of multicellular rosettes in the PR to that of the germ-band extension. In germ-band extension, rosettes form and resolve in directional fashion under the regulation of planar polarity proteins allowing for tissue elongation [20]. However, during SC rotation the progression between configurations (T1, T2, and T3) is bidirectional resulting in rosettes resolving in multiple directions and effectively preventing net elongation of tissue in any particular direction. This shows that cell intercalation is playing a different role during SC rotation.

Conclusion

In short, our result demonstrates that change in cell size and cell shape closely correlate with the length of SC, suggesting a possible association between surface tension and SC rotation. Our study provides important evidence of cell dynamics that warrant further investigation of the role of surface mechanics, as well as the remodeling of cytoskeleton and intercellular junctions in SC development. To confirm the association between epithelial remodeling and SC rotation, further studies characterizing epithelial changes in lines of flies with varying length of non-rotating SCs could be done in comparison with our study. To elucidate the cause and effect relationship between epithelial remodeling and SC rotation, future studies could attempt to selectively reducing the number of cells in the DR and PR using approaches such as laser ablation to observe the effect on SC rotation [11]. Finally, to study the association between surface tension and SC rotation, further experiments could focus on measuring temporal and spatial patterns of intercellular surface tension using approaches such laser-induced cell fusion, or tissue surface tension using tensiometers. Alternatively, experiments could also focus on characterizing the activity of molecular regulators of cytoskeleton and intercellular adhesion in the tarsal epithelium, since surface tensions are ultimately controlled by cortical tension and cell-cell adhesion.

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