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Oscillatory behaviors and hierarchical assembly of contractile structures in intercalating cells

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Abstract

Fluctuations in the size of the apical cell surface have been associated with apical constriction and tissue invagination. However, it is currently not known if apical oscillatory behaviors are a unique property of constricting cells or if they constitute a universal feature of the force balance between cells in multicellular tissues. Here, we set out to determine whether oscillatory cell behaviors occur in parallel with cell intercalation during the morphogenetic process of axis elongation in the *Drosophila* embryo. We applied multi-color, time-lapse imaging of living embryos and SIESTA, an integrated tool for automated and semi-automated cell segmentation, tracking, and analysis of image sequences. Using SIESTA, we identified cycles of contraction and expansion of the apical surface in intercalating cells and characterized them at the molecular, cellular, and tissue scales. We demonstrate that apical oscillations are anisotropic, and this anisotropy depends on the presence of intact cell-cell junctions and spatial cues provided by the anterior-posterior patterning system. Oscillatory cell behaviors during axis elongation are associated with the hierarchical assembly and disassembly of contractile actomyosin structures at the medial cortex of the cell, with actin localization preceding myosin II and with the localization of both proteins preceding changes in cell shape. We discuss models to explain how the architecture of cytoskeletal networks regulates their contractile behavior and the mechanisms that give rise to oscillatory cell behaviors in intercalating cells.

1. Introduction

Mechanical forces play a central role in generating the cell movements and cell-shape changes that sculpt tissues and in coordinating these behaviors during morphogenesis (Fernandez-Gonzalez *et al* 2009, Landsberg *et al* 2009, Monier *et al* 2010). At the tissue level, forces can lead to bending, invagination, or fusion of tissues in normal development (Thompson 1917) and during wound healing (Martin and Lewis 1992, Bement *et al* 1993). The forces required for morphogenesis are generated by specific behaviors at the cellular level, including apical constriction (Leptin and Grunewald 1990), cell stretching (Young *et al* 1993, Martin-Blanco *et al* 2000), and coordinated contraction (Wood *et al* 2002). These cell behaviors rely on contractile forces produced by non-muscle myosin II, a molecular

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motor that can translocate and exert tension on actin filaments (Vicente-Manzanares *et al* 2009). Force generation at the molecular, cellular, and tissue scales must be coordinated to ensure proper morphogenesis. Cell–cell junctions are required for force transmission across tissues (Gorfinkiel and Arias 2007, Martin *et al* 2010). However, the mechanisms that translate actomyosin contractility and force generation at the molecular level into morphogenetic events at the tissue level are still poorly understood.

Axis elongation is a conserved morphogenetic process that extends the anterior–posterior (AP) axis of the embryo. In *Drosophila*, axis elongation is accomplished through the extension of the germband, an epithelial monolayer that gives rise to ectodermal tissues. Germband elongation is driven by intercalary cell movements (Irvine and Wieschaus 1994), oriented cell divisions (da Silva and Vincent 2007), and cell-shape changes (Butler *et al* 2009). During cell intercalation, the contraction of one or more cell–cell interfaces oriented parallel to the dorsal–ventral (DV) axis (convergence) leads to the formation of a vertex where four or more cells meet (Bertet *et al* 2004, Blankenship *et al* 2006). This vertex is systematically resolved by the assembly of new cell interfaces along the AP axis of the embryo (extension). Filamentous actin (F-actin) and non-muscle myosin II are specifically enriched in cell interfaces parallel to the DV axis of the embryo, resulting in planar-polarized contractile behaviors (Zallen and Wieschaus 2004, Bertet *et al* 2004, Blankenship *et al* 2006).

A population of myosin II at the medial cell cortex has been shown to play a role in apical constriction during tissue internalization and cell–sheet fusion. During mesoderm invagination, the ventral-most cells form dense medial myosin meshworks that assemble in periodic, cumulative steps and are associated with cycles of contraction and stabilization during constriction of the apical cell surface (Martin *et al* 2009). Periodic behaviors are also associated with apical constriction in the amnioserosa, an extraembryonic epithelium that covers a dorsal gap in the embryonic epidermis. In the amnioserosa, medial actomyosin structures are periodically assembled and disassembled (David *et al* 2010, Blanchard *et al* 2010), associated with cycles of contraction and limited expansion of the apical cell surface (Solon *et al* 2009). Apical constriction in the amnioserosa reduces the size of the gap, contributing to cell–sheet fusion and the establishment of epidermal continuity.

It is currently not clear if medial cytoskeletal meshworks at the apical cell cortex and the oscillatory behaviors associated with them represent an exclusive feature of apically constricting tissues. Medial myosin II is downregulated in germband cells by the JAK/STAT signaling pathway to prevent apical constriction (Bertet *et al* 2009), and other studies suggest that the remaining medial myosin II promotes contraction of the junctional domain (Rauzi *et al* 2010). Here we use quantitative imaging to investigate the role of medial myosin structures in the *Drosophila* germband. We use dual-color, time-lapse imaging in combination with SIESTA, a tool for Scientific Image Segmentation and Analysis that we have developed for the high-throughput extraction of morphological and molecular features of cells from imaging data. We show that oscillations in apical area and medial myosin dynamics occur in the absence of apical constriction during axis elongation and analyze their spatial and temporal regulation.

2. Materials and methods

2.1. Markers and mutants

For live imaging, we used the following markers *spider:GFP* (gift of Alain Debec), *sqh-sqh:GFP* (Royou *et al* 2004), *sqh-sqh:mCherry* (Martin *et al* 2009), *ubi-E-cadherin:GFP* (Oda and Tsukita 2001) and *sqh-GFP:moesin* (Kiehart *et al* 2000). Mutants were *halo^{Df(2L)dpp[s7-dp35]} snail^{IIIG05}*; *sqh-sqh:GFP* (gift from Adam Martin) and the progeny of *ubi-Ecadherin:GFP*; *bcd^{E1} nos^{L7} tsl¹⁴⁶* females.

2.2. Time-lapse imaging

Embryos were collected for 1 h, dechorionated in 50% bleach, transferred to a drop of halocarbon oil 27 (Sigma) on a cover glass, and mounted on an oxygen-permeable membrane (YSI). GFP was excited with an Argon line (488 nm, 100mW, Melles Griot). mCherry was excited with a Krypton laser (568 nm, 1 mW, Melles Griot). A 63× oil immersion lens (Zeiss, N.A. 1.4) on an UltraView RS spinning disk system (Perkin Elmer) was used for imaging, except in figures 6(A) and (B) where a 40× oil immersion lens (Zeiss, N.A. 1.3) was used. 16 bit, 2-color Z-stacks were acquired at 0.5 μm steps every 15 s and projected for analysis (15 slices per stack).

2.3. Injections

Embryos from 30–60 min collections were dechorionated for 2 min in 50% bleach, lined up on an agarose pad, transferred to a stripe of heptane glue on a cover glass, dehydrated for 7 min, and covered in 1:1 halocarbon oil 27:700. For α -catenin knockdown, double-stranded RNA against α -catenin was injected ventrally in syncytial embryos (stage 3) at 2 $\mu\text{g } \mu\text{l}^{-1}$. Templates to produce double-stranded RNA were generated by PCR from genomic DNA with the following primer pairs, containing the T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGACCAC-3') at the 5' end:

α -catenin T7-forward 5'-ATGAGTCAAAAAGATAACAGCCCAG-3'

α -catenin T7-reverse 5'-CTTGATCTCGTAGAACTTTGACTGC-3'.

Embryos were incubated in a humidified chamber at 25 °C and imaged at stages 7 or 8. For myosin knockdown, Rho-kinase inhibitor (Y-27632 dihydrochloride, Tocris Bioscience, 100 mM) was injected ventrally into the perivitelline space of stage 7 or stage 8 embryos and imaged immediately (Fernandez-Gonzalez *et al* 2009). Injected solutions are predicted to be diluted 50-fold in the embryo.

2.4. Cell segmentation, tracking and quantification

Algorithms described in this section were developed in Matlab (Mathworks) and DIPImage (TU Delft), and integrated in SIESTA. To identify the outlines of cells expressing E-cadherin:GFP, we implemented a fully automated approach based on the watershed algorithm (figure 1(A)). We used an adaptive threshold to identify medial versus junctional pixels in 16 bit projections of the original Z-stacks. The threshold value was the mean pixel value of a 30 × 30 pixel window around each pixel (MacAulay and Palcic 1988). A distance transform was applied to the resulting binary image to compute the distance from medial

pixels to the closest cell interface. The local maxima of the distance transform (pixels at least $0.24\ \mu\text{m}$ away from edges) were selected as seeds. Seed size, shape, and position were used to identify exactly one seed per cell. Seeds were expanded on a Gaussian-smoothed version of the projected image (Gaussian width was $0.13\ \mu\text{m}$) using the watershed algorithm (Beucher 1992). When manual correction was necessary, it was generally at the level of the seeds, which were rapidly edited with the annotation tools provided by SIESTA.

Sqh:GFP and GFP:Moe have a nonuniform distribution and a strong medial component. Therefore, automated segmentation of these signals required extensive manual correction. In addition, when junctional integrity was lost, cells detached from their neighbors, impeding the automated detection of cell boundaries. For these reasons, we developed a semi-automated segmentation strategy based on the LiveWire algorithm (Mortensen *et al* 1992). The user moves the mouse around the cell outline and the brightest path between consecutive points is automatically identified and displayed on the image (movie S1 available at stacks.iop.org/PhysBio/8/045005/mmedia). This path is updated in real time as the user moves the cursor, allowing immediate correction of segmentation errors. In the LiveWire implementation described here, a 16 bit projection of the original Z-stack was cropped around the initial and the current positions of the cursor to increase computational speed and allow real-time user interaction. The cropped image was inverted so that cell edges were dark and medial regions were bright. A weighted graph was built based on the inverted image, with one node per pixel, and bidirectional connections between each pixel and its eight immediate neighbors. Connections were weighted by the pixel value corresponding to the end node, such that it was 'lighter' to move toward dark pixels (cell edges) and 'heavier' to move toward bright pixels (medial regions). Finally, Dijkstra's minimal cost path extraction algorithm (Dijkstra 1959) was used to calculate the 'lightest' path between the nodes representing the initial and the current cursor positions, and this path was displayed on the original, uncropped image.

Cells were tracked over time using a maximum-proximity algorithm based on the position of their corresponding watershed seeds. Because intercalating cells in the germband do not dramatically change position within 15 s, the maximum interval in our time-lapse sequences, cell tracking was done simply by projecting each seed onto the next time point and identifying the closest neighbor. In rare cases in which two seeds mapped onto the same seed in the following time point, the user was asked to identify the correct connections.

The length of a cell along the AP and DV axes was calculated in two ways. In one method we superimposed on the cell outline an array of lines 1 pixel ($0.2\ \mu\text{m}$) apart and parallel to the axis in question (figure 1(B)). Cell length was defined as the mean length of the intersecting segments. In the second method, the cell outline was fitted with an ellipse (Fitzgibbon *et al* 1999). The resulting ellipse was shifted until it was centered at the coordinate origin, and the intersection points with the AP and DV axes were used to determine the length of the cell along those two axes. Both methods produced similar results. Here we show results obtained using the first method.

To quantify junctional and medial protein levels, each cell was divided into two compartments. The junctional compartment was determined by a 3-pixel-wide ($0.6\ \mu\text{m}$)

dilation of the cell outline identified using watershed or LiveWire segmentation. The medial compartment was obtained by inverting a binary image representing the junctional compartment. Protein concentrations were quantified as the mean pixel value in each compartment. Changes in cell area and protein concentration were defined as

$$\Delta \text{area}(t) = \text{area}(t) - \text{area}(t - 60 \text{ s}) \quad (1)$$

$$\Delta \text{protein}(t) = \text{protein}(t) - \text{protein}(t - 60 \text{ s}) \quad (2)$$

When necessary, the effect of photobleaching was corrected by dividing the results at each time point by the mean pixel value in the entire image.

2.5. Laser ablation

An N₂ Micropoint laser (Photonic Instruments) tuned to 365 nm was used to ablate junctional and medial cell domains using *ubi-E-cadherin:GFP* to guide the ablations. The laser delivers 2–6 ns, 120 μJ pulses, producing wounds $\sim 1 \mu\text{m}$ in diameter. Cells outside the site of ablation were not damaged under our experimental conditions (10 pulses, 50% attenuation).

Laser ablation induces local relaxation of mechanical tension, displacing nearby cell vertices. The initial velocity of displacement can be used to quantify the tension released (Hutson *et al* 2003, Fernandez-Gonzalez *et al* 2009), under the assumption that local viscoelastic properties are homogeneous throughout the tissue. Cell outlines were automatically segmented using SIESTA. The positions of cell vertices were extracted by scanning a 3×3 pixel kernel across the segmented image to detect small regions where three or more cells met. Cell vertices were tracked over time using a maximum proximity algorithm after subtraction of the average vertex movement in each time point. The peak radial velocity of displacement of vertices within $6 \mu\text{m}$ of the ablation site was used to measure the local relaxation of forces. The directionality of force propagation was assessed by measuring the peak velocity of vertex displacement according to vertex position with respect to the AP axis.

2.6. Monte Carlo simulations

At each time point, cells were classified as contracting or expanding based on tracking of their apical area. The spatial pattern of contracting and expanding cells at time t was quantified as the average fraction of neighbors of a contracting cell that are also contracting:

$$\text{contracting_neighbors}(t) = \frac{1}{N_C} \sum_{i=1}^{N_C} \frac{n_{i_C}}{n_i} \quad (3)$$

where N_C is the number of cells that are contracting their apex at time t , i indexes over all those cells, n_{i_C} is the number of neighbors of cell i that are also contracting, and n_i is the total number of neighbors of cell i . Two types of Monte Carlo simulations were carried out, obtaining similar results. In one case, the temporal sequences of cell area values were randomized between cells, such that cells oscillate independently while maintaining the in

vivo period of oscillation (not shown). In a second approach, cells were randomly reassigned as contracting or expanding at each time point based on the frequencies observed *in vivo*. This is equivalent to assuming that the period of oscillation is random and that the cells oscillate independently from their neighbors. 2000 simulations were run for each image analyzed.

2.7. Statistical analysis

Sample variances were compared using the *F*-test (Glantz 2002). Sample means were contrasted using Student's *t*-test, applying Holm's correction when three or more groups were considered. The significance of correlation coefficients was evaluated by transforming the correlation value into a *t* statistic using the Matlab function *corrcoef* (Mathworks). The Kolmogorov–Smirnov test was used to compare sample distributions. Errors indicate the standard error of the mean unless otherwise noted. $P < 0.05$ was used to determine significant differences.

To determine the probability of erroneously reporting no correlation between cell area and medial myosin II changes, we randomly selected *n* cells in figure 4(K) and computed the average significance of the correlation between the cell area and medial myosin II for those *n* cells. This process was repeated 1000 times (trials) for each value of *n* tested. For each value of *n*, the probability of erroneously reporting no correlation was the ratio between the number of trials in which the significance was $P > 0.05$ and the total number of trials.

3. Results and discussion

3.1. Cells undergo oscillatory behaviors during axis elongation

Oscillations of apical area are associated with apical constriction (Martin *et al* 2009, Solon *et al* 2009), but have not been reported in cells that maintain a constant area. To ask if cell-shape fluctuations are present in embryonic tissues where cells do not constrict apically, we used spinning-disk confocal microscopy in combination with SIESTA, our custom image analysis software, to examine cell-shape changes during axis elongation in living *Drosophila* embryos expressing a fusion of E-cadherin to GFP (Oda and Tsukita 2001). SIESTA uses a watershed-based image segmentation algorithm to automatically detect and quantify cell shape and behavior in time-lapse image sequences (section 2, figure 1(A)).

Morphometric analysis during axis elongation revealed that intercalating cells undergo cycles of apical contraction and expansion that result in fluctuations of the apical cell area (figure 1(C) and movie S2 available at stacks.iop.org/PhysBio/8/045005/mmedia). Oscillatory behaviors were evident when the absolute apical area or the rate of area change was measured for individual cells (figures 1(D) and (E)). In contrast to the mesoderm or the amnioserosa, cell-shape fluctuations in the germband were not associated with significant apical constriction (figure 1(H)), and in some cells we observed expansion of the apical area (figures 1(D) and (E), red line). The average apical cell area in the germband was $40.3 \pm 11.2 \mu\text{m}^2$ (mean $\pm$ s.d.). The duration of a large majority of oscillations (81.5%, $n = 470$) was distributed between 90 and 180 s, with a mean oscillation period of 147.0 ± 43.5 s (mean $\pm$ s.d.) (figure 1(F)). By contrast, the period of oscillation is 82.8 ± 48 s in the mesoderm (Martin *et al* 2009), and 230 ± 76 s in the amnioserosa (Solon *et al* 2009). Apical

cell areas are smaller in the mesoderm (10–40 μm^2) and larger in the amnioserosa (100–200 μm^2). The correlation between the increased apical cell area and longer oscillation period suggests that the rates of contraction and expansion of the apical domain depend on the size of this domain.

In the mesoderm, the Snail transcription factor is required for oscillatory cell behaviors associated with apical constriction and invagination (Martin *et al* 2009). Although *snail* expression is restricted to the mesoderm, the oscillations of mesoderm cells could influence cell behavior in the germband, as the tissues are mechanically coupled (Butler *et al* 2009). To test this possibility, we imaged germband cells in *snail* mutant embryos. In the absence of oscillations in the mesoderm, germband cells underwent cell-shape fluctuations (figure 1(G)) and the period of oscillation was unchanged (141.9 ± 38.0 s, mean $\pm$ s.d., $n = 26$, $P = 0.56$, figure 1(F)). These results indicate that the presence of oscillatory behaviors in germband cells does not require signals from the mesoderm.

3.2. Anisotropic oscillatory behavior requires AP patterning

In addition to cell rearrangement, cell-shape changes also contribute to axis elongation (Butler *et al* 2009). We found that cell-shape anisotropy increased as axis elongation proceeds, with cells elongating parallel to the AP axis of the embryo (figure 1(H)). The oscillations of germband cells were also moderately anisotropic, with the largest amplitude of oscillation parallel to the AP axis (figure 1(I), $P = 0.02$). These oscillations did not cause a net gain of length along either axis ($P = 0.71$ and $P = 0.56$, respectively). In embryos maternally mutant for the *bicoid* (*bcd*), *nanos* (*nos*) and *torso-like* (*tsl*) patterning genes (*bcd nos tsl* mutants), where AP spatial differences are lost and germband elongation is disrupted, oscillations were no longer appreciably anisotropic (figure 1(K), $P = 0.62$). However, cell-shape anisotropy still increased over time in *bcd nos tsl* mutants (figures 1(H) and (J)), despite the fact that the oscillations were not anisotropic. Consistent with this, we found no correlation on a per cell basis between the rate of area change and the rate of change of cell-shape anisotropy in wild type ($R = 0.04 \pm 0.34$, mean $\pm$ s.d.). These results show that the anisotropic oscillations of intercalating cells are oriented by the AP patterning system but are not required for cell-shape anisotropy.

3.3. Anisotropic oscillatory behavior requires junctional integrity

Tissue-wide forces influence cell behavior in the *Drosophila* embryo (Butler *et al* 2009, Martin *et al* 2010). To determine whether tissue-wide forces generate anisotropic oscillatory behavior, we quantified cell-shape fluctuations in embryos injected with double-stranded RNA to α -catenin, a component of the cadherin–catenin complex. In embryos in which α -catenin was disrupted, we observed a progressive detachment of intercalating cells, accompanied by the appearance of membrane tethers (movie S3 available at stacks.iop.org/PhysBio/8/045005/mmedia). However, cells continued to display fluctuations in apical area (figures 2(A) and (B)), suggesting that oscillatory behaviors in the germband are cell autonomous and do not require stable contact between cells.

To investigate whether cell–cell contacts impose constraints on the mode of oscillation, we quantified the amplitude of oscillation along the AP and DV axes in α -catenin knockdown

embryos. Oscillations were isotropic when α -catenin was disrupted (figure 2(C), $P = 0.43$). These results demonstrate that, although oscillatory behavior is cell autonomous in intercalating cells, the anisotropy of these oscillations requires an intact junctional system.

3.4. Intercalating cells exhibit complex spatiotemporal regulation of oscillatory behaviors

Because cell adhesion is necessary for anisotropic oscillations, we hypothesized that the oscillatory behaviors of neighboring cells might be coupled. To test this idea, we identified pairs of cells that were neighbors for at least 6 min ($n = 969$ cell pairs in 6 embryos), and measured the correlation between the rates of area change for each pair (R_0 , figures 3(A) and (B)). We found a broad distribution of correlation values, indicating that intercalating cells can oscillate in phase, anti-phase and independently from their neighbors.

To determine if any relationship was dominant, we calculated the correlation (R_t) between the rates of area change of neighboring cells after shifting one cell forward or backward in time in 15 s increments up to 4 min (figure 3(A)). Maximum correlation when the shift was close to zero would indicate in-phase oscillation, while minimum correlation with zero shift would suggest anti-phase oscillations. We found that the minimum correlation was more frequently observed with small temporal shifts (0–30 s, figure 3(C)), while the maximum correlation occurred with a modest bias toward longer shifts (30–60 s, figure 3(D)). These results indicate that neighboring cells predominantly oscillate in anti-phase.

We visualized the spatial organization of intercalating cells that were expanding or contracting (movie S4 available at stacks.iop.org/PhysBio/8/045005/mmedia) and identified scattered cells that were completely surrounded by neighbors oscillating in anti-phase (figure 3(E), asterisks, 0.008 occurrences per cell per minute), as well as groups of neighboring cells that contracted or expanded in phase (figure 3(E)). The emergence of clusters of cells that oscillate in phase could result randomly, or could reflect an active mechanism that coordinates cell behavior. To distinguish between these models, we used Monte Carlo simulations to test if the frequency of association of cells that oscillate in phase *in vivo* could be recapitulated by chance. Random distributions of the period and the phase of oscillation could reproduce the clusters of neighboring cells oscillating in phase observed *in vivo* (figures 3(F) and (G), $P = 0.96$), suggesting that these clusters are random and do not represent an active mechanism of cell coordination.

3.5. Cell-shape oscillations are associated with medial myosin II dynamics

Oscillatory behaviors in the mesoderm and the amnioserosa are associated with a medial meshwork of contractile myosin II (Martin *et al* 2009, 2010, Blanchard *et al* 2010, David *et al* 2010). To determine if myosin II activity is required for oscillatory behaviors in intercalating cells, we injected embryos with Y-27632, an inhibitor of the myosin activator, Rho-kinase. We found that the amplitude of oscillation was significantly reduced in embryos injected with Y-27632 compared to control embryos injected with water (figures 4(A)–(C) and movies S5 and S6 available at stacks.iop.org/PhysBio/8/045005/mmedia, $P = 1.2 \times 10^{-6}$). Thus, myosin II activity is necessary for oscillatory behavior.

In the germband, both junctional and medial myosin structures generate force during axis elongation (Rauzi *et al* 2008, 2010, Fernandez-Gonzalez *et al* 2009). Consistent with these results, we identified distinct bending sites at cell–cell junctions following the condensation of medial myosin II (figure 4(E)), suggesting that medial myosin II structures are anchored to and exert forces on discrete sites at cell–cell junctions. To determine the relative tension sustained by medial and junctional contractile structures, we used ultraviolet laser ablation followed by time-lapse imaging and node tracking (Hutson *et al* 2003, Fernandez-Gonzalez *et al* 2009). We found that the tension sustained by the medial cortex was significantly smaller than the tension sustained by myosin-rich, AP interfaces connected in cable-like structures ($P = 0.002$) and greater than the tension sustained by myosin-depleted, DV cell interfaces ($P = 0.019$) (figures 4(F)–(H)). Furthermore, the forces generated at AP interfaces propagate with a preferred orientation parallel to the DV axis of the embryo, while forces generated medially propagate isotropically (figure 4(H)). Based on the recoil velocities after laser ablation, the relative tension sustained by medial contractile structures was T_{DV} : $T_{\text{medial}}:T_{\text{AP}} = 1.0:1.7:2.9$.

We used dual-color, time-lapse spinning disk confocal microscopy in combination with SIESTA to analyze the dynamics of medial and junctional myosin in embryos that express E-cadherin:GFP and Sqh:mCherry (figure 4(I) and movie S7 available at stacks.iop.org/PhysBio/8/045005/mmedia). Apical cell contraction was associated with the assembly of medial myosin structures, while apical expansion followed the disassembly of medial myosin (figure 4(J)). This association was significantly stronger for medial myosin than for junctional myosin ($P = 2 \times 10^{-74}$, figures 4(K) and (L)). If medial myosin dynamics generate cell-shape fluctuations, then changes in myosin levels or activity should precede changes in cell shape. To test this, we calculated the correlation between medial myosin and cell area changes after shifting the myosin data forward or backward in time. We found that the strongest anti-correlation between changes in medial myosin II and cell area occurred when medial myosin was not shifted or when it was shifted 15 s forward (figure 4(L)), indicating that assembly and disassembly of medial myosin structures precede cell contraction and expansion, respectively. We used cubic spline interpolation (Bartels *et al* 1987) and found that the lag between changes in medial myosin II and the subsequent change in cell area was 7.3 s. Taken together, these results strongly suggest that oscillatory cell behaviors during axis elongation result from contractile behaviors at the medial cell cortex.

Contraction of the apical surface during oscillations could occur through the condensation or disassembly of medial myosin. We found that the concentration of medial myosin remained constant during oscillation, with values ranging between 0.995 and 1.005 (average of the minimum and maximum normalized medial myosin concentrations, respectively, $n = 401$ cells), while normalized cell area values ranged between 0.8 and 1.2 (figure 4(M)). Total medial myosin II displayed large fluctuations that correlated with cell area changes ($R = 0.62 \pm 0.19$, mean $\pm$ s.d., figure 4(N)). Thus, as the cell area expands, the total myosin at the medial cortex increases, and as cells contract, the total medial myosin decreases, suggesting that cell contraction occurs through the disassembly of medial myosin structures.

3.6. Actin localization precedes myosin II in medial contractile structures

Actin filaments are subject to dynamic turnover both at cell–cell junctions (Yamada *et al* 2005) and in medial contractile meshworks (Rauzi *et al* 2010), while myosin II forms stable filaments that turn over more slowly (Fernandez-Gonzalez *et al* 2009, Rauzi *et al* 2010). To ask if actin and myosin dynamics are regulated differently during the assembly and disassembly of medial meshworks in intercalating cells, we imaged living embryos expressing Sqh:mCherry and a GFP fusion to the actin binding domain of Moesin (Kiehart *et al* 2000). The assembly of medial myosin II structures was accompanied by the coalescence of actin into these structures (figure 5(A)–(A'')).

To quantify the dynamics of F-actin and myosin II, we used a semi-automated segmentation tool based on the LiveWire algorithm (Mortensen *et al* 1992) to examine a large number of cells while minimizing user interaction (section 2, movie S1 available at stacks.iop.org/PhysBio/8/045005/mmedia). Changes in the cell area were anticorrelated with changes in medial F-actin and myosin levels (figures 5(B)–(D)). Correlation analysis showed that changes in F-actin preceded cell-shape fluctuations by 14.1 s, while changes in myosin preceded cell-shape fluctuations by 8.6 s (figure 5(D)), suggesting that medial contractile structures are assembled by the accumulation of actin into medial foci followed by the recruitment of myosin.

3.7. Increasing the ratio of medial to junctional myosin affects oscillatory behavior

The assembly and disassembly of medial actomyosin structures precedes and may be required for oscillatory behavior during axis elongation. However, it is difficult to establish a causal relationship between medial myosin and cell-shape fluctuations, as methods that specifically target medial cytoskeletal structures while leaving junctional structures intact are lacking. Nevertheless, it is possible to alter the medial-to-junctional ratio in *bcd nos tsl* mutant embryos, which displayed increased medial myosin II relative to junctional levels (figures 6(A) and (B)). Laser ablation experiments demonstrated significantly increased contractility at the medial cortex in *bcd nos tsl* mutants ($P = 0.03$, figures 6(C) and (D)). Germband cells continued to oscillate (figure 6(E)) with a similar period to wild-type cells (149.0 ± 35.9 s, $n = 47$), but the distribution of amplitudes of oscillation was bimodal, compared to the unimodal distribution in wild type (figure 6(F), $P = 0.012$). The average amplitude of oscillation was significantly greater in *bcd nos tsl* ($17.2 \pm 8.5\%/min$, mean $\pm$ s.d.) than in wild type ($14.0 \pm 7.6\%/min$, $P = 0.0063$) and was associated with ectopic apical constriction (figure 1(J)). These results suggest that changing the balance of medial to junctional myosin II affects the basic parameters of oscillation, modifying cell behavior.

3.8. Using quantitative imaging methods to analyze cell behavior

Automated image analysis makes it possible to analyze large numbers of cells, but the statistical advantage of this approach has not been systematically compared to smaller-scale measurements. To determine the minimum sample size needed to identify the correlation between medial myosin II and decreasing apical cell area (figure 4(K), $n = 401$ cells in 6 embryos), we calculated the probability of erroneously reporting no correlation when subsets of cells were randomly selected from our data set. A sample size of 110 cells or greater was required to reduce the probability of reporting no correlation to below 0.05

(figure 4(O)). This example provides a framework for considering the sample sizes necessary to discern specific behaviors associated with contractile networks in the presence of heterogeneity.

4. Conclusions and outlook

In this study we use multi-spectral, time-lapse imaging of living *Drosophila* embryos, combined with SIESTA, an image analysis platform that we have developed for the high-throughput quantification of molecular and cellular changes in living tissues. We report for the first time that intercalating cells undergo cycles of contraction and expansion of their apical surface. The oscillatory behavior of intercalating cells is anisotropic, with the major axis of oscillation parallel to the AP axis of the embryo, and this anisotropy requires adherens junctions and AP patterning. Oscillatory behaviors are closely associated with medial actomyosin structures that generate force as measured by laser ablation. Our data show that the assembly of medial contractile structures is a hierarchical process, with actin preceding myosin II localization, and the localization of both proteins preceding cell-shape change.

Oscillatory cell behaviors associated with medial actomyosin structures have been observed in the *Drosophila* mesoderm (Martin *et al* 2009) and the amnioserosa (Solon *et al* 2009, Blanchard *et al* 2010, David *et al* 2010). In these tissues, cell-shape fluctuations have been linked to apical constriction. By contrast, intercalating cells in the germband do not constrict apically, and therefore, the finding that they undergo oscillatory behavior is unexpected. Cycles of contraction and expansion in intercalating cells are associated with the assembly and disassembly of medial cytoskeletal structures. This mode of oscillation is similar to amnioserosa cells (Solon *et al* 2009), and in contrast with the mesoderm, where the medial myosin pool becomes denser as cells constrict apically, and cell-shape fluctuations do not display a phase of expansion (Martin *et al* 2009). In addition, we found that peak actin accumulation precedes peak myosin accumulation, reminiscent of the molecular hierarchy established for junctional myosin in germband cells (Blankenship *et al* 2006). By contrast, in the amnioserosa and the follicular epithelium, medial actin and myosin accumulate and disappear from medial structures simultaneously, with no delay with respect to the changes in cell area (Blanchard *et al* 2010, He *et al* 2010), indicating that intercalating cells can be better time-resolved despite their shorter period of oscillation.

The period of oscillation in intercalating cells is intermediate between the period of oscillation in mesoderm and amnioserosa cells, suggesting that cells with larger apical surface area have longer periods of oscillation. Consistent with this idea, the period of oscillation in apically constricting cells decreases in parallel with the apical surface of the cells (Martin *et al* 2009, Blanchard *et al* 2010). This is in contrast with the dynamics of cleavage furrow constriction during cytokinesis, in which an actomyosin ring forms at the furrow and contracts to complete cell division. In this process, cells of different sizes complete division in the same amount of time (Carvalho *et al* 2009). It has been proposed that contractile units of constant initial size and rate of disassembly drive actomyosin ring contraction (Carvalho *et al* 2009). The number of these units per ring would be proportional to the size of the ring, resulting in faster overall contraction rates for larger rings (Carvalho

et al 2009) (figure 7, top). We propose that medial myosin meshworks are composed of actin filaments that are polymerized and depolymerized at a constant rate (figure 7, bottom). The time required to assemble and disassemble a network that spans the medial cortex would be proportional to the apical surface area, allowing smaller cells to oscillate faster than larger cells (figure 7, bottom). This model predicts that not only the period but also the amplitude of oscillation will scale with the surface area of the cells, consistent with the reduction in the amplitude of oscillation experienced by mesoderm and amnioserosa cells as they constrict their apical surface (Blanchard *et al* 2010, Martin *et al* 2009, Solon *et al* 2009). Thus, actomyosin contractility may be regulated differently in cytoskeletal structures that display different architectures.

In both intercalating cells and the adjacent mesoderm, cell-shape fluctuations do not require the presence of intact adherens junctions (Martin *et al* 2010) (and this work), suggesting that oscillatory behavior is hardwired into the cells. By contrast, increasing cell-shape anisotropy in both tissues is statistically independent of oscillatory behavior, suggesting that tissue-wide forces rather than individual cell behaviors establish mechanical constraints that generate cell-shape asymmetry. The nature of the forces that lead to increasing cell-shape anisotropy is unknown. Our analysis of AP patterning mutants indicates that cell-shape anisotropy in the germband does not require AP spatial information. It has been suggested that in the germband, extrinsic forces oriented along the AP axis of the embryo result from the invagination of the neighboring mesoderm (Butler *et al* 2009). Alternatively, tissue-wide forces could result if the rate of tissue convergence is greater than the rate of tissue elongation, or if intercalating cells are squeezed by the isotropic growth of dividing cells ventral and dorsal to the germband.

Our results, together with recent data published by Sawyer and colleagues (Sawyer *et al* 2011), demonstrate that oscillatory behaviors are not exclusive to cells undergoing apical constriction. Furthermore, these data suggest that cell-shape fluctuations may be a general feature of tissues where cells have a pool of medially localized contractile proteins. However, the fundamental question of how oscillatory behaviors are generated remains unanswered. One possibility is that myosin is transported in small ‘packages’ across the cell for delivery to cell interfaces that contract during intercalation, as myosin II has been shown to flow directionally across the medial cortex, toward cell interfaces where E-cadherin is reduced (Rauzi *et al* 2010). Alternatively, the assembly of medial contractile structures could be an active response of the cells to counteract the strain generated by intercalary behavior. Actin filaments in cells align parallel to the direction of major strain in response to tension (Kolega 1986). During elongation, external tension could align actin filaments parallel to the AP axis, driving anisotropic contraction. The use of photoconvertible fluorochromes and high-throughput computational methods to quantitatively track protein movement and cytoskeletal reorganization in response to mechanical stress will shed light on the mechanisms that regulate myosin dynamics and cell behavior.

In this study we have introduced SIESTA, a computational tool that integrates image processing and analysis for unbiased automated measurement, tracking, and statistical assessment of cell behaviors and molecular dynamics over time. The use of quantitative imaging has allowed us to identify statistically significant properties of intercalating cells,

including anisotropic oscillatory behaviors and the stepwise temporal assembly of medial contractile structures. These tools should be generally applicable to other developmental stages, morphogenetic processes, and model organisms to elucidate the subcellular and cellular events that drive profound tissue changes during morphogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nomenclatures

AP	anterior–posterior
<i>bcd</i>	<i>bicoid</i>
DV	dorsal–ventral
<i>nos</i>	<i>nanos</i>
s.d.	standard deviation
SIESTA	scientific image segmentation and analysis
<i>sqh</i>	<i>spaghetti squash</i>
<i>tsl</i>	torso-like

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Figure 1.

Intercalating cells undergo cycles of expansion and contraction during *Drosophila* axis elongation. (A) Algorithm for the automated identification of cell outlines from time-lapse image sequences. (B) Method to compute cell length along AP (left) or DV (right) axes. The average length of the blue or red segments was used to calculate cell length along the corresponding axis. (C) Confocal sequences showing three cycles of contraction and expansion for two intercalating cells (top and bottom) expressing E-cadherin:GFP. Scale bar = 5 μm . Anterior left, ventral down. (D) Apical area versus time for three cells in wild type. Red line corresponds to the top cell in (C), blue line corresponds to the bottom cell in (C). (E) Rate of change of the apical area for the cells in (D). (F) Distribution of the period of oscillation in wild-type embryos (blue, $n = 470$ cycles) and in *snail* (red, $n = 26$ cycles). (G) Rate of change of apical area for three cells in a *snail* mutant. (H) Changes in the apical cell area and cell-shape anisotropy (AP/DV length) with respect to the initial values and averaged across cells in wild type ($n = 401$ cells in six embryos). (I) Amplitude of oscillation of the rate of cell-length change along the AP (blue, $n = 472$ cycles) and DV (red, $n = 441$ cycles) axes in wild type, normalized to mean cell length along the AP and DV axes respectively. Asterisk indicates a significant difference ($P = 0.02$). Amplitude was half of the absolute distance between the maximum and minimum that defined each cycle. (J) Changes in the apical cell area and cell-shape anisotropy (AP/DV length) with respect to the initial values and averaged across cells in *bcd nos tsl* ($n = 67$ cells in three embryos). (K) Amplitude of oscillation of the rate of cell-length change along the AP (blue, $n = 52$ cycles) and DV (red, $n = 53$ cycles) axes in *bcd nos tsl* ($P = 0.62$).

Figure 2.

Anisotropic oscillatory behavior requires intact adherens junctions. (A) Confocal sequence demonstrating three cycles of contraction and expansion of a cell expressing Spider:GFP in an embryo injected with α -catenin double-stranded RNA. Scale bar = 5 μ m. Anterior left, ventral down. (B) Rate of change of the apical area for three cells in embryos injected with α -catenin double-stranded RNA. Green line corresponds to the cell in (A). (C) Amplitude of oscillation of the rate of cell-length change along the AP (blue, $n = 58$ cycles) and DV (red, $n = 70$ cycles) axes in embryos injected with α -catenin double-stranded RNA ($P = 0.43$).

Figure 3.

Intercalating cells oscillate independently from their neighbors, with a slight bias toward anti-phase oscillations. (A) Algorithm to determine whether cells oscillate in phase or in anti-phase. (B) Correlation coefficient between the rate of change of the apical area of neighboring cells. Each dot represents one pair of neighbors ($n = 969$). The value of the X coordinate was arbitrarily assigned to facilitate data visualization. (C) and (D) Distribution of the time shift necessary to obtain the minimum (C) or maximum (D) correlation between neighbors. (E) Output of segmentation and tracking algorithms depicting the oscillation of cells during axis elongation. Red cells are contracting, blue cells are expanding. Asterisks indicate cells surrounded by neighbors in anti-phase. (F) Monte Carlo analysis of clusters of contracting cells in a representative embryo. The red line indicates the average fraction of neighbors of a contracting cell that are also contracting *in vivo*. The shaded area delimits the

95% confidence interval for the range of values obtained by randomly assigning cells as contracting or expanding at each time point according to the *in vivo* frequencies of these two cell types. (G) Monte Carlo analysis of the distribution of contracting cells. Red bars indicate the *in vivo* distribution of the fraction of neighbors of contracting cells that are also contracting ($n = 188$ cells at 16 time points selected 3 min apart in six embryos). Blue bars display the distribution obtained after randomly assigning cells as contracting or expanding ($n = 376\,000$ cells in 32 000 simulations). The number under each pair of bars indicates the lower limit of the bin represented by those bars, with the upper limit being the number under the pair of bars to the right (or 1.0 for the rightmost pair).

Figure 4.

Medial myosin II pulses are strongly correlated with oscillatory cell behavior in intercalating cells. (A) Rate of cell-area change in control embryos injected with water. Each line represents a single cell ($n = 15$ cells in three embryos). (B) Rate of cell-area change in embryos injected with the Rho-kinase inhibitor Y-27632 ($n = 15$ cells in 3 embryos). (C) Amplitude of oscillation of the rate of cell area change in embryos injected with water (blue, $n = 29$ cycles) or Y-27632 (red, $n = 15$ cycles). (D) Myosin II localization (Sqh:mCherry, red) in cells labeled with E-cadherin:GFP (green). Arrows indicate junctional myosin, triangles indicate medial myosin. (E) Confocal sequence demonstrating a discrete bending site on a cell-cell junction (arrow) after assembly of a medial myosin II structure (triangle). Cells express E-cadherin:GFP (green) and Sqh:mCherry (red). (F) Overlay of confocal images acquired immediately before (cyan) and 5 s after (red) laser ablation of the medial cortex. (G) Overlay of confocal images acquired immediately before (cyan) and 5 s after (red) laser ablation of an interface between anterior and posterior cells that is part of an

aligned contractile structure. (*F*) and (*G*) Colocalization is white. Cells are labeled with E-cadherin:GFP. Yellow circle indicates the $6\ \mu\text{m}$ radius region around the ablation site (white cross) used to quantify the tension relieved by ablation. (*H*) Relative magnitude and mode of propagation of forces generated by different contractile structures in myosin-rich AP interfaces (blue, $n = 13$), the medial cortex (red, $n = 22$), and myosin-depleted DV interfaces (green, $n = 11$). Left: peak recoil velocity after ablation within a $6\ \mu\text{m}$ radius of the ablation site. Right: peak recoil velocity after ablation at different orientations with respect to the AP axis of the embryo. (*I*) Confocal sequence demonstrating three cycles of contraction and expansion of an intercalating cell expressing E-cadherin:GFP (green) and Sqh:mCherry (red). (*J*) Rate of change of apical area (red) and medial myosin (blue) for the cell shown in (*I*). (*K*) Correlation coefficient between the rate of apical area change and the rate of change of junctional (blue) or medial (red) myosin. Each dot represents one cell ($n = 401$ cells in six embryos). The value of the X coordinate was arbitrarily assigned to facilitate data visualization. (*L*) Mean correlation coefficient ($n = 401$ cells) between the rate of apical area change and the rate of junctional (blue) or medial (red) myosin change. The X -axis indicates the time offset applied to the myosin values. An offset of 0 corresponds to the average of the data shown in (*K*). (*M*) Changes in normalized medial myosin II concentration with respect to the normalized apical surface. Normalization is to the average value per cell. Small dots represent single cells at individual time points ($n = 123\ 508$ measurements in 401 cells). Large dots and error bars indicate the mean and standard deviation of measurements binned every 0.05 normalized area units. (*N*) Rate of change of the apical area (red) and total medial myosin (blue) for a representative cell. (*O*) Probability of erroneously reporting no correlation between the cell area and medial myosin II changes as a function of the number of cells analyzed. Cells were randomly selected from (*K*). Scale bars = $5\ \mu\text{m}$. Anterior left, ventral down.

Figure 5.

Medial actin dynamics are associated with the assembly of medial contractile structures. (A) Confocal sequence demonstrating three cycles of contraction and expansion of an intercalating cell expressing GFP:Moe (green in (A), grayscale in (A')) and Sqh:mCherry (red in (A), grayscale in (A'')). Scale bar = 5 μm . Anterior left, ventral down. (B) Rate of change of the apical area (black), medial actin (green) and medial myosin II (red) for the cell shown in (A). (C). Correlation coefficient between the rate of apical area change and the rate of change of medial actin (green) or medial myosin II (red). Each dot represents one cell ($n = 69$ cells in 5 embryos). The value of the X coordinate was arbitrarily assigned to facilitate data visualization. (D) Mean correlation coefficient ($n = 69$ cells) between the rate of apical area change and the rate of change of medial actin (green) or medial myosin II (red). The X-axis indicates the time offset applied to the actin and myosin values. An offset of 0 corresponds to the average of the data shown in (C).

Figure 6.

Increased medial/junctional myosin ratio is associated with abnormal oscillatory behaviors in *bcd nos tsl* mutants. (A) Myosin II localization in germband cells in a wild-type embryo. Sqh:GFP demonstrates the localization of myosin II predominantly at the junctional domain of the cells. (B) Myosin II localization in germband cells in an embryo mutant for *bcd nos tsl*. Sqh:GFP demonstrates the localization of myosin II, with a more diffuse pattern than in wild type. (A) and (B). Scale bar = 10 μm . Anterior left, ventral down. (C) Overlay of confocal images immediately before (cyan) and 5 s after (red) laser ablation of the medial cortex in wild type (top) or *bcd nos tsl* (bottom). Colocalization is white. Cells labeled with E-cadherin:GFP. Scale bar = 5 μm . Anterior left, ventral down. (D) Peak recoil velocity after ablation at the medial cortex in wild type (blue, $n = 22$ cells), and *bcd nos tsl* (red, $n = 15$). (E) Rate of change of the apical area for three cells in *bcd nos tsl* mutants. (F) Distribution of amplitudes of oscillation in wild type (blue bars, $n = 470$ cycles) and *bcd nos tsl* (red bars, $n = 47$ cycles). The number under each pair of bars indicates the lower limit of the bin represented by those bars, with the upper limit being the number under the pair of bars to the right (or 30 for the rightmost pair). Amplitude was normalized to the mean area of the corresponding cell.

Figure 7.

Architecture-based regulation of contractile actomyosin networks. The cartoon displays a model previously proposed for the regulation of the contractile ring at the cleavage furrow of dividing cells (top, (Carvalho *et al* 2009)) and the model proposed here to link medial meshwork dynamics with the patterns of oscillatory behavior observed in intercalating, amnioserosa and mesoderm cells (bottom). In the cleavage furrow model, the contractile ring is assembled by contractile units which have initially the same size. The number of contractile units per ring is proportional to the size of the ring, and the rate of contraction of each unit is constant. Thus, the rate of contraction of the ring scales inversely with its diameter, and the time necessary for contraction of the ring is independent of its initial size. In the medial meshwork model, actomyosin filaments are polymerized and depolymerized at constant rates. The time necessary to assemble and disassemble a contractile meshwork that spans the medial cortex is proportional to the apical surface of the cell, allowing smaller cells to oscillate faster than larger cells.