1 **A feedback mechanism mediated by myosin II-dependent apical**
2 **targeting of Rab11 vesicles reinforces apical constriction**

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9 **Summary:**

10 During tissue morphogenesis, cell shape changes resulting from cell-generated forces
 11 often require active regulation of intracellular trafficking. How mechanical stimuli
 12 influence intracellular trafficking and how such regulation impacts tissue mechanics
 13 are not understood. We identify a mechanosensitive mechanism involving Rab11
 14 recycling endosomes in regulating apical constriction in the *Drosophila* embryo.
 15 During *Drosophila* mesoderm invagination, apical actin and myosin II (actomyosin)
 16 contractility induces accumulation of Rab11-marked vesicles near the apical
 17 membrane by promoting a directional bias in vesicle transport, which is mediated by
 18 the microtubule motor dynein. At the apical domain, Rab11 vesicles are targeted to
 19 the adherens junctions (AJs). The apical accumulation of Rab11 vesicles is essential
 20 to prevent fragmented apical AJs, breaks in the supracellular actomyosin network and
 21 a reduction in the apical constriction rate, which is separate from the function of
 22 Rab11 in promoting apical myosin II activation. These findings provide evidence for a
 23 mechanosensitive feedback mechanism between actomyosin-mediated apical
 24 constriction and Rab11-mediated intracellular trafficking that regulates the force
 25 generation machinery during tissue folding.

26

27 **Key word:**

28 Apical constriction, actomyosin contractility, Rab11, adherens junction,
 29 mechanosensitive feedback

Introduction:

Cell-generated mechanical forces play a central role in tissue morphogenesis. Genetically prescribed cellular forces can drive cell shape change and cell motion as a direct physical outcome, thereby mediating spatially and temporally defined tissue deformation (Collinet and Lecuit, 2021; Gilmour et al., 2017). In addition, mechanical forces can impact morphogenesis by triggering various cellular activities ranging from cytoskeleton reorganization to changes in gene expression (Fletcher and Mullins, 2010; Kirby and Lammerding, 2016; Sun and Irvine, 2016; Uhler and Shivashankar, 2017). Such active processes allow cells and tissues to respond to mechanical inputs adaptively and thereby increase the robustness of tissue morphogenesis. However, our knowledge of the active response of cells to mechanical forces remains limited.

In this work, we used mesoderm invagination during *Drosophila* gastrulation as a model to study how cells respond to mechanical forces generated by actomyosin contractions. Immediately before gastrulation, *Drosophila* embryos undergo an atypical cleavage called cellularization, during which peripherally localized syncytial nuclei are partitioned into a monolayer of epithelial cells (Mazumdar and Mazumdar, 2002). Mesoderm invagination is initiated by actomyosin mediated apical constriction in a 12-cell wide region in the ventrally localized mesodermal primordium (Leptin and Grunewald, 1990; Sweeton et al., 1991). These cells subsequently invaginate as

the epithelium bends inwards, resulting in the formation of a furrow on the ventral side of the embryo. The upstream signaling pathway that activates apical constriction has been well characterized (reviewed in Gilmour et al., 2017; Martin, 2020; Gheisari et al., 2020). In response to the dorsal-ventral patterning information, the prospective mesodermal cells express two transcription factors, Twist and Snail, which in turn trigger the apical recruitment and activation of RhoGEF2 through GPCR signaling. RhoGEF2 further activates the small GTPase Rho1 (the *Drosophila* homologue of RhoA) and leads to the activation of Rho1's effector Rho-associated protein kinase (Rok). Phosphorylation of the regulatory light chain of non-muscle myosin II by Rok activates myosin II at the apical cortex, where it forms an actomyosin network that is physically connected to apical AJs (Amano et al., 1996; Kimura et al., 1996; Winter et al., 2001; Nikolaidou and Barrett, 2004; Dawes-Hoang et al., 2005; Mason et al., 2013; Vasquez et al., 2014). Within individual cells, the apical actomyosin network undergoes pulsed contractions, pulling the AJ sites inwards and reducing apical cell area in a ratchet-like manner (Martin et al., 2009). At a tissue level, the contractile forces are integrated through AJs, resulting in progressive apical area reduction in the presumptive mesoderm (Martin et al., 2010; Mason et al., 2013; Martin and Goldstein, 2014).

Apical constriction can drive reorganization from subcellular to tissue levels through both active and passive means. Apical constriction drives a tissue-scale viscous flow

at the tissue interior, which mediates cell shape changes across the depth of the tissue (He et al., 2014). Certain organelles, such as the nucleus, appear to move passively with the flow by advection (Gelbart et al., 2012). Other subcellular structures, in contrast, undergo active reorganization in response to apical forces. For example, apical actomyosin contractility has been shown to mediate an apical shift of the AJs at the beginning of gastrulation and protects these AJs from Snail-mediated disassembly (Weng and Wieschaus, 2016). More recently, apical constriction was shown to promote the formation of a medioapically-localized noncentrosomal microtubule-organization center that mediates microtubule reorganization during ventral furrow formation (Ko et al., 2019). It remains unclear whether other subcellular structures also undergo active remodeling in response to apical constriction.

Rab11, a small GTPase of the Rab family, is a well-established marker for recycling endosomes (Welz et al., 2014). Like typical small GTPases, Rab11 switches between active and inactive states depending on the phosphorylation state of its bound guanine nucleotide. In addition to recycling endosomes, Rab11 has also been reported to localize to the trans-Golgi network (TGN) and post-Golgi vesicles. It functions in both exocytic and endocytic recycling pathways by regulating vesicular transport from TGN and recycling endosomes to the plasma membrane (Benli et al., 1996; Jedd et al., 1997; Ullrich et al., 1996; Chen et al., 1998; Pelissier et al., 2003; Takahashi et al., 2012; Welz et al., 2014). At the molecular level, Rab11 regulates multiple

93 vesicular trafficking steps, including vesicle formation, transport and tethering. The
 94 functions of Rab11 are mediated through its downstream effectors. For example,
 95 Rab11 directly interacts with the actin motor myosin V (Lapierre et al., 2001;
 96 Lipatova et al., 2008). It can also form complexes with microtubule motors dynein
 97 and kinesins through a group of effector proteins called Rab11-FIPs (Rab11-family
 98 interacting proteins) (Welz et al., 2014). These interactions allow Rab11 vesicles to be
 99 transported along both actin and microtubule filaments (Horgan et al., 2010;
 100 Schonteich et al., 2008; Schuh, 2011; Wang et al., 2008). Rab11 also interacts with the
 101 Sec15 subunit of the exocyst, a protein complex critical for the tethering of secretory
 102 vesicles to the plasma membrane (Mei and Guo, 2018). This interaction is important
 103 for directing Rab11 vesicles to specific sites of the plasma membrane for exocytosis
 104 (Zhang et al., 2004; Wu et al., 2005; Langevin et al., 2005; Oztan et al., 2007;
 105 Takahashi et al., 2012).

106

107 Here, we found that apical constriction induces biased transport of Rab11-positive
 108 vesicles towards the cell apex in a dynein- and microtubule-dependent manner,
 109 resulting in apical accumulation of the vesicles. The bias in transport depends on
 110 apical myosin II activity and quickly diminishes upon acute inactivation of apical
 111 actomyosin contractility, indicating the involvement of a rapid mechanosensitive
 112 response. At the apical side, Rab11 vesicles are targeted to AJs and function to
 113 reinforce them during apical constriction, which in turn promotes the structural

114 integrity of the supracellular actomyosin network as tissue tension increases. This
 115 mechanosensitive function of Rab11 is separate from its role in apical myosin II
 116 activation and instead serves as a feedback regulation for apical constriction.
 117 Together, our findings reveal a mechanosensitive interplay between actomyosin-
 118 mediated apical constriction and Rab11-mediated vesicular trafficking that regulates
 119 the actomyosin force generation machinery during epithelial folding.

120

Results:

Rab11 accumulates apically in the constricting cells during ventral furrow formation

To probe potential changes in subcellular organization in cells undergoing apical constriction, we examined a number of fluorescently tagged protein markers for different intracellular compartments during ventral furrow formation (Figure S1). Among all the markers we examined, Rab11 labeled compartments showed the most striking morphological and positional changes during apical constriction (Figure 1A, B; Movie 1). Before gastrulation started, Rab11 mainly localized to a large perinuclear compartment at the apical side of each nucleus (hereafter “perinuclear Rab11 compartment”; Figure 1C, green arrows). In addition, some vesicle-like structures were present near the basal side of the perinuclear Rab11 compartments (hereafter “Rab11 vesicles”; Figure 1C, red arrows). As the cells constricted apically, perinuclear Rab11 compartments moved basally following the basal movement of the nuclei and became moderately elongated apicobasally (Figure 1C, blue outlines). Meanwhile, Rab11 vesicles appeared near the cell apices and accumulated apically (Figure 1B-D). No apical accumulation of Rab11 vesicles occurred in the surrounding, non-constricting cells (Figure 1B-D). The Rab11 vesicles were highly dynamic, yet at any given time the majority accumulated either adjacent to or slightly basal to the apical actomyosin network (Figure 1E). We did not observe obvious apical enrichment for the other organelles we examined (Figure S1). For example, the

Golgi compartments, which also appeared as punctate structures, were depleted from the apical region of the constricting cells during ventral furrow formation (Figure S1A). The apical enrichment of Rab11 vesicles could not be readily explained by advection by cytoplasmic flow driven by apical constriction (He et al., 2014) and suggests the involvement of active regulations.

Apical accumulation of Rab11 vesicles depends on myosin II activity

The spatiotemporal correlation between the apical constriction of cells and the apical accumulation of Rab11 vesicles prompted us to examine whether vesicle accumulation is induced by apical constriction. To this end, we inhibited apical constriction by injecting embryos with the Rok inhibitor Y-27632 (Narumiya et al., 2000). Injection of Y-27632 at the end of cellularization completely prevented apical myosin II activation and abolished apical Rab11 vesicle accumulation (Figure 2A). Next, we adapted a previously described on-stage injection protocol (Coravos and Martin, 2016) to test the effect of Y-27632 injection on Rab11 vesicles after they accumulated apically. After drug injection, myosin II immediately (< 1 min) dissociated from the apical cortex, which was associated with a relaxation of the furrow (Figure 2B') and a moderate reduction of apical Rab11 vesicles (Figure 2B). About 4 minutes after injection, Rab11 vesicles were largely depleted from the apical surface (Figure 2B). The morphology of perinuclear Rab11 compartments were not obviously affected by Y-27632 injection (Figure 2B, 3 – 6 μ m), indicating that the

drug did not cause a general disruption of the Rab11 positive membrane structures.

These observations indicate that the induction and maintenance of apical

accumulation of Rab11 vesicles depends on apical myosin II activity.

To further test the relationship between apical myosin II activation and apical

accumulation of Rab11 vesicles, we examined embryos in which the myosin heavy

chain Zipper was depleted by RNA interference-mediated maternal knockdown.

Consistent with the Y-27632 treatment, Zipper knockdown also inhibited apical

Rab11 vesicle accumulation (Figure 2C). Conversely, we asked whether ectopic

activation of myosin II would induce apical accumulation of Rab11 vesicles. For this

purpose, we examined the mutant embryos for *Spn27A*. *Spn27A* encodes a serine

protease inhibitor that regulates dorsal-ventral patterning in early embryos by

inhibiting the Toll-Dorsal pathway (Ligoxygakis et al., 2003). Consistent with

previous reports, *Spn27A* mutant embryos showed a ventralized phenotype with

expanded apical myosin II activation domain during gastrulation (Figure 2D;

Ligoxygakis et al., 2003). In these embryos, Rab11 vesicles were also enriched

apically in cells with ectopic activation of apical myosin II (Figure 2D, D'). Taken

together, we conclude that apical actomyosin contractility is both necessary and

sufficient to induce apical accumulation of Rab11 vesicles.

Apical Rab11 vesicles are not derived from endocytosis

We next examined how Rab11 vesicles accumulate at the apical surface during apical constriction. Rab11 vesicles may be derived from endocytosis of apical membranes. Apical endocytosis has been shown to play an important role in apical constriction as the apical membrane area decreases over time (Lee and Harland, 2010; Mateus et al., 2011; Miao et al., 2019). To test this possibility, we blocked endocytosis by using a temperature sensitive mutant of *shibire* (*shi^{ts}*), the *Drosophila* homolog of dynamin (van der Bliek and Meyerowitz, 1991). At the restrictive temperature of 32°C, *shi^{ts}* mutant embryos exhibited ventral furrow defects with different levels of severity, ranging from slower apical constriction (mild group, N = 10 embryos) to failure in invagination (severe group, N = 7 embryos) (Figure S2A). Embryos in the mild group showed comparable apical Rab11 vesicle accumulation to the wildtype controls (Figure S2B). Embryos in the severe group showed a modest accumulation of apical Rab11 vesicles before gastrulation, which was distinct from the wild type and suggests the presence of early defect due to a strong loss of dynamin function. Nevertheless, when gastrulation started, the amount of apical Rab11 vesicles further increased in the constricting cells, resulting in more apical vesicles than the wildtype controls (Figure S2B). These results indicate that endocytosed apical membrane is not the major source of apical Rab11 vesicles.

Apical constriction induces apically biased transport of Rab11 vesicles

An alternative possibility is that the apical Rab11 vesicles are supplied from the more

basal side of the cell by directional transport. To capture the rapid movement of Rab11 vesicles in the apical-basal direction, we focused on the constricting cells located near the medial-lateral boundaries of the constricting domain. These cells are tilted towards the ventral midline at the late stage of apical constriction, making it possible to follow the apical-basal movement of Rab11 vesicles by fast imaging of a single focal plane (Figure 3A). Using this approach, we observed constant transport of Rab11 vesicles in the axial direction in the constricting cells (Figure 3B; Movie 2). There were both apically and basally directed movements, with a bias towards the apical direction. Vesicle tracking revealed that the apically directed movement accounted for approximately three quarters of the total transport events ($74.5\% \pm 5.7\%$; $n = 255$ trajectories from 3 embryos, Figure 3C-E). Other than the bias in directionality, there was no significant difference in other aspects of the trajectories between apical and basal transport, including the average velocity, the duration and the travel distance of the tractable trajectories (Figure 3F). These results suggest that the apical bias in the direction of Rab11 vesicle transport results in the apical enrichment of these vesicles.

Because the apical enrichment of Rab11 vesicles depends on apical constriction, we hypothesized that the biased basal-to-apical transport of Rab11 vesicles also depends on apical actomyosin contractility. To test this hypothesis, we used an optogenetic tool we recently developed to acutely inhibit myosin II activity through light-sensitive

recruitment of a dominant negative form of Rho1 (Rho1DN) to the plasma membrane (“Opto-Rho1DN”, Guo et al. in preparation). The approach is based on the CIBN-CRY2 system (Kennedy et al., 2010; Guglielmi et al., 2015). Blue-light dependent recruitment of a CRY2-Rho1DN fusion protein to plasma membrane-anchored CIBN blocks activation of endogenous Rho1, causing rapid inactivation of myosin II (Figure 3G, H). Using the same imaging configuration as shown in Figure 3A, we examined the immediate impact of myosin II inhibition on the transport of Rab11 vesicles. As expected, blue light stimulation resulted in rapid loss of apical myosin II (within ~30 seconds; Figure 3I). The loss of apical myosin II at this stage of ventral furrow formation did not cause furrow relaxation or any obvious changes in cell orientation. Strikingly, blue light stimulation resulted in a rapid change in the transport of Rab11 vesicles. Within the first 23 seconds after stimulation, the proportion of apical transport was 62.5% ($62.5\% \pm 5.0\%$; $n = 257$ trajectories from 3 embryos), lower than that in the wildtype controls (Figure 3J). This proportion dropped to close to 50% ($52.3\% \pm 1.6\%$; $n = 280$ trajectories from 3 embryos) in the next 23-second time window, indicating that the directional bias was completely abolished (Figure 3J; Movie 3). There was no significant difference between the two time windows regarding other aspects of transport (Figure 3K). Therefore, the main effect of myosin II inhibition on Rab11 vesicle transport is to eliminate the apical bias of the transport. The strong dependence of the biased transport on apical myosin II activity suggests that the alteration in directionality of Rab11 vesicle trafficking is a rapid

mechanosensitive response to apical actomyosin contractility.

The transport of Rab11 vesicles also depends on microtubules and dynein

Because the saltatory and directional movement of Rab11 vesicles was reminiscent of microtubule-dependent transport, we examined the spatial relationship between Rab11 vesicles and microtubules by two-color imaging with mCherry-Rab11 and the microtubule marker Jupiter-GFP (Karpova et al., 2006). In constricting cells, abundant apical-basally oriented microtubules were present between the cell apex and the nucleus. As Rab11 vesicles moved apically or basally, most of them persistently associated with microtubule fibers, which represented either individual microtubules or microtubule bundles (Figure 4A; Movie 4, before injection).

To further test whether the transport of Rab11 vesicles requires microtubules, we performed on-stage colchicine injection during apical constriction. In less than a minute after injection, most microtubules disappeared or became greatly shortened. Meanwhile, the number of directional transport events significantly decreased. The small number of remaining transport-like events occurred only in regions where some residual microtubules remained, which provided further evidence that the transport of Rab11 vesicles in normal cells is microtubule dependent (Figure 4B; Movie 4).

To further test our hypothesis, we examined the impact of colchicine injection on the

apical accumulation of Rab11 vesicles. Injection of embryos at late cellularization (N = 5 embryos) resulted in a significant reduction in the apical accumulation of Rab11 vesicles compared to control embryos (N = 4 embryos) (Figure 4C-F). Importantly, the activation of apical myosin II was normal in these embryos, which is consistent with a recent report (Ko et al., 2019). Therefore, the impact of microtubule disruption on Rab11 vesicle accumulation is not due to an indirect effect of disrupting apical myosin II activation. These results support our hypothesis that the biased transport of Rab11 vesicles along microtubules leads to their apical accumulation.

Unexpectedly, injection of colchicine at or after the onset of gastrulation (N = 5 embryos) resulted in a wider range of phenotypes, and on average there was no significant reduction of vesicle numbers compared to the control embryos (Figure 4C-F). We speculate that disruption of microtubules after the onset of gastrulation not only reduces the apical-basal transport of Rab11 vesicles but also inhibits the turnover of the vesicles that are already accumulated apically. This combined effect may obscure the net effect of loss of microtubules.

Next, we sought to test the role of microtubule motors on the transport of Rab11 vesicles. We found that injection of antibody against the dynein intermediate chain (DIC) (Yi et al., 2011) during apical constriction resulted in a rapid reduction of vesicle transport events in both directions, whereas injection of a control GST

antibody had no effect (Figure 4G, Movie 5). Furthermore, the apical accumulation of Rab11 vesicles was greatly reduced in dynein antibody-injected embryos but not in the control antibody-injected embryos (Figure 4H). Whereas we were not able to test the role of kinesin motors due to a lack of functional reagents, the pronounced impact of dynein inhibition on Rab11 vesicle transport and accumulation suggests that dynein plays a predominant role in the apical-basal transport of Rab11 vesicles during apical constriction. When apical constriction starts, the centrosomes in the constricting cells move basally together with the nuclei (Ko et al., 2019). Microtubules originating from these centrosomes are expected to have their plus ends pointing toward the apical side. Meanwhile, the microtubule minus end binding protein Patronin is enriched at the medio-apical cortex (Ko et al., 2019), where it organizes a microtubule population with their minus ends pointing to the apical side. Our observation that inhibiting dynein abolishes both apical and basal transport of Rab11 vesicles may reflect the bidirectional nature of the microtubule populations.

Rab11 reinforces apical AJs during apical constriction

We next sought to determine the function of apically accumulated Rab11 vesicles. Single plane fast imaging analysis revealed that Rab11 vesicles remained very dynamic after they arrived at the cell apices, with a substantial fraction delivered to apical AJs (Figure 5A; Movie 6). The enrichment of Rab11 vesicles at AJs was particularly obvious during the early stage of apical constriction when apical AJs had

not yet formed a continuous belt and instead were observed as discrete foci. Intensity profiling of Rab11 and E-cadherin along the apical cell-cell boundaries in constricting cells revealed a positive correlation. In contrast, the intensity profiles of Rab11 and a general membrane marker P4M, a PI(4)P binding protein, showed no significant correlation (Figure 5B-D). It remains unclear whether the targeting of Rab11 vesicles to the cell periphery is followed by fusion of vesicles with the plasma membrane, as demonstrated in some other systems (Grünfelder et al., 2003; Takahashi et al., 2012). We were not able to detect an obvious plasma membrane-associated signal for Rab11. One possibility is that Rab11 is only transiently associated with the plasma membrane due to GTP hydrolysis. Consistent with this view, a constitutively active form of Rab11 (Rab11CA), which is locked in its GTP-bound state, showed a strong cell membrane localization in addition to the vesicle form, and the cell membrane signal was enhanced when the cells constricted apically (Figure S3).

The enrichment of Rab11 vesicles near the apical AJs prompted us to propose that Rab11 vesicles regulate the structure and function of AJs. To test this hypothesis, we inhibited Rab11 activity by injecting embryos with a purified dominant negative Rab11 mutant (Rab11DN), which blocks the activation of endogenous Rab11 by binding to and sequestering the guanine nucleotide exchange factors (GEFs) for Rab11 (Ullrich et al., 1996). Importantly, the injection approach allows stage-specific inhibition of Rab11, which is critical for bypassing the requirement for Rab11 in pre-

gastrulation stages (Pelissier et al., 2003; Riggs et al., 2003; Tiwari and Roy, 2008). Injection of Rab11DN at mid/late cellularization completely prevented the accumulation of apical Rab11 vesicles during apical constriction (N = 5/5); whereas injection of Rab11DN close to the onset of apical constriction either eliminated apically accumulated Rab11 vesicles (in 4.35 ± 1.31 min, N = 24/31; Figure 5E; Movie 7) or decreased their number (N = 7/31). In contrast to Rab11 vesicles, the size and morphology of perinuclear Rab11 compartments were not immediately affected by injection (Figure 5E, green arrows). These results suggest that both the initiation and maintenance of the apical accumulation of Rab11 vesicles require active Rab11.

To determine whether the apical accumulation of Rab11 vesicles is important for the formation or maintenance of AJs, we examined the effects of Rab11DN injection on E-cadherin-GFP. In control GST-injected embryos, apical AJs became more continuous and belt-like as apical constriction progressed (Figure 5F). In contrast, Rab11DN- injected embryos showed fragmented junctions at comparable stages (Figure 5F). Furthermore, the junctional component Jub, an Ajuba LIM protein that localizes to the AJs (Razzell et al., 2018), was greatly depleted from AJs upon Rab11DN injection (Figure 5G). In addition to the junctional defects, the cell area within the constriction domain became more heterogeneous after Rab11DN injection, raising the possibility that defects in AJ organization disrupt apical constriction (Figure 5F; see below). Together, these results suggest a role for apical Rab11 vesicles

in reinforcing apical AJs. Such a mechanosensitive reinforcement of AJs might be important for maintaining tissue integrity as tension increases in the constriction domain due to contractions of the actomyosin network.

Rab11 regulates myosin II organization and promotes apical constriction

Given the important role of apical AJs in the spatial organization of the supracellular actomyosin network (Martin et al., 2010; Sawyer et al., 2009), we examined whether inhibition of Rab11 activity would cause defects in apical constriction. Injection of Rab11DN at mid/late cellularization (< 20 min before gastrulation) resulted in an overall slower rate of apical constriction compared to GST injected embryos (Figure S4A, B). The difference between control and Rab11DN injection groups was relatively small in the first ~ 5 minutes after the onset of gastrulation but became much more prominent after 5 minutes (Figure S4B). In the control group, the average cell area continued to decrease from 2 min to 8 min. In contrast, in Rab11DN injection group, whereas the average cell area noticeably decreased from 2 min to 5 min, the area reduction was greatly attenuated from 5 min to 8 min (Figure S4C-D). As a result, the average apical area in the Rab11DN injection group became substantially larger than that in the control group at 8 min despite the similarity between the two groups at 2 min (Figure S4E). These results suggest that the apical accumulation of Rab11 vesicles is important for preventing apical constriction defect at late stages of apical constriction.

373

374 To investigate the cause of the apical constriction defect, we examined the dynamics
 375 and organization of apical myosin II in embryos injected with Rab11DN around the
 376 onset of gastrulation. Despite the prompt elimination of Rab11 vesicles, Rab11DN
 377 injection did not cause any obvious defect in the apical activation of myosin II. The
 378 width of the tissue that showed apical myosin II accumulation, as well as the average
 379 intensity of apical myosin II during apical constriction, were comparable between
 380 control and Rab11DN injected groups (Figure 6A-C, GST vs. Rab11DN late
 381 injection; Movie 8). However, quantification of the rate of myosin flow towards the
 382 ventral midline at approximately 6 – 7 minutes into gastrulation showed a
 383 significantly lower rate of apical constriction in Rab11DN injected embryos compared
 384 to control embryos (Figure 6D-G), which was consistent with the results obtained by
 385 analyzing cell membrane markers (Figure S4A-E). Interestingly, the apical
 386 constriction defect in Rab11DN injected embryos was associated with frequent breaks
 387 within the apical actomyosin network, which was rarely seen in the control embryos
 388 (Figure 6H, J; Movie 9). These myosin breaks occurred randomly across the
 389 constriction domain and were usually promptly reconnected, which prevented the
 390 complete tearing apart of the network. The higher frequency of myosin breaks, even
 391 though transient, can slow down apical constriction and cause temporary force
 392 imbalances between neighboring cells that increase heterogeneity in the apical cell
 393 area (Figure 5F).

394

395 The lack of defects in apical myosin activation upon Rab11 inhibition apparently
 396 contradicted with previous findings that Rab11 is required for apical myosin
 397 activation in several other apical constriction-mediated tissue folding processes
 398 (Ossipova et al., 2014, 2015; Le and Chung, 2021). In these studies, Rab11 was
 399 typically inhibited at a stage much earlier than the onset of apical constriction. We
 400 therefore tested whether Rab11 also has an earlier function that is required for apical
 401 myosin II activation during ventral furrow formation. We found that injection of
 402 Rab11DN proteins during early cellularization (> 30min before the onset of
 403 gastrulation) indeed resulted in a strong reduction or lack of apical myosin
 404 accumulation during gastrulation (Figure 6A, B). Therefore, through injection
 405 mediated acute inhibition of Rab11, we were able to resolve the early and late
 406 functions of Rab11 in apical constriction and identify a previously unappreciated,
 407 mechanosensitive function of Rab11 in regulating the spatial organization of the
 408 actomyosin network after myosin II activation.

409

410 We then investigated what caused myosin breaks in Rab11DN injected embryos. We
 411 noticed that AJs were often pulled to the opposite side from where myosin breaks
 412 happened, indicating the decoupling of actomyosin network and AJs from one side of
 413 the cell-cell boundary (Figure 6I, red arrows). This observation suggests that the
 414 increased frequency of myosin breaks is due to impaired connections between

junction and the actomyosin network as a consequence of altered junction organization. Supporting this view, we observed similar myosin breaks in embryos defective in *canoe*, the *Drosophila* Afadin homolog that regulates the linkage of the actin cytoskeleton to AJs during apical constriction (Movie 10). We hypothesized that the consequences of weakening AJ-actomyosin connections would likely become more prominent during later phases of apical constriction when the tissue tension becomes high. In line with this prediction, myosin breaks in the Rab11DN-injected embryos became prominent only during later phases of apical constriction (Movie 8), which resulted in a stage-specific defect in apical constriction (Figure S4C-D).

In addition to myosin breaks, we observed a second phenotype in the spatial organization of apical myosin II. Instead of forming a supracellular meshwork with medioapically enriched myosin foci connected by myosin fibers, myosin II instead formed ring-like circular structure at the apical domain of each constricting cell (Figure 6A). These apical myosin rings appeared to be connected with each other, suggesting that actomyosin contractions could remain integrated across the mesodermal tissue. Interestingly, a recent study showed that loss of anisotropy in apical tension led to similar myosin ring formation in the constricting cells (Chanet et al., 2017). In Rab11DN-injected embryos, most myosin breaks occurred in the anterior-posterior (AP) orientation (Figure 6K), which is the direction in which tension is higher (Martin et al., 2010). These breaks may dissipate tension in the AP

436 direction, thereby reducing tension anisotropy of the actomyosin network and causing
 437 myosin ring formation. In support of this view, the constricting cells exhibited no
 438 obvious increase of anisotropy in their apical geometry at the late stages of apical
 439 constriction in Rab11DN injected embryos (from 5 min to 8 min). This was in sharp
 440 contrast to control embryos in which anisotropy continued to increase during apical
 441 constriction (Figure S4F). Together, these findings indicate that apical constriction
 442 induced apical targeting of Rab11 vesicles is essential for proper spatial organization
 443 and structural integrity of the supracellular actomyosin network, a function distinct
 444 from Rab11's function in promoting apical myosin II activation. This
 445 mechanochemical feedback mechanism allows the tissue to respond and adapt to
 446 altered mechanical environment during apical constriction and maintain the integrity
 447 of the force generation machinery during tissue folding.

448 ***Discussion:***

449 Our work identifies a Rab11-dependent mechanosensitive feedback mechanism that
 450 promotes proper spatial organization of apical actomyosin network during apical
 451 constriction. Using a combination of genetic, optogenetic and pharmacological
 452 approaches, we present evidence that during ventral furrow formation, apical
 453 constriction induces dynein- and microtubule-dependent, biased transport of Rab11
 454 vesicles towards the cell apices and results in enrichment of these vesicles at the
 455 apical AJs. The bias in transport depends on apical actomyosin contractility and is
 456 rapidly diminished upon acute myosin II inactivation. Acute elimination of apical
 457 Rab11 vesicles through Rab11DN injection resulted in fragmented apical AJs, which
 458 impaired the integrity of the apical supracellular actomyosin network as tension builds
 459 up in the network. Together, our findings suggest that the mechanosensitive regulation
 460 of Rab11-mediated vesicular trafficking provides a mechanism to allow the tissue to
 461 promptly adapt to rapid increases in tissue tension and maintain the integrity of the
 462 force generation machinery during tissue deformation (Fig. 7).

463

464 In recent years, it has been increasingly appreciated that mechanical forces can impact
 465 the process of intracellular membrane trafficking. For example, increased membrane
 466 tension can suppress the formation of endocytic vesicles (Wu et al., 2017) and
 467 conversely promote vesicle fusion with the lipid bilayer (Shillcock and Lipowsky,
 468 2005; Staykova et al., 2011). Mechanical force-induced stimulation of exocytosis has

been observed in various contexts, including cells undergoing spreading, being stretched or experiencing shear stress (Boycott et al., 2013; Gauthier et al., 2011; Khandelwal et al., 2013). The impact of mechanical force on vesicle transport and vesicle accumulation has also been observed. For example, mechanical tension can promote synaptic vesicle accumulation at the presynaptic terminals in *Drosophila* neuromuscular synapses and modulate the transport of large dense core vesicles in cultured *Aplysia* neurons (Siechen et al., 2009; Ahmed et al., 2012; Ahmed and Saif, 2014). Our work demonstrates that vesicle transport can also be regulated by endogenous contractile forces in embryonic epithelium to facilitate morphogenesis. The striking observation that the directionality of Rab11 vesicle transport can be modulated nearly instantaneously upon acute alteration of apical actomyosin contractility demonstrates that mechanical regulation of vesicle transport can take effect very rapidly and suggests the involvement of a fast mechanotransduction mechanism.

How does apical constriction affect vesicle transport inside of the cell? The directional bias of Rab11 vesicle transport could arise from the orientation of the cytoskeletal tracks. However, we do not favor this possibility considering the bias of vesicle transport diminished within one minute upon inhibition of myosin II activity, which is too short for significant changes in overall microtubule polarity to happen. Alternatively, apical constriction may cause changes in the activity of the motor

complexes that drive the motion of Rab11 vesicles. Our data indicate that the apically biased transport of Rab11 vesicles is predominantly mediated by dynein. Regulation of dynein-mediated transport can occur at multiple levels, including Rab11-adaptor binding, adaptor activities, adaptor-motor binding, dynein recruitment/binding to microtubules and motor activity itself, and these regulations often involve protein phosphorylation (Dillman and Pfister, 1994; Vaughan et al., 2002; Horgan et al., 2010; Otani et al., 2011; Fu and Holzbaur, 2013; Moughamian et al., 2013; Wang et al., 2019). It would be of interest to determine whether apical constriction regulates Rab11 vesicle transport by inducing alterations in the phosphorylation status of proteins mediating this transport. Finally, apical constriction may bias the direction of transport by generating a “sink” that retains the vesicles at the apical side. For example, the F-actin meshwork enriched at the apical cortex during apical constriction could serve as scaffolds to entrap the Rab11 vesicles or as tracks for vesicle dissemination at the apical domain, thereby inhibiting the reverse transport of vesicles.

Our results suggest a function of Rab11 vesicles in facilitating apical constriction after myosin II activation. Apical enrichment of Rab11 vesicles has also been observed in several other apical constriction mediated processes, including gastrulation and neural tube formation in *Xenopus* and salivary gland invagination in *Drosophila* (Ossipova et al., 2014, 2015; Le and Chung, 2021). Inhibition or mistargeting of Rab11 during

these processes results in impaired apical myosin II accumulation and defects in apical constriction, demonstrating the important function of Rab11 in mediating apical myosin II activation. In our study, while disrupting the “early” function of Rab11 inhibited apical myosin II accumulation, disrupting the “late” function of Rab11, which completely abolished apical Rab11 vesicles, did not affect the accumulation of myosin II at the cell apex but instead impaired the spatial organization of the actomyosin network. These findings, for the first time, demonstrate that Rab11 has separate “early” and “late” functions in regulating apical constriction during tissue folding.

In Rab11DN injected embryos, the appearance of frequent myosin breaks was associated with severe fragmentation of apical AJs. We propose that the late function of Rab11 in maintaining the integrity of the actomyosin network is attributed to its role in regulating apical AJs. Breaks in the actomyosin network have also been observed in other conditions where junctions are impaired (Sawyer et al., 2009; Martin et al., 2010; Movie 10). In addition, disruption of microtubules during apical constriction, which we show inhibited the apical-basal transport of Rab11 vesicles, has been shown to cause dynamic separation of actomyosin fibers from AJs (Ko et al., 2019). Notably, the elevated frequency of myosin breaks in Rab11DN-injected embryos was most prominent at late stages of apical constriction as tension increases. Furthermore, these breaks mainly occurred along anterior-posterior direction, where

the tension is higher (Martin et al., 2010). These observations suggest that Rab11-mediated reinforcement of junction-actomyosin connections is important for the supracellular actomyosin network to resist increased tissue tension as apical constriction proceeds.

The mechanisms by which Rab11 reinforces AJs is unclear. We propose that this function is mediated by apical Rab11 vesicles for the following reasons. Firstly, at the apical side, Rab11 vesicles were enriched at AJs. And secondly, when these vesicles were eliminated, AJs became more fragmented and the level of the junctional component Jub was substantially reduced. Rab11 recycling endosomes have been shown to regulate the delivery of E-cadherin to the AJs in a number of other cell and tissue contexts (Lock and Stow, 2005; Langevin et al., 2005; Yashiro et al., 2014; Le Droguen et al., 2015; Woichansky et al., 2016). However, we did not detect E-cadherin-GFP signal on Rab11 vesicles during apical constriction. It is possible that the signal was too weak to be detected by the method we used. Alternatively, these vesicles may function to transport other junction components or regulators. Finally, Rab11 vesicles may regulate the connection between AJ and actomyosin network by delivering actin regulators to AJs, as dynamic actin turnover at AJs has been shown to impact the detachment and reattachment of actomyosin network to AJs (Jodoin et al., 2015). Future studies identifying the cargos of Rab11 vesicles during apical constriction will be the key for understanding the molecular function of the vesicles.

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Author Contributions

W.C. and B.H. designed the study. W.C. performed the experiments and analyzed the data. W.C. and B.H. wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

STAR ★ Methods

Lead Contact and Materials Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bing He (bing.he@dartmouth.edu).

Experimental Model and Subject Details

Drosophila melanogaster flies were grown and maintained at 18°C and crosses were raised and maintained at room temperature (21 – 23°C) unless otherwise mentioned.

All flies were raised on standard fly food. For embryo collection, flies with corresponding genotype were used to set up cages and maintained at 18°C, and embryos were collected from apple juice agar plate containing fresh yeast paste.

For most experiments, embryos expressing endogenously tagged YFP::Rab11 (Dunst et al., 2015) (BDSC Stock#62549) were imaged to visualize Rab11 vesicles. We also generated UAS-mCherry::Rab11 transgenic flies for dual-color imaging with other GFP-tagged markers. For lines with UAS-driven transgenes or shRNA, we used GAL4 driver lines carrying *matα4*-GAL-VP16 (denoted as “mat67” on the 2nd chromosome and “mat15” on the 3rd chromosome) to drive maternal expression in the embryo through either direct cross or recombination.

The following fly lines were generated for dual-color imaging of Rab11 with other markers in this study:

- Sqh::mCherry; YFP::Rab11
- Gap43::mCherry/Cyo; YFP::Rab11
- mat67 mCherry::Rab11; mat15 GFP::Jupiter
- mat67 mCherry::Rab11; mat15 E-cadherin::GFP
- mat67 mCherry::Rab11; mat15 mCherry::P4M

Figure S1: To examine other organelle behavior during apical constriction, the following lines were either directly used or first crossed to mat67; mat15 flies in order to obtain embryos expressing the corresponding fluorescent marker:

- UAS-Arf79F::GFP (BDSC Stock#65850)
- UAS-KDEL::GFP (BDSC Stock#9898)
- UAS-YFP::Rab7 (BDSC Stock#23641)
- YFP::Rab5 (BDSC Stock#62543)
- YFP::Rab8 (BDSC Stock#62546)

Figure 2C: To knock down myosin II, female flies of a TRiP *zipper*; YFP::Rab11 stock generated from a TRiP *zipper* RNAi line (BDSC Stock#37480) were crossed to male flies from stock mat67 Sqh::mCherry; mat15 YFP::Rab11 to obtain F1 TRiP *zipper*/mat67 Sqh::mCherry; YFP::Rab11/mat15 YFP::Rab11 flies, and embryos from

613 these F1 flies were collected for imaging.

614

615 Figure 2D: To generate ventralized embryos, *Spn27A1/Cyo*; *Sqh::mCherry*

616 *YFP::Rab11/TM3* was crossed to *Df(2L)BSC7/Cyo*; *Sqh::mCherry YFP::Rab11/TM3*

617 to obtain *Spn27A1/ Df(2L)BSC7*; *Sqh::mCherry YFP::Rab11* flies, and embryos from

618 these flies were collected for imaging.

619

620 Figure 2E: The fly stock used for temperature shift experiment is *shi^{ts}/ shi^{ts}*;

621 *Sqh::mCherry*; *YFP::Rab11*.

622

623 Figure 5 and S3: Female flies from stock *CRY2::mCherry::Rho1DN*

624 *YFP::Rab11/TM6C*; *CIBNpm/FM7* were crossed to male flies from stock *mat67*

625 *Sqh::mCherry*; *mat15 YFP::Rab11* to generate *mat67 Sqh::mCherry/+*;

626 *CRY2::mCherry::Rho1DN YFP::Rab11/mat15 YFP::Rab11*; *CIBNpm/+* flies, and

627 embryos from these flies were collected for imaging.

628

629 Figure 6: *Sqh::mCherry E-cadherin::GFP* was used for *Rab11DN* injection

630 experiment for examining adherens junction phenotype and quantifying apical area

631 change. *Sqh::mCherry mat67*; *mat15 UAS-Jub::GFP* was used for figure 6G.

632

633 Figure S3: To visualize the localization of constitutively active *Rab11*, *UAS-*

YFP::Rab11Q70L (BDSC Stock#9791) was crossed to *mat67*; *mat15* males, and embryos from F1 females were used for imaging.

Movie 10: A TRiP *canoe* RNAi line (BDSC Stock#38194) was used for *canoe* knockdown. Female flies from the TRiP *canoe* stock were crossed to male flies from stock *mat67* Sqh::mCherry; *mat15* E-cad::GFP to obtain F1 TRiP *canoe*/*mat67* Sqh::mCherry; +/-*mat15* E-cad::GFP flies, and embryos from these F1 flies were collected for imaging.

Method details

Molecular cloning and generation of transgenic fly lines

To make construct for in vitro expression of recombinant dominant negative Rab11 (S25N) protein, dominant negative Rab11 coding sequence was PCR amplified from genomic DNA of UAS-YFPRab11DN (BDSC Stock#9792) and inserted into pGEX-6p-1 vector (a gift from the Griffin lab, Dartmouth College) using BamHI and XhoI restriction sites.

To make constructs for transgenic fly lines, mCherry-P4M double strand DNA was synthesized in vitro (Integrated DNA Technologies) and inserted into a fly transformation vector (pTiger, courtesy of S. Ferguson, State University of New York at Fredonia, Fredonia, NY, USA) using NotI and NheI restriction site to generate the

655 pTiger-mCherry-P4M plasmid.

656

657 To make pTiger-mCherry-Rab11 construct, wild type Rab11 coding sequence was

658 PCR amplified from genomic DNA of UAS-YFP::Rab11 (BDSC Stock# 9790) and

659 cloned into pTiger-mCherry-P4M construct using SpeI and NheI restriction sites to

660 replace the P4M sequence.

661

662 The resulting pTiger-mCherry-P4M and pTiger-mCherry-Rab11 constructs were sent

663 to BestGene, Inc., for integration into either attP2 or attP40 site using the phiC31

664 integrase system (Groth et al., 2004).

665

666 **Live imaging and optogenetics**

667 Embryos were dechorionated in 3% bleach, rinsed with water 12 times and mounted

668 in water in a 35 mm MatTek glass-bottom dish (MatTek Corporation). Unless

669 otherwise mentioned, all images were obtained using a Nikon inverted spinning disk

670 confocal microscope equipped with the perfect focus system and Andor W1 dual

671 camera, dual spinning disk module. An CFI Plan Apo Lambda 60×/1.40 WD 0.13 mm

672 Oil Objective Lens objective was used for imaging at room temperature. YFP and

673 GFP tagged proteins were imaged with a 488-nm laser and mCherry tagged proteins

674 were imaged with a 561-nm laser. Images in Figure 1A, S1A and B were obtained

675 using an upright Olympus FV-MPERS multiphoton microscope equipped with the

InSight Deep Laser System with an Olympus 25X/1.05 water dipping objective (XLPLN25×WMP2). Images in Figure 1D, 2A, 2D, S2, S3 were obtained using a Zeiss Axio Observer laser scanning confocal microscope (LSM 880).

For optogenetic experiments, flies were kept in the dark and live sample preparation was performed under red light. Embryos were first imaged with 561-nm laser to visualize myosin II signal to determine the developmental stage of the embryo. Once the embryo reached the desired stage, two-color imaging with both 488-nm and 561-nm lasers were carried out, where the 488-nm laser was used for both optogenetic stimulation and YFP-Rab11 visualization.

For the temperature shift experiment with *shi^{ts}* mutants, a Zeiss Axio Observer laser scanning confocal microscope (LSM 880) with an incubation chamber was used. Embryos subjected to restrictive temperature were prepared at room temperature and then transferred to an incubation chamber, which was preheated to 32°C. A 40X/1.3 numerical aperture oil-immersion objective, and 488-nm argon laser and 561-nm laser were used for imaging.

Rab11 dominant negative protein expression and purification

pGEX-6p-1-Rab11DN plasmid was transformed into E.coli (BL21(DE3), New England Biolabs). An empty vector with only GST coding sequence was also

transformed as control. After IPTG induction, bacteria were resuspended in 32 mL lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 1 mM DTT) and lysed with 0.25 mg/mL lysozyme incubation on ice for 2 h followed by sonication (6 rounds of 10 pulses every 1 minute). GST and GST-Rab11DN were purified from the supernatant with Glutathione Sepharose 4B GST-tagged protein purification resin (GE17-0756-01, Sigma-Aldrich) using batch method. Eluted protein was dialyzed using slide-A-Lyzer dialysis cassettes (Cat#66380, Thermo Scientific) with 50 mM Tris buffer (pH8.0) and further concentrated to a final concentration of ~200 μ M with Amicon Ultra-0.5mL centrifugal filters (Cat#UFC501024, Millipore Sigma).

On-stage drug / protein injection

Embryos at cellularization stage were prepared as previously described, then mounted ventral side down on a 50 x 25 mm glass coverslip pre-covered with a thin layer of glue. Embryos were dried for 10-15 minutes in a desiccator. Embryos were then covered with halocarbon oil (halocarbon 700/halocarbon 27 = 3:1). A homemade injection device mounted next to the spinning disk confocal microscope was used for on-stage injection. Embryos were injected laterally into the ventral part of the embryo. 50 mM Y-27632 (Enzo Life Sciences) and 25 mg/mL colchicine (Sigma-Aldrich) were used. For Rab11DN injection, Triton X-100 was added to a final concentration of 0.1% to prevent the solution from clogging the needle. For dynein

and GST antibody injection, commercial monoclonal antibodies against cytoplasmic dynein intermediate chain (Cat#sc-13524, Santa Cruz Biotechnology) and monoclonal antibody against GST (Cat#sc-138, Santa Cruz Biotechnology) were first concentrated to 2 mg/mL with Amicon Ultra-0.5 mL centrifugal filters before injection (Cat#UFC501024, Millipore Sigma).

Quantification and Statistical Analysis

Image processing and analysis

All images were processed using ImageJ (NIH) and MATLAB (MathWorks). For the following figures, due to laser power fluctuation and the extent of photobleaching with different imaging duration, the contrast was adjusted to make the cytoplasmic signal comparable: Figure 2A, 2B (Rab11), 2C (myosin II), 2D (myosin II and Rab11), S2(myosin II and Rab11), 4C (Rab11), 4H (myosin II and Rab11), 5E, 5F, 6A.

For vesicle tracking (Figure 3C-F, J, K), a 13.8 μm x 6.9 μm region of interest including approximately one constricting cell at 50% egg length was selected, and Rab11 vesicles were manually marked frame by frame for each trajectory using a multi-point tool in ImageJ. The x-y coordinates of individual vesicles over time were then exported. A MATLAB script was written to reconstruct the trajectories based on the coordinate information. The direction of each trajectory was defined by the

relative translocation of the vesicle along apical basal axis between trackable start and end positions. All trajectories were grouped into two categories based on their direction (apical or basal) for further analysis of parameters, including trajectory count, average velocity, trajectory length and trajectory duration.

For quantification of vesicle density in 2D (Figure 4D-F), 3 separate ROIs covering most of the constriction region were selected, with each ROI enclosing a relatively flat piece of tissue surface (based on the apical myosin II signal). For each ROI, a Z position near the apical surface where most vesicles were accumulated was selected for quantification. Vesicles were detected using the Find Maxima function in ImageJ in combination with manual correction to ensure accuracy of the counting. For vesicle counting in 3D (Figure 4D-F), the FIJI plugin 3D Maxima Finder was used and the noise tolerance parameter was determined based on average intensity of the entire image stack (Ollion et al., 2013).

The colocalization between Rab11-mCherry and E-cadherin-GFP was quantified in embryos at the stage of early ventral furrow formation (Figure 5B-D). For each embryo, 5 non-neighboring constricting cells were manually segmented using a multi-point tool to mark the vertices of the cell outline. A MATLAB script was written to import the coordinates of these vertices to create the cell outline. For each cell outline, a series of closely spaced and evenly distributed sampling points were generated

along the outline. For each sampling point, E-cadherin-GFP signal intensity on that single pixel was extracted as the junction signal, and average Rab11-mCherry signal intensity within a radius of 5 pixels ($\sim 0.54 \mu\text{m}$) was calculated as Rab11 signal for the same point. For each cell, a scatter plot of E-cadherin intensity over Rab11 intensity for all the sampling points was generated, and a correlation coefficient was calculated. A similar analysis was performed between P4M-GFP and Rab11-mCherry as a control.

For myosin II intensity quantification (Figure 6B), 100-pixel by 100-pixel ROIs ($10.8 \mu\text{m} \times 10.8 \mu\text{m}$) were taken from the apical myosin II domain. Average intensity R was measured inside the ROI on that plane, and background noise at the same plane (R_{bg}) was measured as the mean intensity of a random ROI outside the embryo. To assess cytoplasmic myosin intensity (C), we used the same ROI position but at a slightly deeper Z below the apical myosin network, the background noise at that plane (C_{bg}) was measured as described above. The normalized myosin II intensity $I = (R - R_{bg}) / (C - C_{bg})$. Embryos at $\sim 6 - 7$ min after the onset of gastrulation were used for quantification.

For quantification of myosin breaks (Figure 6J, K), a maximum projection over $3 - 5 \mu\text{m}$ thick Z stacks that include all apical myosin II signal was generated. A ~ 100 -sec time window starting from the time point when the myosin II domain at the ventral

surface exhibits a relatively clear domain boundary (~6 – 7 min after the onset of gastrulation) was selected to count the number of myosin break events. At the site where myosin break occurs, the myosin fiber usually snaps and the two ends rapidly retract, which creates a clear gap between neighboring myosin clusters.

To quantify the rate of apical constriction based on the movement of apical myosin II structures (Figure 6D-G), kymographs were generated from the same dataset of the above-mentioned 100-sec myosin movies. For each embryo, a series of myosin kymographs were generated by average projection over every 30 pixels (3.2 μm) along anterior posterior axis. Bandpass filter in ImageJ were used to remove noise. A MATLAB script was used for automatic segmentation of individual myosin traces and fitting with lines. Rate of myosin II moving towards ventral midline was extracted by calculating the slope of each myosin trace. Velocity of myosin II at ventral midline was subtracted to correct for the drift of ventral midline itself. The rate of myosin movement was then plotted against the distance of the corresponding myosin structure to ventral midline at the time when tracing started.

To quantify the rate of apical constriction from E-cadherin-GFP movies (Figure S4A, B), images at 6 – 7 μm below the apical surface were used to measure the cell area change over time. A group of constricting cells at the ventral most region of the embryo were outlined frame by frame using multi-point tool in ImageJ. The area of

the same cell group over time was then calculated and plotted using MATLAB.

To analyze the distribution of cell area in the same embryo at 2 min, 5 min and 8 min after onset of gastrulation (Figure S4C-E), individual constricting cell was segmented using Embryo Development Geometry Explorer (EDGE), a MATLAB-based software (Gelbart et al., 2012). Individual cell area was then calculated, and the distribution histogram of cell area was plotted. Cell anisotropy was calculated based on the same segmented cell dataset (Figure S4F). Two orthogonal (anteroposterior and mediolateral) lines passing the centroid of the cell were drawn across the segmented cell, and the cell anisotropy was calculated as the ratio between the anteroposterior intercept by the cell boundary and the mediolateral intercept.

Statistics

Statistical comparisons were performed using two tailed one-sample t test or two-tailed Student's t tests. Sample sizes can be found in figure legends. *p* values were calculated using MATLAB ttest (Two tailed one-sample t test) and ttest2 function (Two tailed Student's t test).

819 Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals and Antibodies		
Y-27632	Enzo Life Sciences	270-333-M005
Colchicine	Sigma-Aldrich	C9754-500MG
Dynein IC1/2 mouse monoclonal antibody	Santa Cruz Biotechnology	Sc-13524
GST(B-14) mouse monoclonal antibody	Santa Cruz Biotechnology	Sc-138
Experimental Models: Organisms/Strains		
D.melanogaster: YFP-Rab11	Bloomington Stock Center	BDSC:62549
D.melanogaster: UAS-mCherry-Rab11	This study	N/A
D.melanogaster: mat α Tub-Gal4VP16 67 (mat67)	Hunter and Wieschaus, 2000 (Wieschaus Lab)	N/A
D.melanogaster: mat α Tub-Gal4VP16 15 (mat15)	Hunter and Wieschaus, 2000 (Wieschaus Lab)	N/A
D.melanogaster: Sqh-mCherry	Martin et al 2009	N/A

	(Wieschaus Lab)	
D.melanogaster: UAS-GFP-Jupiter	Bloomington Stock Center	BL6836
D.melanogaster: E-cadherin-GFP	Oda, H. & Tsukita, S. J. Cell Sci. 114, 493–501 (2001).	N/A
D.melanogaster: UAS-mCherry-P4M	This study	N/A
D.melanogaster: UAS-Arf79F-GFP	Bloomington Stock Center	BDSC: 65850
D.melanogaster: UAS-KDEL-GFP	Bloomington Stock Center	BDSC: 9898
D.melanogaster: UAS-YFP-Rab7	Bloomington Stock Center	BDSC: 23641
D.melanogaster: YFP-Rab5	Bloomington Stock Center	BDSC: 62543
D.melanogaster: YFP-Rab8	Bloomington Stock Center	BDSC: 62546
D.melanogaster: UASp-YFP-Rab11Q70L	Bloomington Stock Center	BDSC: 9791
D.melanogaster: UASp-YFP-	Bloomington Stock	BDSC: 9792

Rab11S25N	Center	
D.melanogaster: UASp-YFP-Rab11	Bloomington Stock Center	BDSC: 9790
D.melanogaster: Jub-GFP/TM2	Bloomington Stock Center	BDSC: 56086
D.melanogaster: UASp-CRY2-mCherry-Rho1DN	Guo et al In preparation	N/A
D.melanogaster: UASp-CIBNpm	Guglielmi et al., 2015	N/A
D.melanogaster: TRiP Zipper	Bloomington Stock Center	BDSC: 37480
D.melanogaster: <i>shi^{ts}</i>	Bloomington Stock Center	BDSC: 7068
D.melanogaster: <i>Spn27A1/Cyo</i>	http://flybase.org/reports/FBrf0151901.html	N/A
D.melanogaster: Df(2L)BSC7/Cyo	Bloomington Stock Center	BDSC: 6374
D.melanogaster: TRiP cno	Bloomington Stock Center	BDSC: 38194
Oligonucleotides		

Forward primer for making mCherry-P4M construct: AAAAGCGGCCGCATGGTGA GCAAGGGCGAG		N/A
Reverse primer for making mCherry-P4M construct: TTTGTCTAGCTTATTTTATCT TAATGGTTTGTCTTTCTTG		N/A
Forward primer for making mCherry-Rab11 construct: AAAAACTAGTATGGGTGCA AGAGAAGACGA		N/A
Reverse primer for making mCherry-Rab11 construct: aaaaGCTAGCTCACTGACAGC ACTGTTTGCG		N/A
Forward primer for pGEX6p-1- Rab11DN cloning: AAAAGGATCCATGGGTGCA AGAGAAGACGA		N/A
Reverse primer for pGEX6p-1-		N/A

Rab11DN cloning: AAAAC TCGAGTCACTGACA GCACTGTTTGCG		
Software and Algorithms		
Matlab	MathWorks	https://www.mathworks.com/?s_tid=gn_logo; RRID:SCR_001622
FIJI	ImageJ	http://fiji.sc; RRID:SCR_002285
Embryo Development Geometry Explorer (EDGE)	Gelbart et al., 2012	https://github.com/mgelbart/embryo-development-geometry-explorer
Inkscape		https://inkscape.org/

820

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Figure 1

Figure 1. Rab11-positive vesicle-like structures accumulate apically in

constricting cells during ventral furrow formation

(A) Apical constriction during ventral furrow formation. Scale bar, 10 μ m. The bottom panel shows the imaging setup for acquiring the en face view of constricting cells.

(B) 3-D reconstruction of the ventral part of an embryo expressing YFP-Rab11 and mCherry-Sqh during ventral furrow formation. Magenta box indicates the apical region of the constricting cells.

(C) Rab11 compartments in an embryo at the onset (0 min) and 9 min into ventral furrow formation. Top panel, maximum projection over 2 μ m depth from en face view; middle panel, a single slice cross section view; bottom panel, maximum projection of the cross section view. Red box indicates the Z position of the corresponding en face view. Red arrows: perinuclear Rab11 vesicles; green arrows and blue outlines: perinuclear Rab11 compartments; yellow outline: nucleus.

(D) Zoom-in view of a small ROI in the ventral region of the embryo.

(E) Montage showing YFP-Rab11 and mCherry-Sqh distribution over depth during apical constriction.

All scale bars are 5 μ m unless mentioned otherwise.

Figure 2

Figure 2. Apical accumulation of Rab11 vesicles depends on apical constriction

(A) Inhibition of myosin II activation by Y-27632 injection at late cellularization

prevents apical Rab11 vesicle accumulation. Shown are maximum projections of a ~3

– 4 μm -stack below apical surface of the constriction domain (same for the other en

face view images in this figure). The zoom-in view of regions marked by red boxes is

shown to the right. 0 sec marks the onset of gastrulation. For Y-27632 injection: N = 4

embryos; H₂O injection: N = 3 embryos. Contrasts were adjusted to make the

background comparable.

(B) Injection of Y-27632 during apical constriction causes rapid diminishing of apical

Rab11 vesicles. N = 4 embryos. (B') Cross section view of the same embryo. Yellow

dotted lines mark the apical outline highlighting tissue relaxation after drug injection.

(B'') Zoom-in view of panel B.

(C) Knockdown of myosin heavy chain Zipper inhibits apical accumulation of Rab11

vesicle. N = 4 embryos for each genotype. (C') Zoom-in view of panel C.

(D) *Spn27A/Df* mutant embryos exhibit ventralized phenotype with expanded domain

of apical myosin II activation. The domain of apical Rab11 vesicle accumulation also

expands accordingly (N = 3 embryos), as shown in (D') with zoom-in view of regions

marked by red boxes in (D). Images were Gaussian filtered with a radius of 0.5 pixel.

To improve the signal/noise, fewer apical z-slices were used to generate the projected

images for the zoom-in view.

All scale bars for zoom-in view, 2 μm ; scale bars for the rest, 10 μm .

Figure 3

Figure 3. Rab11 vesicles are transported apical-basally with a strong bias in the

apical direction, which depends on activation of apical actomyosin contractility

(A) Imaging configuration to capture the movement of Rab11 vesicles along the apical-basal direction. Red dotted line indicates the imaging plane. Two constricting cells being imaged are marked in green.

(B) Left: surface view of an embryo expressing Sqh-mCherry and YFP-Rab11. Scale bar, 10 μ m. Right: zoom-in view of the marked region showing the movement of two Rab11 vesicles (white circles) towards apical myosin II. Scale bar, 2 μ m.

(C) Vesicle trajectories in 3 wildtype embryos in a ~35-sec time window. Red and blue mark apical- and basal-bound trajectories, respectively.

(D) Counts of apically and basally oriented trajectories.

(E) Rab11 vesicles exhibit directional bias towards the apical side. Two tailed one-sample t-test against 50%; N = 3 embryos. In all figures, Error bar stands for s.d.; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; n.s.: $p > 0.05$.

(F) Comparison of average trajectory length, velocity and duration (mean $\pm$ s.d.) shows no significant difference between apically and basally directed transport. Two tailed Student's t-test; N = 3 embryos.

(G) Upstream signaling pathway that activates myosin II driven apical constriction.

(H) Diagram depicting optogenetic inhibition of myosin activation by Opto-Rho1DN.

(I) Upon continuous blue light (488 nm) stimulation, Rho1DN is rapidly recruited to the plasma membrane (yellow arrows). Apical myosin II (red boxes) disappears from

1121 cell apices ~ 30 sec after stimulation. A wildtype embryo at similar stage of furrow
 1122 formation is shown as a control. Scale bar, 10 μ m.
 1123 (J) The apical bias of the transport is abolished in less than one minute after
 1124 stimulation in Opto-Rho1DN embryos (two tailed one-sample t-test against 50%).
 1125 Two tailed Student's t test was used for all other statistical comparisons.
 1126 (K) Average trajectory velocity, length and duration of apical and basal bound
 1127 transports (mean $\pm$ s.d.). There is no significant difference between 0 – 23 sec and 35
 1128 – 58 sec post stimulation time windows. Two tailed Student's t test; N = 3 embryos.

Figure 4

Figure 4. Rab11 vesicle transport requires microtubules and dynein

(A) An example of Rab11 vesicle movement (yellow circle) along the microtubules (Jupiter-GFP). Scale bar, 1 μm .

(B) Acute disruption of microtubules via on-stage injection of colchicine results in immediate inhibition of Rab11 vesicle transport. Images showing the same region before and one minute after injection. Magenta arrowheads indicate residual microtubules after colchicine injection. Yellow dotted circle marks a region where microtubules are completely disrupted. Scale bar, 2 μm .

(C) En face view of the constricting domain shortly (< 2 min) after on-stage injection of water or colchicine. Injection of colchicine shortly before but not after the onset of gastrulation ($N = 5$ embryos for each condition) results in reduced Rab11 vesicle accumulation compared to water injected controls ($N = 4$ embryos). Scale bar, 10 μm . Zoom-in view of regions marked by red boxes are shown in the bottom. Scale bar, 2 μm .

(D-F) Quantification of Rab11 vesicle density after water or colchicine injection within a 3.5 μm -stack from apical surface (D) or in a single z plane near apical surface with the highest vesicle density (E). The two approaches are well consistent with each other (F).

(G) On-stage injection of dynein antibody rapidly abolishes both apical and basal transport of Rab11 vesicles. Images showing the overlay between the Rab11 channel and the apically or basally targeted vesicle trajectories within a 20-sec time window at

1152 the indicated time after injection. N = 5 embryos for both DIC antibody and GST
 1153 antibody injection. Scale bar, 2 μ m.
 1154 (H) En face view showing inhibition of apical accumulation of Rab11 vesicles upon
 1155 on-stage injection of dynein antibody immediately before the onset of gastrulation. N
 1156 = 5 and 6 embryos for GST antibody and dynein antibody injection, respectively.
 1157 Scale bar, 10 μ m. Zoom-in view of regions marked by red boxes are shown in the
 1158 bottom. Scale bar, 2 μ m.

Figure 5

Figure 5. Rab11 reinforces the apical adherens junctions (AJs) during ventral furrow formation

(A) Movie stills showing Rab11 vesicles moving towards an AJ spot. Scale bar, 1 μ m.

(B-D) Analysis of spatial correlation between Rab11 vesicles and AJs. (B) A

constricting cell is outlined in green. Magenta marks the circle within which Rab11

signal is measured. Right panel: the intensity profiles of E-cadherin and Rab11 along

the indicated cell outline. (C) E-cadherin intensities for all points along the cell

outline plotted against the corresponding Rab11 intensities. (D) Rab11 shows a

significantly higher correlation with E-cadherin than with a general membrane marker

P4M (N = 3 embryos for each genotype, 5 cells per embryo). Two tailed Student's t-

test was used for statistical comparison. E-cad: E-cadherin.

(E) Injection of dominant negative Rab11 (Rab11DN) eliminates apical Rab11

vesicles but does not immediately affect the perinuclear Rab11 compartments (green

arrows). Scale bar, 10 μ m. (E') Zoom-in view of regions marked by red boxes. Scale

bar, 2 μ m.

(F) Rab11DN injection affects the integrity of apical AJs in the constricting cells. 14

out of 19 Rab11DN injected embryos show fragmented apical AJs while 8 of 8 GST

injected embryos show relatively continuous AJs. Scale bar, 5 μ m.

(G) Upon Rab11DN injection, Ajub is more sparsely distributed at the apical AJs

during apical constriction (N = 2 embryos for no injection control, N = 4 embryos for

Rab11DN injection). Scale bar, 5 μ m.

Figure 6

Figure 6. Acute Inhibition of Rab11 results in defects in myosin II organization

and apical constriction

(A-C) Stage-dependent effect of Rab11DN injection on apical myosin network. (A):

Injection of Rab11DN at early cellularization (> 30 min before onset of gastrulation)

impairs apical myosin II activation (N = 3 embryos), whereas injection around the

onset of gastrulation does not affect myosin II activation but causes defects in apical

myosin organization (N = 4 embryos). GST injection: N = 3 embryos. Scale bar, 10

μm. (B, C): Quantification of apical myosin II intensity (B) and the width of myosin

domain (C) at ~6-7 min after the onset of gastrulation. Two tailed Student's t-test was

used for statistical comparison.

(D) Example kymograph showing the movement of apical myosin II towards ventral

midline at ~6-7 minutes into gastrulation. Only the late Rab11DN injected embryos

were analyzed in (D) – (K). Scale bar, 5 μm.

(E-G) Quantification of apical constriction rate in embryos injected with GST and

Rab11DN. (E, F) Rate of myosin movement towards ventral midline is plotted against

the distance of myosin structures from ventral midline at 0 sec. Red asterisks

represent the average velocity at the corresponding position and the red curve is the

polynomial fit of the average velocity along the medial-lateral direction. (G) The rate

of movement for myosin structures located 10-15 μm away from the ventral midline

is compared between GST and Rab11DN-injected embryos. Two tailed Student's t test

was used for statistical comparison.

1205 (H-K) Injection of Rab11DN increases the frequency of myosin breaks during apical
 1206 constriction. (H) Left, representative surface views of the supracellular myosin
 1207 network in Rab11DN- and GST-injected embryos. Scale bar, 10 μ m. Right, zoom-in
 1208 view of the highlighted region (cyan boxes) showing myosin dynamics over time. Red
 1209 arrowheads show separation of neighboring myosin structures after a break event
 1210 happens. Scale bar, 2 μ m. (I) Apical AJ is pulled inward (red arrows) when myosin
 1211 break (yellow asterisks) occurs in the adjacent cell. Scale bar, 2 μ m. (J) Quantification
 1212 of the number of myosin breaks over a ~100-sec time window at ~6 – 7 min after
 1213 apical constriction starts. Two tailed Student's t test was used for statistical
 1214 comparison. (K) Quantification of myosin break orientation in GST- and Rab11DN-
 1215 injected embryos.

Figure 7

Figure 7. Schematics of proposed mechanosensitive feedback mechanism

During *Drosophila* ventral furrow formation, actomyosin contraction mediated apical constriction results in biased transport of Rab11 vesicles towards the apical surface, which leads to the apical accumulation of these vesicles. At the apical region, the Rab11 vesicles are targeted to apical AJs and function to reinforce the structural integrity of AJs. This mechanosensitive function of Rab11 is important for securing the anchoring sites for the contractile machinery at the cell-cell boundaries, which ensures proper organization of the supracellular actomyosin network and efficient apical constriction. When apical accumulation of Rab11 vesicles is inhibited, the constricting cells exhibit fragmented AJs, frequent myosin breaks and myosin ring formation, which reduces the rate of apical constriction.