Title
Coordination of protrusion dynamics within and between collectively migrating border cells by myosin II

Permalink
https://escholarship.org/uc/item/35c8t375

Journal
Molecular Biology of the Cell, 30(19)

ISSN
1059-1524

Authors
Mishra, Abhinava K
Mondo, James A
Campanale, Joseph P
et al.

Publication Date
2019-09-01

DOI
10.1091/mbc.e19-02-0124

Peer reviewed
Coordination of protrusion dynamics within and between collectively migrating border cells by myosin II

Abhinava K. Mishra, James A. Mondo, Joseph P. Campanale, and Denise J. Montell*
Molecular, Cellular, and Developmental Biology Department, University of California, Santa Barbara, Santa Barbara, CA 93106

ABSTRACT Collective cell migration is emerging as a major driver of embryonic development, organogenesis, tissue homeostasis, and tumor dissemination. In contrast to individually migrating cells, collectively migrating cells maintain cell–cell adhesions and coordinate direction-sensing as they move. While nonmuscle myosin II has been studied extensively in the context of cells migrating individually in vitro, its roles in cells migrating collectively in three-dimensional, native environments are not fully understood. Here we use genetics, Airyscan microscopy, live imaging, optogenetics, and Förster resonance energy transfer to probe the localization, dynamics, and functions of myosin II in migrating border cells of the Drosophila ovary. We find that myosin accumulates transiently at the base of protrusions, where it functions to retract them. E-cadherin and myosin colocalize at border cell-border cell contacts and cooperate to transmit directional information. A phosphomimetic form of myosin is sufficient to convert border cells to a round morphology and blebbing migration mode. Together these studies demonstrate that distinct and dynamic pools of myosin II regulate protrusion dynamics within and between collectively migrating cells and suggest a new model for the role of protrusions in collective direction sensing in vivo.

INTRODUCTION Collective cell migration is essential for normal embryonic development and tissue homeostasis. It is also emerging as a major mechanism facilitating tumor metastasis (Friedl and Gilmour, 2009; Ewald et al., 2012; Cheung et al., 2013, 2016; Cheung and Ewald, 2014; Aceto et al., 2014; Richardson et al., 2018). Border cells in the Drosophila ovary provide an excellent model for studying fundamental mechanisms of collective cell migration in vivo (Friedl and Gilmour, 2009; Montell et al., 2012). Border cells are a group of six to eight epithelial follicle cells that migrate roughly 150 µm in between large cells called nurse cells during stage 9 of oogenesis (Figure 1, A and B). The border cell cluster is composed of a central pair of nonmigratory polar cells, which secrete the cytokine Unpaired (Upd). Upd activates Jak/STAT signaling and motility in surrounding cells (Silver and Montell, 2001). Once specified, border cells round up and surround the polar cells. One or two border cells extend Rac-dependent protrusions between the nurse cells to initiate migration (Murphy and Montell, 1996; Fulga and Rørth, 2002; Prasad and Montell, 2007). Over the course of 3 to 6 h, the cluster migrates posteriorly toward the oocyte, arriving at the border by stage 10 (Figure 1C). There, they cooperate with other cells to form an eggshell structure called the micropyle (Montell et al., 1992), which is the site of sperm entry. Thus, border cell migration is essential for fertility.

Some mechanisms of collective cell migration differ from those of single cells. For example, E-cadherin (Ecad) acts as a migration-suppressor in the context of the epithelial to mesenchymal transition (Onder et al., 2008). However, the gene coding for E-cad, CDH1, is rarely deleted in human cancer (cbioportal.org). E-cad expression is maintained and required in collectively invasive mammary tumors (Rakha et al., 2013; Shamir and Ewald, 2015;
Distributions of E-cad and F-actin (labeled by phalloidin) before, during, and after border cell migration. (A–C) Maximum intensity projections of wild-type egg chambers labeled with E-cad (magenta), F-Actin (green), and Hoechst (blue) during stages 9 and 10 of Drosophila egg chamber development. (A) Border cells (white arrows) initiating migration. (B) Border cells in mid-migration between nurse cells. Dashed yellow line indicates their migration path. (C) Border cells reach the oocyte border by stage 10. (D–I) Zoomed stills from time-lapse images of border expressing Lifeact-GFP driven by an upd enhancer (green) and polar cell nuclear DsRed (DsRed.nls, magenta) driven by an slbo enhancer. Polar cells marked with “p.” Border cells (D–F) extend and retract protrusions (white arrows), prior to a single leader cell forming a dominant protrusion to lead the cluster in G–I delaminating from the anterior epithelium (white arrows). Numbers in G–I denote hours and minutes. All images are oriented anterior on the left and posterior on the right. Scale bars in A–C and D–I are the same. All scale bars are 20 µm.

**FIGURE 1:**

During border cell migration, E-cad performs three essential functions (Niewiadomska et al., 1999; Cai et al., 2014). E-cad-mediated adhesion between polar cells and outer migratory cells maintains cluster cohesion and thus collective motility (Cai et al., 2014), which is important because individual cells migrate less efficiently than clusters (Cai et al., 2016). E-cad-mediated adhesion between the lead border cell and nurse cells participates in a positive feedback loop with guidance receptor signaling, the small GTPase Rac, and actin polymerization to generate the large leading protrusions that initiate and guide border cell migration. Finally, E-cad-mediated adhesion between individual border cells communicates direction from the leader to the following cells of the cluster to maintain coordinated movement (Wang et al., 2010; Cai et al., 2014). Mechanical coupling between leader and follower cells by classical cadherins has subsequently also been proposed for collectively invading tumor cells and tumor/stroma cell groups (Veracini et al., 2015; Labernadie et al., 2017).

The work by Cai et al. (2014) predicts that as the lead border cell protrudes and moves forward, it pulls on the following cells. Furthermore, the proposed model predicts that E-cad-mediated adhesions between border cells transmit force from cell to cell leading to inhibition of Rac activity in followers and thus reducing their probability of protrusion. One candidate for force transduction is the actomyosin cable that connects individual cells through cell–cell junctions. Therefore we set out to test the function of nonmuscle myosin II (hereafter myosin II) in communication of direction between border cells. Other roles for myosin in border cell migration have previously been described, including detachment of the cluster from the anterior end of the egg chamber (Majumder et al., 2012) and maintenance of cluster morphology during migration (Aranjuez et al., 2016). Here, we report previously unrecognized localizations and functions for myosin II during border cell migration. We use Airyscan microscopy, live imaging, RNA interference (RNAi), photoactivatable Rac, and a Rac FRET activity sensor to probe the diverse [AQ 2] and dynamic effects of myosin II on collective cell migration. We propose an integrated model for the multiple roles of dynamic myosin II activity in coordinating collective border cell migration.

**RESULTS**

**Dynamic localization of myosin**

Using confocal microscopy, we observed border cell migration live and at high spatio-temporal resolution during delamination (Figure 1; Supplemental Movie S1). The first morphological changes after border cell fate specification are that the cluster rounds up and multiple cells extend and retract actin-rich protrusions for ∼1 h (Figure 1, D–F). Eventually a single leader cell with a dominant forward protrusion emerges (Figure 1G). As the lead cell moves forward, additional cells delaminate from the epithelium (Figure 1H), ultimately detaching from the epithelial cells that remain behind (Figure 1I).

Since myosin II assembles cooperatively on contractile filaments, accumulating to its highest levels at sites where it is active (Lehara et al., 2010), we examined its localization together with F-actin during border cell migration. We used a fluorescently tagged form of myosin light chain, known in Drosophila as Spaghetti squash (Sqh). The Sqh-mCherry fusion protein is expressed under the endogenous genomic regulatory sequences and is fully functional (Martin et al., 2009). Like E-cad (Cai et al., 2014), Sqh-mCherry accumulates to higher levels in somatic cells than in the germline (Figure 2, A–D) even though the germline contains high levels of F-actin (Figure 2, A and B). The Sqh-mCherry protein is present in border cells throughout their migration and is enriched near the apical surfaces of all follicle cells (Figure 2, A–D) including polar cells.
FIGURE 2: Spaghetti squash (sqh) distribution during border cell migration. (A–D) Maximum intensity projections of fixed egg chambers at stages 9 (A, C) and 10 (B, D) labeled with phalloidin for F-actin (green), Hoechst (blue), and expressing Sqh-mCherry expressed from its endogenous promoter and stained using an anti-mCherry antibody. Arrow indicates border cell position and arrowhead indicates apical surfaces of posterior follicle cells (C). (E–M) High-magnification maximum intensity projections of Sqh-mCherry localization during delamination (E, H, K), midmigration (F, I, L), and at the completion of migration (G, J, M). White arrows indicate the accumulation of Sqh-mCherry at the base of protrusions (E), at the cluster periphery (F), and the polar cell (p) apical surfaces facing the oocyte after docking at stage 10 (G). Scale bars in A–D and E-M are same. All scale bars are 20 µm.

(Figure 2E). A similar pattern is observed with a Sqh-GFP and Sqh-TS::GFP (Supplemental Figure S1), although the apical polar cell labeling is more prominent with the mCherry fusion. Knockdown of Sqh by RNAi in polar cells does not result in a detectable phenotype (Majumder et al., 2012), but the Sqh accumulation serves as a useful marker of polar cell position and the apical side of the cluster.

In fixed images of outer, migratory border cells, the pattern of Sqh-mCherry only partially overlaps with F-actin (Figure 2, H–M). The Sqh-mCherry pattern is not identical from one cluster to the other, suggesting it is dynamic. We noted prominent accumulation of Sqh-mCherry at the base of protrusions (Figure 2, E and K). In addition, patches of Sqh-mCherry are evident at the periphery of some clusters during migration (Figure 2, F and L), consistent with a previous report of dynamic Sqh flashes during migration (Aranjuez et al., 2016). At the end of migration, myosin accumulates apically in the cluster at the border cell/oocyte interface (Figure 2, G and M).

To determine to what extent myosin colocalizes with E-cad in migrating border cells, we labeled Sqh-mCherry–expressing clusters with anti–E-cad antibody and imaged them using confocal microscopy. E-cad and Sqh-mCherry colocalized extensively (Figure 3, A–C). Furthermore, Airyscan imaging of fixed samples with amplified GFP and mCherry signals at high lateral and axial resolution revealed a high degree of E-cad and Sqh colocalization at cell–cell junctions within the border cell cluster and the apical surfaces of polar cells (Figure 3, D–I; Supplemental Figure S2).

To compare their dynamics we took z-stacks of Sqh and E-cad for 8 min (Supplemental Movies 2 and 3). To limit phototoxicity, low laser intensities were used, so only the brightest pools of myosin and E-cad were detected (Figure 3, J, K, M, and N). Kymographs from representative clusters show junctional Sqh-mCherry puncta that appear and disappear with a half-life of 30 s (Figure 3L), whereas E-cad is stable for at least 20 min (Figure 3O and unpublished data). Thus, live imaging revealed myosin to be more dynamic than E-cad.

Requirement for myosin II in cell–cell communication
Since Sqh and E-cad colocalize, and E-cad is proposed to mechanically couple border cells, we tested whether myosin also contributes to mechanical coupling. To test the hypothesis that myosin activity mechanically couples lead cells to followers, we inhibited myosin expression or activity in three different ways (Figure 4, A–E). First, we knocked down expression of the light chain using RNAi (Figure 4B). Second, we expressed a nonphosphorylatable form of the light chain (Figure 4C), which likely acts as a dominant-negative (Jordan and Karess, 1997). Finally, we blocked expression of the myosin heavy chain by temperature-sensitive alleles of the heavy chain (Majumder et al., 2012).
chain, known as Zipper (Zip) using RNAi (Figure 4D). Each of these manipulations resulted in a significant fraction of clusters displaying multiple ectopic protrusions (Figure 4E). If myosin-mediated contractility couples the lead cells to the followers, then we would expect that wild-type follower cells in contact with a Sqh-deficient leader would exhibit excess protrusions. To test this, we generated border cell clusters composed of a mixture of wild-type and Sqh RNAi-expressing cells (Figure 4, F–L). Wild-type follower cells indeed exhibited excess protrusions when in contact with Sqh-depleted lead cells (Figure 4, G and L; Supplemental Figure S3; Supplemental Movies S4 and S5). Live imaging of clusters with reduced Sqh showed an overall higher frequency of ectopic side protrusions that are longer lived than those observed in wild-type controls (Supplemental Figure S4; Supplemental Movies S6–S8).

Normally, protrusions from the lead cell (the cell closest to the oocyte) are longer and longer-lived than protrusions from other cells of the cluster (Prasad and Montell, 2007). The small GTPase Rac is essential for border cell protrusion and migration (Murphy and Montell, 1996), and its activity is highest in protruding cells (Wang et al., 2010). Moreover, focal stimulation of a photoactivatable form of Rac (PA-Rac) in one cell is sufficient to steer the entire cluster (Wang et al., 2010). PA-Rac in the lead cell accelerates forward-directed movement, whereas Rac activation in the rear cell reverses the direction of cluster movement (Wang et al., 2010) (Figure 5, A and B). In both cases, Rac activation in one cell stimulates protrusion in the activated cell and inhibits protrusion of other cells (Wang et al., 2010) (Figure 5, A and B). Thus, the protruding cell steers the whole cluster. E-cad is essential for this cell–cell communication (Cai et al., 2014). To test the hypothesis that myosin is similarly required, we expressed PA-Rac together with sqh RNAi and photoactivated Rac in the rear cell. Protrusions were defined and quantified as previously described (Wang et al., 2018). Inhibition of Sqh resulted in multiple protrusions, not only in the stimulated cell but also from other cells (Figure 5, C and E). We conclude that the protruding cell inhibits protrusion in following cells in a myosin II-dependent manner. Results were similar for clusters imaged near the beginning of their migration (Figure 5, C–E) or near the end (Supplemental Figure S5).
Myosin coordinates collective migration

To investigate the mechanism of this myosin-mediated protrusion restriction, we evaluated the effects of altering myosin expression or activity on the pattern of Rac activation in border cell clusters (Figure 5, F–L). In wild-type clusters, Rac activity is highest in protrusions (Wang et al., 2010), specifically while they are extending. Lead cell protrusions typically show the highest Rac activity (Figure 5F), whereas nonprotruding clusters do not exhibit higher Rac activity in the lead cell (Figure 5G). To test the effect of myosin on the distribution of Rac activity, we expressed the established Rac FRET probe in migratory control clusters to those with reduced E-cad expression (Figure 6, A–H). Multiple validated RNAi lines which have varying potencies are available for E-cad (Supplemental Table S1). Since border cells with complete E-cad knockdown rarely migrate (Niewiadomska et al., 1999; Cai et al., 2014), we carried out experiments at low temperature (18°C) that produces a mild migration defect to analyze the effect on myosin. Although this approach risks underestimating the phenotypic effect, we thought it was important to compare migratory control and knockdown clusters rather than compare migratory control clusters to immobile E-cad knockdown clusters. Upon partial E-cad knockdown, we observed both protrusive clusters and rounded clusters, as in controls (Figure 6, A–H; Supplemental Movies S9–S14). Clusters of both morphologies exhibited significantly reduced cortical myosin compared with controls (Figure 6I). To quantify the effect, we used laser scanning confocal imaging to capture time-lapse movies of migrating border cells labeled with Lifeact-GFP and Sqh-mCherry. Using Imaris image analysis software, we segmented the border cell cluster based on the Lifeact-GFP channel and then isolated the cluster perimeter (Supplemental Movie S15). We used the Sqh-mCherry channel to measure cortical myosin levels normalized to the Sqh-mCherry signal in the nurse cells adjacent to the border cell cluster, to correct for photo-bleaching. Owing to the normal dynamic fluctuations of cortical myosin, there is great variation in the cortical myosin intensity in wild-type clusters (Figure 6I). E-cad knockdown reduced the overall levels and fluctuations of cortical myosin (Figure 6I). Figure 6J illustrates schematically the effects of low versus high cortical myosin both in control and E-cad knockdown clusters. Together these results show that recruitment of myosin requires E-cad–mediated adhesion between border cells.

To assess whether myosin also exerts an effect on E-cad and/or cell-cell contacts, we compared the localization of E-cad in control and sqh RNAi–expressing border cells (Figure 6, K–N). We used the FLPout technique (see Materials and Methods) so clusters were composed of a mixture of GFP-positive and GFP-negative cells. In control clusters in which both GFP+ and GFP− cells express normal levels of Sqh, E-cad labeling of the apical polar cell domain and border cell/border cell contacts are evident (Figure 6, K and L). In clusters in which the GFP+ cells also express sqh RNAi, border cell/border cell contacts appeared irregular in temporal regulation of myosin activity is essential to establish asymmetric Rac activity.

**Myosin distribution depends on E-cad**

Since myosin and E-cad colocalize and function in cell–cell communication, we asked whether myosin is recruited to cell–cell contacts in an E-cad-dependent manner. We compared the distribution of Sqh-mCherry in control clusters to those with reduced E-cad expression (Figure 6, A–H). Multiple validated RNAi lines which have varying potencies are available for E-cad (Supplemental Table S1). Since border cells with complete E-cad knockdown rarely migrate (Niewiadomska et al., 1999; Cai et al., 2014), we carried out experiments at low temperature (18°C) that produces a mild migration defect to analyze the effect on myosin. Although this approach risks underestimating the phenotypic effect, we thought it was important to compare migratory control and knockdown clusters rather than compare migratory control clusters to immobile E-cad knockdown clusters. Upon partial E-cad knockdown, we observed both protrusive clusters and rounded clusters, as in controls (Figure 6, A–H; Supplemental Movies S9–S14). Clusters of both morphologies exhibited significantly reduced cortical myosin compared with controls (Figure 6I). To quantify the effect, we used laser scanning confocal imaging to capture time-lapse movies of migrating border cells labeled with Lifeact-GFP and Sqh-mCherry. Using Imaris image analysis software, we segmented the border cell cluster based on the Lifeact-GFP channel and then isolated the cluster perimeter (Supplemental Movie S15). We used the Sqh-mCherry channel to measure cortical myosin levels normalized to the Sqh-mCherry signal in the nurse cells adjacent to the border cell cluster, to correct for photo-bleaching. Owing to the normal dynamic fluctuations of cortical myosin, there is great variation in the cortical myosin intensity in wild-type clusters (Figure 6I). E-cad knockdown reduced the overall levels and fluctuations of cortical myosin (Figure 6I). Figure 6J illustrates schematically the effects of low versus high cortical myosin both in control and E-cad knockdown clusters. Together these results show that recruitment of myosin requires E-cad–mediated adhesion between border cells.

To assess whether myosin also exerts an effect on E-cad and/or cell-cell contacts, we compared the localization of E-cad in control and sqh RNAi–expressing border cells (Figure 6, K–N). We used the FLPOut technique (see Materials and Methods) so clusters were composed of a mixture of GFP-positive and GFP-negative cells. In control clusters in which both GFP+ and GFP− cells express normal levels of Sqh, E-cad labeling of the apical polar cell domain and border cell/border cell contacts are evident (Figure 6, K and L). In clusters in which the GFP+ cells also express sqh RNAi, border cell/border cell contacts appeared irregular in temporal regulation of myosin activity is essential to establish asymmetric Rac activity.
8/36 samples examined (Figure 6, M and N) compared with the relatively smooth contacts between control cells in 29/29 clusters (Figure 6, K and L). The remaining 28 sqh RNAi samples were not clearly distinguishable from wild type. These results suggest that there is normally actomyosin-mediated tension maintaining border cell-border cell contacts, as in epithelial monolayers in vitro (Warner and Longmore, 2009; Acharya et al., 2018; Charras and Yap, 2018).

Myosin functions in retraction of lead protrusions

We noted prominent but transient accumulation of Sqh-mCherry at the base of protrusions (Figures 2E and 7A; Supplemental Movies S16 and S17), which has not previously been described. We carried out live imaging and found that, as protrusions approach their maximal extension, Sqh-mCherry accumulates and is followed by protrusion retraction. To quantify this effect, we measured the change in protrusion length ($\Delta L$) per unit time (Figure 7B). Positive $\Delta L$ indicates...
FIGURE 6: Mutual requirement for E-cad and Sqh. (A–H) Stills from time-lapse imaging of clusters coexpressing Lifeact-GFP under the control of the slbo enhancer and Sqh-mCherry from its endogenous promoter. (A–D) Control clusters expressing UAS-white RNAi in protrusive (A, B) and round (C, D) clusters. (E–H) Border cells expressing UAS-E-cad RNAi in protrusive (E, F) and round (G, H) clusters. All genotypes include c306-Gal4 and were incubated at 18°C. (B, D, F, H) Myosin-only channel. (I) Box plot comparing the average cortical myosin intensity in control vs. E-cad RNAi–expressing clusters. n refers to the total number of frames measured from six control and nine E-cad RNAi time-lapse movies. (J) Schematic representation of the effects of varying the level of myosin in both protrusive and round clusters. (K–N) Flipout-Gal4–expressing cells (labeled by GFP antibody in green) in clusters expressing control, UAS-white RNAi (K, L), or UAS-sqh RNAi (M, N) stained for E-cad (magenta). Arrow indicates a discontinuity in the junction as observed in the indicated fraction of clonal clusters. Scale bars in A–H and K–N are the same. All scale bars are 20 µm. Data were analyzed using **p < 0.0001.
protrusion while negative values of $\Delta L$ represent retraction. Plotting myosin intensity as a function of the rate of change of protrusion length demonstrates a positive correlation between myosin accumulation and retraction and a negative correlation with protrusion (Figure 7C).

This observation forces a reevaluation of the functions of protrusions. On the basis solely of imaging fixed tissue, Fulga and Rørth (2002) suggested that protrusions function as a grapple to pull the cluster forward (Schober and Perrimon, 2002). However such a model implies that the tip of the protrusion adheres strongly to the substrate and would not retract but rather would be subsumed into the advancing cluster. We observed that 130/162 protrusions retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and that nonprotruding clusters will not advance. We quantified cluster advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grappleples. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grapplers. The grapple and pull model further predicts that clusters will advance most rapidly when protrusions are maximally extended and retracted, suggesting that most protrusions are not effective grappler.
implying that the junctions are normally under tension. Myosin also cooperates with E-cad to communicate direction from the lead cell to followers. The nonautonomous function and the autonomous function within protrusions cooperate to restrict follower cells from protruding. Together with the previously published role for myosin in detachment of the cluster from the anterior, these findings suggest an integrated model (Figure 9).

**Rethinking the functions of protrusions**

A well-accepted general model for cell motility (Lauffenburger and Horwitz, 1996), based primarily on observations of fibroblast-like cells migrating on hard surfaces in vitro, is that cells protrude via actin polymerization at the front and adhere through nascent adhesions with the substrate, which mature into focal adhesions that anchor F-actin stress fibers. Actomyosin-mediated contraction of the stress fibers then leads to retraction of the cell rear. Some difference between the front and the back must exist so that rear adhesions are preferentially broken. Calpain-mediated cleavage of talin, for example (Cox and Huttenlocher, 1998), specifically at the rear, may account for such differences at least in some cells and circumstances. Quantitative studies of actin dynamics led to a refinement of this model (Ponti et al., 2004). Cells cultured on hard flat surfaces produce a leading lamellipodium composed of treadmilling actin filaments that rapidly polymerize and depolymerize, causing random protrusion and retraction but not substantial adhesion. One to three micrometers behind the leading edge, the lamellum forms where focal adhesions serve as a clutch to anchor F-actin filaments. It is in the lamellum where productive adhesions form, which are necessary for the cell to advance.

It has been unclear how these mechanisms relate to those of cells like border cells that move in three-dimensional environments, surrounded on all sides by pliable matrix or other cells. The shapes of the border cell protrusions are clearly different from the broad and flat lamellipodia and lamella that Ponti studied using speckle microscopy (Ponti et al., 2004). An earlier border cell study based only on fixed tissue analyses proposed a grapple and pull model for initiation of border cell migration by forward-directed protrusions (Fulga and Rørth, 2002). This idea is similar to the original protrusion/adhesion/retraction model for fibroblast migration in vitro (Lauffenburger and Horwitz, 1996). However, the results presented here rule out the grapple and pull model as the major mechanism of forward advancement and suggest an alternative. The grapple and pull model proposes that the border cells extend a long protrusion and grip the substrate at the tip. Then the cell body pulls itself toward the tip, absorbing the protrusion into the cell body in the process. According to such a model, protrusions should not retract and the cluster should reach the furthest extent of each
protrusion in one step, accelerating as they do. By contrast, the results reported here show that border cells accumulate myosin at the base of a fully extended protrusion and then retract the great majority of protrusions before crawling into the space vacated by them. The cluster continues to crawl forward at a relatively constant speed even as the protrusion retracts. We propose that rather than functioning as a grapple or attachment site, the tip of the protrusion functions as a sensory organ. In this model, protrusions in any direction probe the external environment. Protrusions that encounter a more favorable environment, such as a higher concentration of chemoattractants and/or physical openings, are able to extend further before retracting. Consistent with this idea, all border cells actively crawl to propel the cluster forward. For example, clusters composed of mixtures of wild-type and motility-deficient cells migrate better the more wild-type cells they possess (Silver et al., 2005; Xiang et al., 2016). If only the lead cell pulled the followers forward, one wild-type cell should suffice. Moreover, “cryptic” lamellipodia have been observed in between border cells (Cliffe et al., 2017). These may serve a crawling function similar to the basal protrusions found in all follicle cells at earlier stages, which drive collective migration of the entire epithelium (Barlan et al., 2017).

Similar to the lamellipodium and lamellum described for individual cells migrating on two-dimensional hard surfaces (Ponti et al., 2004), border cells exhibit dynamic protrusion and retraction at the very leading edge. However, the cells are surrounded by other cells and the protrusion is shaped more like a spear than the fan-like lamellipodium/lamellum of single cells migrating unopposed on glass. Nevertheless, though superficially disparate structures, both spear- and fan-shaped protrusions appear to be divided into functionally similar domains of related function: an actin-rich/myosin-poor tip and, a few micrometers further back, a region where actomyosin accumulates and generates contractile force. While we propose that the protrusions probe the microenvironment, productive adhesion between border cells and nurse cells may occur at the base of the protrusion where the actomyosin accumulates, analogous to the lamellum region of a fibroblast. A few differences, in addition to shape, are that the tip of the border cell protrusion must find or make space in between tightly apposed cells, and the whole spear-shaped structure appears and disappears dynamically.

Myosin in cell-cell communication

The observations that myosin colocalizes with E-cad at border cell-border cell contacts, depends on E-cad and is required for communication of direction between the leader cell and followers demonstrate that myosin and E-cad cooperate in collective direction sensing. These findings support the model that mechanical coupling between leaders and followers mediates collective direction sensing (Cai et al., 2014), though the mechanism by which Rac activity and protrusion are inhibited in follower cells remains to be clarified. Since adhesions between collectively invading cancer cells are also under mechanical load (Labernadie et al., 2017), it is likely that the role of myosin II described here also applies in that context.

Peripheral myosin flashes

Aranjuez et al. (2016) noted seemingly random flashes of myosin at the border cell periphery and proposed that myosin activity somehow pushes outward to resist inward forces from nurse cells. These authors also proposed that myosin pulls inward to maintain the cluster’s rounded morphology. The results presented here are more consistent with the latter idea than the former. The experiments with photoactivatable Rac show that in the absence of myosin, the border cells are capable of extending protrusions in between nurse cells, despite any opposing forces that nurse cells might exert. The myosin knockdown experiments show that myosin normally prevents protrusion in follower cells and mechanically couples leaders and followers to keep all the cells moving in one direction in a coordinated manner. These phenotypes are similar to knockdown of E-cad (Cai et al., 2014, and this study) or of moesin (Ramel et al., 2013), which is an F-actin-binding protein that couples cortical F-actin to the plasma membrane. Together these observations suggest that mechanical coupling between border cells mediated by E-cad and actomyosin coupling keep the cells moving in one direction. The peripheral myosin flashes apparently constrain protrusion, which is consistent with, and a refinement of, the interpretation of Aranjuez et al. (2016) that the myosin flashes maintain cluster shape.

Mesenchymal to amoeboid transition in vivo

Cells can migrate using diverse modes (Friedl and Wolf, 2010). They can move as individual cells or as collectives. They can move in a mesenchymal mode or an amoeboid mode. Mesenchymal migration refers to a mode in which cells extend lamellar protrusion and an elongated morphology. They extend protrusions, translocate the cell body, and pull up the rear. In contrast, so-called amoeboid migration is initiated by contraction of the actomyosin cortical cytoskeleton. When the weakest point on the cortex breaks, cytoplasm shoots out forming a bleb. Actin polymerizes into the bleb to stabilize the protrusion and then the rest of the cell moves forward. Transitions between mesenchymal and amoeboid migration have been reported for cancer cells cultured in vitro (Wolf et al., 2003; Wolf and Friedl, 2006; Friedl, 2004; Panková et al., 2010; Yilmaz and Christofori, 2010; Liu et al., 2015). However whether or not normal cells in a native environment can undergo such transitions has been unclear.

Although border cells retain epithelial apical/basal polarity as they move the protrusion morphologies and Rac-dependence, this suggests a "mesenchymal-like" mode of migration. The observation [AQ 8] that border cells undergo a dramatic transition to amoeboid...
migration following expression of a SqhE20E21 establishes that such transitions can occur in normal cells in vivo and that hyperactive myosin in the absence of manipulation of Rho or its downstream kinase Rock is sufficient to drive the transition. The high level of myosin activity also causes the cluster to break apart suggesting that while one level of tension on E-cad junctions is essential to maintain mechanical coupling of the cells, excessive myosin-mediated contraction breaks the cell–cell adhesions. This may be why blebbing-based motility is associated primarily with individually migrating cells such as primordial germ cells (Blaser et al., 2006) rather than collectively migrating cells. These observations suggest that collective, epithelial/mesenchymal border cell migration requires tuning of the magnitudes and vectors of forces generated by Rac, E-cad and myosin. Altering the balance changes their morphologies and behaviors. The distinct morphologies and behaviors of other collectively migrating cells such as the zebrafish lateral line and cranial neural crest may thus emerge from modulating the balance of cadherin-mediated cell–cell adhesion, Rho/Rock/myosin-mediated contractility, and Rac/Cdc42-mediated protrusion.

**MATERIALS AND METHODS**

**Drosophila strains and genetics**

The sqh-mcherry (59024), UAS-Rac-FRET (32050), UAS-mCherry-PA-RacG61L (32049), UAS-sqh.A20A21 (64114), UAS-sqh.E20E21 (64411), E-Cad-GFP (60584), slbo-Lifeact-GFP (58364), UAS-Rho1V14 (8144), and UAS-PLC1-PH-GFP (39693) fly strains used in this study were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The UAS-sqh RNAi (line 7917), UAS-zip RNAi (330299), and UAS-E-cad RNAi (103962) were obtained from Vienna Drosophila Resource Center (Vienna, Austria). The sqh;A3; sqh-Sqh:GFP (III) flies were a gift from Jocelyn McDonald (Kansas State University), and Adam Martin (Massachusetts Institute of Technology) kindly provided sqh-Ts::GFP lines (Vasquez et al., 2014). The upd-Gal4, UAS-DsRed.nls described previously (Xiang et al., 2016) was combined with slbo-Lifeact-GFP to perform time-lapse imaging shown in Figure 1, D–I. The slbo-Gal4 (Reth et al., 1998) driver was used to perform all the FRET and photoactivation experiments. The c306-Gal4; UAS-Lifeact-GFP, tub-GAL80ts line was combined using c306-Gal4; Manseau et al., 1997) driver with UAS-Lifeact-GFP (Cai et al., 2014) and tub-GAL80ts (Dai et al., 2017) for RNAi experiments shown in Figures 4, A–D, and 8, A–H, and Supplemental Figure S4, A–L. This driver activates UAS transgene expression early in development, allowing time for the RNAi to take effect. The hsFLP, AyGal4, UAS-sqh RNAi (described previously (Xiang et al., 2016) and hsFLP, AyGal4, UAS-RFP were used to generate clonal expression of sqh in border cells. Desired progeny for live imaging of sqh RNAi clones were obtained by crossing slbo-Lifeact-GFP, UAS-sqh RNAi to hsFLP, AyGal4, UAS-RFP fly stock (Supplemental Figure S3, A–H, and Supplemental Movies S4 and S5).

The upd-Gal4, UAS-DeRed.nls, sqh-mcherry and E-Cad-GFP flies were reared at 25°C and shifted to 29°C for 16–20 h prior to dissection for fixed and live imaging. The tub-GAL80ts were set up at 18°C. One-day-old progeny were collected and shifted to 29°C for 3 d before dissection. All the crosses for FRET and photo-manipulation experiments were set at 25°C. One-day-old progeny were collected, transferred on to a dry yeast containing vial, and shifted to 29°C for 16–20 h prior to dissection. For measurement of cortical myosin intensity, control and E-cad-RNAi clones were kept at 18°C to allow partial knockdown of E-Cad and border cell movement. For generating sqh clones in border cells, flies were heat-shocked twice a day for 1 h, −4 h apart, in a 37°C water bath. Flies were then kept at 29°C for 3 d prior to dissection.

**Immunostaining**

Adult female ovaries were dissected in a depression slide containing Schneider’s Drosophila medium supplemented with 20% fetal bovine serum. Individual ovarioles were pulled out of the muscle sheath using forceps as described (Prasad and Montell, 2007), and mature eggs were discarded. The dissected egg chambers were transferred to a microfuge tube and washed gently with fresh medium. Ovarioles were fixed for 20 min on ice in 4% paraformaldehyde. Postfixation ovarioles were washed with phosphate-buffered saline (PBS) containing 0.4% Triton X-100 (PBST) and incubated with primary antibodies overnight at 4°C. The following primary antibodies from Developmental Studies Hybridoma Bank were used: E-cad (DCAD2, 1:5) and mCherry (3A11, 1:100). Rabbit anti-GFP (G10362, Thermo Fisher) was used in 1:1000 dilution. The next day, ovarioles were washed in PBST and incubated in secondary antibodies for 2 h at RT. Alexa Fluor 488– and 568–conjugated secondary antibodies (Thermo Fisher) were used at 1:400 dilution and F-actin was labeled using Phalloidin-Atto 464 (Sigma Aldrich). Samples were washed in PBST and stained with Hoechst (Sigma Aldrich) for 20 min at RT. Egg chambers were washed again in PBST and mounted in Vectashield (Vector Laboratories, Burlingame, CA) before imaging.

**Airyscan imaging of E-cad and Sqh-mCherry**

To observe the colocalization of E-cad and Sqh-mCherry, high resolution airyscan images were collected in Zeiss 800 laser scanning microscope using a 63× Plan-Apochromat (1.4 NA) objective. Images were captured using the Airyscan detector array at 300 nm × 300 nm × 160 nm in the X, Y, and Z dimensions, respectively. Images were Airyscan processed using Zen Blue software (Zeiss).

**FRET imaging and analyses**

FRET images were obtained from cultured living egg chambers using a Zeiss LSM 780 microscope. A 458-nm laser was used for excitation of the sample. CFP and YFP images were collected simultaneously using channel 1 (464–502 nm) and channel 2 (517–570 nm) under a 40×/1.1 numerical aperture (NA) water immersion objective LD C-Apo lens. Single 16-bit optical sections of frame size 512 × 512 and 3.15 μm pixel dwell in the middle of the cluster were collected each time the cluster makes a protrusion or retracts the forward protrusion. CFP and YFP images were then processed using Fiji image analysis software as described before (Wang et al., 2010). Final YFP/CFP ratio image was generated in Fiji and divided into three equal parts, namely front, middle, and back from the front tip to the rear end of the cluster. The FRET index was calculated as the ratio of front to the back for both protrusive and nonprotrusive clusters. All imaging was performed during the first half of border cell migration.

**Photomanipulation of Rac**

Photoactivation of Rac and time-lapse imaging were performed using a Zeiss 780 Laser scanning confocal microscope using a 63×, 1.4 NA oil objective lens at 2× zoom. Photoactivation was done at the rear of the cluster in a 8-μm spot using a 458-nm laser set at 5% power with a pixel dwell of 101 μs. The scan was completed in ~30 s. Five cycles at 60-s intervals were carried out. Further, z-sections of 1.5 μm thickness spanning the entire border cell cluster were collected using a 568-nm laser. These steps were repeated for an entire time-lapse experiment for a period of 30 min. Maximum intensity projection images before and after photoactivation were generated in Fiji software and the protrusions per cluster after photoactivation were counted using the mCherry signal.
Protrusions were counted using the criteria described in Wang et al. (2018.)

Measurement of cortical intensity of myosin
Egg chambers were dissected and mounted as described previously (Prasad and Montell, 2007) and time-lapse imaging was performed using a 40x/1.1 NA water immersion objective. The 1.25-µm-thick z-sections spanning the entire border cell cluster were collected at 150-s intervals using channel 1 (499–553 nm) and channel 2 (570–695 nm) for Lifeact-GFP and Sqh-mCherry, respectively. The z-stack images acquired from time-lapse were then processed in Imaris Image analysis software. Gaussian smooth filter was applied to both channels. Lifeact-GFP channel was used to create a surface and then a mask was applied to collect all Sqh-mCherry signal inside the actin surface. Further, using the cell module in Imaris, a cell of this masked myosin was generated and the surface of this cell was exported for all timepoints. Then, a distance transformation map of this new myosin surface was created for all the inside voxels. A new surface was again created for only the outer 2 µm of cluster periphery. The Sqh-mCherry signal inside the actin surface was then masked to this new surface to obtain only the outer 2-µm signal of Sqh-mCherry. This signal was normalized to the background nurse cell myosin intensity at all timepoints in order to correct for photo-bleaching effects. See Supplemental Movie S15 for more details.

Measurement of colocalization
Colocalization measurements were done using Fiji image analysis software with the coloc2 plug-in. Analysis was done on all pairwise combinations of E-cad, F-actin, and Sqh-mCherry channels. We report the Pearson correlation coefficient (r) for each of these combinations. The analysis was done in a central z-plane of the border cell cluster as a whole (based on the center of the polar cells), border cell-border cell junctions, and the apical cap of the cluster (as determined by E-cad or myosin signal).

Live imaging of cultured egg chambers for phosphomimetic sqh
Dissection and mounting of live egg chambers were performed as described previously (Prasad and Montell, 2007) and time-lapse imaging was performed using a 40x/1.1 NA water immersion objective lens. The 1.25-µm-thick z-sections spanning the entire border cell cluster were collected at 2-min intervals. Maximum intensity projection was made using the Lifeact-GFP in Fiji image analysis software. Migration speed was calculated for time frames showing either protrusions or bleb-based migration. To calculate the migration speed of these two modes, the displacement of the polar cells between the first and last timepoints was divided by the total time elapsed.

Kymograph and myosin intensity during protrusion elongation and retraction
Kymographs for Sqh-mCherry and E-Cad-GFP movies were made in Fiji image analysis software. To quantify myosin intensity during protrusion and retraction, a line scan for the entire protrusion area was drawn using Lifeact-GFP channel to measure the length of protrusion. Myosin intensity was measured in the Sqh-mCherry channel. The average background nurse cell myosin intensity was used to normalize the Sqh-mCherry signal.

Statistical analysis and figure preparation
All statistical analyses ([U](D) test and one-way analysis of variance [ANOVA]) and graph preparations were done in GraphPad Prism software. Figures and illustrations were created in Adobe Creative Cloud 2014.

ACKNOWLEDGMENTS
This work was supported by National Institutes of Health grant 5R01GM46425 to D.J.M. and American Cancer Society postdoctoral fellowship PF-17-024-01-CSM to J.P.C.

REFERENCES

Molecular Biology of the Cell


ETOC:
Using border cells in the fly ovary as a model, we find distinct and dynamic pools of myosin II that regulate protrusion dynamics within and between collectively migrating cells and suggest a new model for the role of protrusion in collective direction sensing in vivo.
Author Queries

[QA 1]: μm as meant by “micron”? OK as set?
[QA 2]: Spell out FRET on 1st use.
[QA 3]: A–D, I–K, M, and N as meant?
[QA 4]: Define *, **.  
[QA 5]: Single asterisk not present in art; please replace with new art or delete single asterisk information here.
[QA 6]: Scale bar only appears in A and K art; okay.
[QA 7]: Correct that Figure is not labels 1, 2, 3?
[QA 8]: First sentence as meant? Please reword if not.
[QA 9]: Define RT at 1st use.
[QA 10]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 11]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 12]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 13]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 14]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 15]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 16]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 17]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 18]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 19]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 20]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 21]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 22]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 23]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 24]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.
[QA 25]: Per MBOC style, QA: give first 10 authors followed by et al. if more than 10.