Cycling Rho for tissue contraction

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Cell contractility, driven by the RhoA GTPase, is a fundamental determinant of tissue morphogenesis. In this issue, Mason et al. (2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201603077) reveal that cyclic inactivation of RhoA, mediated by its antagonist, C-GAP, is essential for effective contractility to occur.

The cell cortex is a complex network of F-actin and myosin filaments that supports the plasma membrane of many eukaryotic cells (Salbreux et al., 2012). Through its capacity to generate contractile force and determine mechanics, the cortex influences key properties such as cell shape and integrity. Moreover, when coupled to cell adhesion, cortical actomyosin networks become the mechanical apparatus that drives tissue-level morphogenesis (Martin et al., 2009; Rauzi et al., 2010). This is exemplified by the phenomenon of epithelial folding, where constriction of the apical poles of cells leads to inward folding (invagination) of the tissue when the cells are linked together by adherens junctions (Fig. 1 A; Sawyer et al., 2010). To understand the cellular basis of such morphogenetic processes, we therefore need to elucidate the mechanisms that regulate actomyosin during development.

One system that has proven especially fruitful for analyzing epithelial folding is the process of ventral furrow formation in the *Drosophila melanogaster* embryo (Sawyer et al., 2010). Here a population of ∼1,000 cells undergoes a coordinated process of apical constriction that leads to the invagination of the presumptive mesoderm. Apical constriction would be expected to require a contractile apparatus located at the apical regions of the cells. Indeed, actomyosin is found both at adherens junctions as well as in the cortex of the apical poles themselves, often described as junctional and medioapical pools, respectively (Martin et al., 2009; Mason et al., 2016; Fig. 1 A). It was first thought that apical constriction might occur by contraction of the junctional pool of myosin, akin to closure of a purse-string. However, high-resolution time-lapse imaging revealed that apical constriction actually occurs in a step-wise fashion, involving pulses of constriction at the apical poles of cells (Martin et al., 2009). This led to the realization that constriction was driven by pulsatile contractions in the medioapical actomyosin network, pulling the adherens junctions inwards like a ratchet (Xie and Martin, 2015). Since then, pulsatile contractility has been identified in many tissues that undergo apical constriction (Roh-Johnson et al., 2012) and in other forms of morphogenetic rearrangements (Rauzi et al., 2010). Indeed, it can be found at cell–cell interfaces that do not constrict (Wu et al., 2014) and may even reflect a more general way for contractility to organize membranes on the nanoscale (Gowrishankar et al., 2012). But how such pulsatile contractility might be generated remained an open question. Nor, indeed, was it clear whether the pulsatile nature of the contractility was itself necessary for productive constriction, as opposed to being a quirky epiphenomenon of the process. These are issues that Mason et al. address in this issue of *The Journal of Cell Biology*.

Ventral furrowing is a developmentally regulated process (Sawyer et al., 2010). A key pathway is initiated by the transcription factor Twist to ultimately activate the RhoA GTPase. RhoA is a canonical regulator of actomyosin, which initiates a cascade of events to promote contractility, including activation of myosin via phosphorylation and stimulating formin-mediated actin assembly. Like many other members of the Ras superfamily, RhoA can cycle between an active, GTP-bound state and an inactive, GDP-bound state (Hodge and Ridley, 2016). GTP-loading and stimulation of RhoA signaling are catalyzed by upstream guanine nucleotide exchange factors (GEFs). Accordingly, much research effort has been devoted to identifying the GEFs that activate RhoA in specific signaling pathways. For the Twist pathway one such GEF is RhoGEF2 (Kölsch et al., 2007), which localizes at the medioapical cortex and is necessary for myosin recruitment and apical constriction (Fox and Peifer, 2007; Mason et al., 2016). Further, Mason et al. (2016) found that RhoGEF2 itself undergoes pulsatile condensations in the medioapical cortex that precede contraction of the actomyosin networks, consistent with its role in activating RhoA and myosin. To further explore how RhoA influences apical constriction, the authors sought to overdrive the system by expressing a constitutively active form of RhoA (CA-RhoA) that is locked in its GTP-loaded state and therefore unable to cycle. CA-RhoA enhanced apical myosin but this did not display pulsatile behavior, consistent with its sustained activation. Strikingly, despite this increased myosin, cells expressing CA-RhoA failed to undergo apical constriction. Together, these findings implied that myosin pulsation, entrained by cycling of RhoA between its active and inactive states, was essential for apical constriction. By implication, the inactivation of RhoA was as necessary for constriction as its activation.

RhoA possesses an intrinsic GTPase activity that converts it from its GTP- to its GDP-loaded state. However, this intrinsic activity is too slow ($t_{1/2}$ of ∼30 min [Zhang and Zheng, 1998]) to account for cycling in dynamic cellular processes (e.g., cortical myosin pulses often exhibit a period of ∼80–100 s [Martin et al., 2009; Rauzi et al., 2010; Wu et al., 2014]). Instead, RhoA inactivation is potentiated by GTPase-activating proteins

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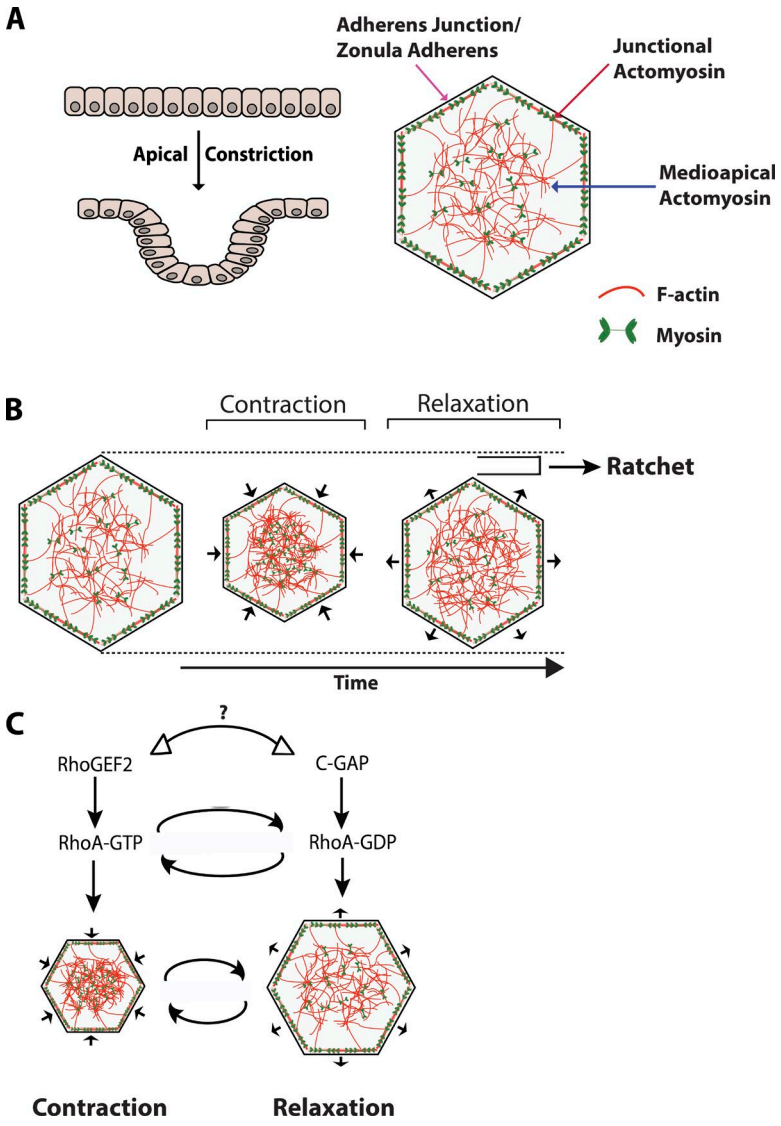

Figure 1. **Regulation of cellular contractility for apical constriction.** (A) Epithelial invagination, as found during ventral furrow formation in the gastrulating *Drosophila* embryo, is a morphogenetic movement where apical constriction of epithelial cells that are joined to one another by cell–cell adhesion leads to inward folding of the epithelial sheet. At the apical poles of cells, actomyosin is found adjacent to the adherens junctions (zonula adherens) and in a medioapical network at the apical cortex. (B) Ratchet-like apical constriction. Apical constriction occurs in a step-wise fashion, driven by cycles of contraction and relaxation in the medioapical actomyosin network. The apical poles do not relax fully, constituting a ratchet for each cycle of contraction. (C) Hierarchical control of medioapical actomyosin via cycling of GTP- and GDP-loaded RhoA through the action of activator (RhoGEF2) and inactivator (C-GAP).

(GAPs). This implies that a GAP might play a key role in apical constriction. Screening the 22 known *Drosophila* GAPs, Mason et al. (2016), found only one that was necessary for ventral furrowing. This was a rather poorly characterized molecule called RhoGAP71E, which the authors renamed Cumberland GAP (C-GAP). They did so because C-GAP/RhoGAP71E-deficient embryos displayed an abnormal, C-shaped ventral furrow, resembling the eponymous passage found in the Appalachian mountains of Tennessee that is famous in American colonial history. C-GAP mRNA was enriched in regions of the embryo that underwent furrow formation and its protein was found at the ventral furrow, localizing with actomyosin both at cell–cell junctions and at medioapical cortices. Further, overexpression of C-GAP reduced apical actomyosin and detectable GTP-RhoA at the apical cortices, evidence that it could regulate the RhoA–Myosin II pathway in the cells.

C-GAP depletion generated a range of phenotypes consistent with abnormal contractility. Some embryos failed to undergo cellularization, whereas in others that passed this developmental step ventral furrow invagination was either delayed or failed completely. Interestingly, all these embryos showed apical actomyosin, indicating that the defect lay not in a failure of recruitment, but in some other feature of the contractile apparatus. One abnormality was evident in the organization of medioapical myosin. Whereas control cells showed nodes or fibers of apical myosin, these were replaced by a diffuse distribution when invagination was retarded or by a single, dense nodule in cells that failed to invaginate. Importantly, pulsatile contractility did not occur in the medioapical networks of C-GAP–deficient cells, which displayed a progressive increase in intensity, consistent with an inability to decondense actomyosin after it had contracted. This identified C-GAP as necessary for pulsatile contraction. It further implied that contractile pulses might reflect a RhoA pacemaker that requires C-GAP for cyclic inactivation of RhoA, such as has been observed in other contractile, morphogenetic processes (Munjal et al., 2015). Mason et al. (2016) investigated this by monitoring the apical distribution of ROCK as a proxy for RhoA signaling because its cortical recruitment requires GTP-RhoA (Simões et al., 2014). They found that ROCK displayed pulsatile condensation at the apical poles, as was seen for myosin II, and this was impaired by C-GAP depletion, supporting the idea that C-GAP participated in cyclic RhoA signaling. One potential confounding factor was that the cortical localization of ROCK can be influenced by association with other proteins, including myosin II itself (Munjal et al., 2015; Priya et al., 2015). However, ROCK

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pulsatility persisted when myosin pulses were abolished by inactivation of myosin phosphatase (which leads to sustained myosin activity). Overall, this identified a key role for RhoA cycling, mediated by the coordinated action of RhoGEF2 and C-GAP, in contractile pulsatility.

Surprisingly, Mason et al. (2016) found that the overexpression of C-GAP did not compromise pulsatility in the medioapical networks. Instead, C-GAP overexpressing embryos displayed a different defect in ventral furrowing. Earlier studies had shown that the presence of contractile pulses was not sufficient to drive apical constriction. Both in *Drosophila* and *Caenorhabditis elegans* embryos pulsatile contractility has been observed to begin before cellular invagination occurs (Roh-Johnson et al., 2012; Xie and Martin, 2015), when it first proceeds without productive constriction of the apical poles, like an idling motor. In *Drosophila* ventral furrowing, the process of invagination coincides with a transition from such ineffectual contractility to the pattern of ratcheted constrictions (Fig. 1 B), where the apical poles do not fully relax after the peak of contraction, resulting in net constriction (Xie and Martin, 2015). This transition to ratcheting constriction was compromised by C-GAP overexpression. One factor that contributes to ratcheting is the degree of residual medioapical myosin that is found after the peak of each pulse (Xie and Martin, 2015), presumably generating persistent contraction that limits the extent to which the apical cortex can relax. Of note, this myosin persistence was reduced by C-GAP overexpression, leading the authors to postulate that the amount of residual active RhoA, determined by the balance between RhoGEF2 and C-GAP, might regulate ratcheting through its impact on myosin persistence. Interestingly, Twist, which specifies the ventral furrow, stimulates ratcheting, leading to the hypothesis that it might alter the GEF/GAP ratio by promoting RhoGEF2. Indeed, the C-GAP overexpression phenotype closely resembled that of the *twist* mutant, and *twist* was necessary for the medioapical accumulation of RhoGEF2, which would be predicted to decrease the GEF/GAP balance.

Together, these findings yield a picture where effective apical constriction arises from a hierarchical network of regulators and effectors, many of whose elements show cyclic behavior (Fig. 1 C). Thus, the presumably sequential action of RhoGEF2 and C-GAP serves as a pulse generator for RhoA signaling. This ultimately drives the pulsatile contractility that is necessary for apical constriction to occur. Further, the balance between RhoGEF2 and C-GAP also influences how much myosin persists after each contractile pulse to support the ratcheting required for effective net constriction. This model opens many avenues for future research. Here, we highlight two of them.

First, why do actomyosin networks need to pulse to achieve apical constriction? Though tightening a purse string might seem enough, cells clearly know better. Specifically, why is it necessary for medioapical actomyosin to relax, albeit transiently and incompletely, for effective net constriction to occur? One possibility is that some degree of relaxation is necessary to optimize contractility. Actin filaments can undergo stress-induced disassembly within contractile networks (Haviv et al., 2008); relaxation may prevent this and also allow architectural reorganization within networks to facilitate force generation (Reymann et al., 2012). In addition, transient relaxation may be necessary for cell–cell adhesion to be reinforced. Here, it is of note that adherens junctions were perturbed in C-GAP–deficient cells that failed to invaginate. Instead of being concentrated in lateral junctions, E-cadherin redistributed over the apical poles of the cells, which also became more rounded. This suggested that cell–cell adhesion may have been unable to resist the forces of sustained contractility. Increasingly, it is apparent that mechanotransduction allows junctions to reinforce upon stress. This requires cell signaling and cytoskeletal responses (Leerberg et al., 2014). Transient relaxation may then provide the time necessary for mechanosensitive junctional compensation to occur in response to stress.

Second, what coordinates the action of RhoGEF2 and C-GAP? Cycling of RhoA activity implies that there is some sequential action of these two proteins. Moreover, how might these be coordinated to control the regular period of RhoA cycling? One, as-yet-untested, way might be through temporal control of their specific activity. Alternatively, and not exclusively, RhoA activity might be induced to cycle if the cortical recruitment of RhoGEF2 and C-GAP were coordinated. For example, if C-GAP were to be recruited after RhoGEF2, then this could support cycles of activation and inactivation. Evidence for this comes from the authors' observation that both these proteins recruit to the medioapical cortex to coaccumulate with myosin puncta. Furthermore, RhoGEF2 exhibited cortical pulsations that seemed to occur through condensation. It would be interesting to know what the cortical dynamics of C-GAP may look like. Cortical condensation might come about by advection (cortical flows) or recruitment of cytosolic proteins to specific sites on the cortex. Which of these may be relevant here remains to be determined. As inhibiting myosin pulsation did not affect ROCK condensation (Mason et al., 2016), it is tempting to speculate that there is an upstream mechanism that coordinates the recruitment of RhoGEF2 and C-GAP to set the period for RhoA cycles. However, oscillatory behavior is often generated by feedback networks that operate within dynamic systems (Kruse and Jülicher, 2005). The clock for RhoA cycling may instead constitute an emergent property of the hierarchical system that Mason et al. (2016) have identified, perhaps influenced by mechanochemical feedback within the tissue itself (Munjal et al., 2015; Priya et al., 2015). What the present study emphasizes is that understanding the role of RhoA's inactivators will be as important as understanding its activators. As devotees of bicycle riding know, good pedal technique is as much about how you release pressure as how you apply it.

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