Rho GTPase and Shroom direct planar polarized actomyosin contractility during convergent extension

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Actomyosin contraction generates mechanical forces that influence cell and tissue structure. During convergent extension in Drosophila melanogaster, the spatially regulated activity of the myosin activator Rho-kinase promotes actomyosin contraction at specific planar cell boundaries to produce polarized cell rearrangement. The mechanisms that direct localized Rho-kinase activity are not well understood. We show that Rho GTPase recruits Rho-kinase to adherens junctions and is required for Rho-kinase planar polarity. Shroom, an asymmetrically localized actin- and Rho-kinase-binding protein, amplifies Rho-kinase and myosin II planar polarity and junctional localization downstream of Rho signaling. In Shroom mutants, Rho-kinase and myosin II achieve reduced levels of planar polarity, resulting in decreased junctional tension, a disruption of multicellular rosette formation, and defective convergent extension. These results indicate that Rho GTPase activity is required to establish a planar polarized actomyosin network, and the Shroom actin-binding protein enhances myosin contractility locally to generate robust mechanical forces during axis elongation.

Introduction

Actomyosin contractility generates mechanical forces that determine cell and tissue structure through its role in regulating cell division, cell shape, and cell rearrangement. Spatially and temporally controlled contractile forces during development are achieved in large part by the regulated activity of protein kinases that phosphorylate myosin and promote its activity. Increasing evidence indicates that Rho-kinase, the primary regulator of myosin activity in epithelial tissues, is a critical determinant of where and when myosin is active within the cell (Riento and Ridley, 2003; Quintin et al., 2008; Amano et al., 2010). Rho-kinase is localized to the apical cell cortex in epithelia (Wang and Riechmann, 2007) and recruits myosin apically to induce apical constriction during epithelial bending and cell invagination (Dawes-Hoang et al., 2005; Mason et al., 2013). A distinct pattern of Rho-kinase localization at cell boundaries promotes cell rearrangements during tissue remodeling. Rho-kinase accumulates in a planar polarized fashion at cell contacts that are disassembled during axis elongation in Drosophila melanogaster (Simões et al., 2010). Rho-kinase activity influences the orientation of cell rearrangements during axis elongation by promoting localized actomyosin contractility and inhibiting cell adhesion by excluding the Baz/Par-3 junctional protein (Simões et al., 2010). Rho-kinase planar polarity has been observed in several epithelial tissues, and Rho-kinase activity is required for planar polarized cell rearrangements and cell shape changes during development (Walters et al., 2006; Nishimura et al., 2012; Robertson et al., 2012; Bulgakova et al., 2013). However, although planar polarized Rho-kinase activity plays a critical role in regulating localized actomyosin contractility, the mechanisms that generate Rho-kinase planar polarity are not well understood.

Rho GTPases are conserved upstream regulators of actomyosin organization and contractility (Riento and Ridley, 2003; Jaffe and Hall, 2005). Several lines of evidence suggest that apical myosin activity is regulated by localized Rho GTPase signaling. Rho and its activator RhoGEF2 are required for apical constriction and apical myosin localization in the Drosophila embryo, and RhoGEF2 protein is apically localized (Barrett et al., 1997; Häcker and Perrimon, 1998; Nikolaidou and Barrett, 2003). In the developing egg chamber, the spatially regulated activity of Rho GTPase coordinate with the localization of Rho kinase to direct apical actomyosin contractility and cell rearrangement. However, the mechanisms that direct the planar polarized activity of Rho GTPases are not well understood.
myosin planar polarity and junctional localization. These results in turn is required to amplify and maintain Rho-kinase and Rho-kinase to adherens junctions and generates a planar polar contractility during apical constriction. However, it is not known whether localized Rho signaling is involved in planar polarized Rho-kinase localization and myosin activity. The Rho activators RhoGEF2 in Drosophila and its chick homologue, PDZ-RhoGEF, are present in a planar polarized distribution during axis elongation and neural tube closure (Levayer et al., 2011; Nishimura et al., 2012; Warrington et al., 2013). However, the defects in RhoGEF2 mutants are weaker than those caused by loss of Rho-kinase or myosin II (Häcker and Perrimon, 1998; Levayer et al., 2011), suggesting that additional mechanisms regulate actomyosin contractility in the plane of the tissue.

Shroom family proteins are actin-associated proteins that bind to Rho-kinase and target it to the apical cell cortex (Hildebrand and Soriano, 1999; Haigo et al., 2005; Hildebrand, 2005; Nishimura and Takeichi, 2008; Bolinger et al., 2010). Shroom proteins are required for neural tube closure in the mouse, frog, and chick (Hildebrand and Soriano, 1999; Haigo et al., 2003; Nishimura and Takeichi, 2008; Massarwa and Niswander, 2013). These defects are thought to be caused by a role for Shroom in apical constriction, as ectopic Shroom is sufficient to promote apical Rho-kinase and myosin localization and induce apical constriction in epithelial cells (Haigo et al., 2003; Hildebrand, 2005; Nishimura and Takeichi, 2008). Moreover, Shroom is required for many developmental processes that involve apical constriction, such as epithelial bending at neural tube hinge points in Xenopus laevis (Haigo et al., 2003; Lee et al., 2007), invagination of the mammalian lens placode (Plageman et al., 2010), and apical constriction of cells in the zebrafish lateral line (Ernst et al., 2012). However, although the effects of ectopic Shroom have been well characterized in culture, the effects of loss of Shroom on cell behavior and myosin dynamics are less well understood.

In the Drosophila embryo, axis elongation is driven by planar polarized cell rearrangements that require spatially regulated actomyosin contractility. Actin and myosin II are enriched at cell boundaries that are progressively disassembled during elongation (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006), and adherens junction proteins stabilize cell contacts in the complementary cellular domain (Blankenship et al., 2006; Simões et al., 2010; Levayer et al., 2011; Tamada et al., 2012). These spatially regulated processes of contraction and adhesion both require Rho-kinase activity, but the mechanisms that generate planar polarized Rho-kinase localization are not known. Here, we identify distinct roles for Rho GTPase and Shroom in regulating Rho-kinase localization and actomyosin contractility during Drosophila axis elongation. Rho recruits Rho-kinase to adherens junctions and generates a planar polarized distribution of the Shroom actin-binding protein. Shroom in turn is required to amplify and maintain Rho-kinase and myosin planar polarity and junctional localization. These results demonstrate that Rho and Shroom regulate planar polarized Rho-kinase localization and actomyosin contractility to generate sustained mechanical forces during axis elongation.

Results

Sequences required for Rho-kinase localization along apical–basal and planar axes

During axis elongation, Rho-kinase is concentrated at adherens junctions between anterior and posterior cells, where it promotes junctional disassembly and oriented cell rearrangement (Simões et al., 2010). To identify the mechanisms that generate localized Rho-kinase activity, we performed structure/function analysis. Rho-kinase has a conserved N-terminal serine/threonine kinase domain, a central coiled-coil (CC) domain that binds to Rho GTPase and the Shroom actin-binding protein, and a C-terminal pleckstrin homology (PH) domain (Fig. 1 A). As overexpression of active Rho-kinase in wild type disrupts embryo morphology, we generated deletions in the context of Venus-tagged, catalytically inactive Rho-kinase that recapitulates the localization of the wild-type protein (Fig. 1 and Fig. S1; Simões et al., 2010).

We identified distinct sequences required for Rho-kinase cortical, junctional, and planar polarized localization (Fig. 1 B). Deletion of the N-terminal and catalytic domains had no effect on Rho-kinase localization (Fig. 1 C). In contrast, Rho-kinase lacking the PH domain (Rok ΔPH) was predominantly cytoplasmic (Fig. 1 F), consistent with previous findings that the PH domain is required for cortical localization (Miyazaki et al., 2006). The PH domain alone was sufficient for cortical localization but displayed no asymmetry along apical–basal or planar cell axes (Fig. 1, D and J), indicating that other domains are required for Rho-kinase junctional localization and planar polarity.

We next tested the effect of deleting the Rho-binding (RB) domain that interacts with Rho GTPase, an upstream regulator of Rho-kinase activity. Rho-kinase lacking its RB domain (Rok ΔRB) localized to the cortex but was no longer enriched at adherens junctions, resulting in increased levels at the lateral membrane (Fig. 1, M and N). In addition, Rok ΔRB protein at adherens junctions did not display planar asymmetry (Fig. 1, C and G). Conversely, a fusion protein containing the RB and PH domains of Rho-kinase (RB:PH) was sufficient for junctional localization (Fig. 1 O) but displayed only a partial asymmetry in the plane of the tissue (Fig. 1, D and K). These results indicate that binding to Rho GTPase recruits Rho-kinase to adherens junctions but is not sufficient for full Rho-kinase planar polarity.

To identify additional sequences in Rho-kinase that are required for its planar polarized localization, we turned our attention to the CC domain. A protein lacking most of the CC domain (Rok ΔCC,SB) displayed significantly less planar polarity, despite the presence of the PH and RB domains, and had slightly reduced accumulation at adherens junctions (Fig. 1, C, H, and P). The CC domain contains a small region that interacts with the Shroom actin-binding protein (Nishimura and Takeichi, 2008; Bolinger et al., 2010). Removing the Shroom-binding (SB) domain alone (Rok ΔSB) disrupted Rho-kinase...
Rho GTPase is required for Rho-kinase junctional localization and planar polarity

Rho GTPase binds directly to Rho-kinase and triggers a conformational change that promotes Rho-kinase activity to phosphorylate its substrates (Riento and Ridley, 2003; Jaffe and Hall, 2005). To ask whether Rho is required for Rho-kinase planar polarity, we inhibited Rho activity during axis elongation. Because Rho is required for early development (Strutt et al., 1997; Magie et al., 1999), we injected embryos in late cellularization with mRNAs encoding wild-type Rho, dominant-negative Rho GTPase, and wild-type, active Rho-kinase expressed in a Rho-kinase mutant (Fig. S2, B, C, and I). Moreover, the SB, RB, and PH domains together fully reproduced the planar asymmetry of full-length Rho-kinase (Fig. 1, D and L). Together, these results indicate that Rho-kinase cortical localization is mediated by its PH domain, Rho-kinase enrichment at adherens junctions requires its RB domain, and the RB and SB domains are required for Rho-kinase planar polarity.
Rho\textsuperscript{N19}, or constitutively active Rho\textsuperscript{V14} to express these proteins during axis elongation. Embryos expressing wild-type Rho displayed the proper planar polarized enrichment of Rho-kinase and myosin II at interfaces between anterior and posterior cells (AP interfaces; Fig. 2, A–C). Rho-kinase and myosin II planar polarity were significantly reduced in embryos expressing dominant-negative Rho\textsuperscript{N19} (Fig. 2, D–F and M). In addition, Rho-kinase failed to concentrate at adherens junctions and was more uniformly distributed along the lateral membrane (Fig. 2, I and J). Adherens junction proteins were correctly localized in embryos expressing dominant-negative Rho\textsuperscript{N19} (Fig. 2, G and H), indicating that the failure of Rho-kinase to localize apically is not caused by a loss of adherens junctions. However, E-cadherin and Baz/Par-3 formed aberrant aggregates (Fig. 2 H), reminiscent of embryos injected with the Rho-kinase inhibitor Y-27632 (Simões et al., 2010), indicating that Rho\textsuperscript{N19} alters E-cadherin localization and planar polarity in intercalating cells. [A–F] Localization of HA:Rho, Venus:Rok\textsuperscript{K116A}, and Myo:GFP in stage 7 embryos expressing wild-type Rho (Rho WT; A–C) or dominant-negative Rho (Rho\textsuperscript{N19}; D–F). [G and H] Localization of E-cadherin and Baz/Par-3 in stage 7 embryos expressing wild-type Rho (G) or Rho\textsuperscript{N19} (H). Anterior is left, and ventral is down. [I–L] Localization of Venus:Rok\textsuperscript{K116A} (I and J) or E-cadherin (K and L) in stage 7 embryos expressing wild-type Rho (I and K) or Rho\textsuperscript{N19} (J and L). Cross sections are shown, with apical up. [M] Planar polarized enrichment of Rho-kinase and myosin II at AP cell boundaries (75°–90° with respect to the AP axis) relative to DV cell boundaries (0°–15°). Rho-kinase and myosin II were significantly less planar polarized in embryos expressing Rho\textsuperscript{N19} compared with embryos expressing wild-type Rho (\(P < 0.01\)). [N] Rok RB:PH was significantly more enriched at AP cell boundaries relative to DV cell boundaries than total Rho protein (\(P < 0.001\)). No planar polarized enrichment was detected for total Rho, GFP:Rho, or the PKN Rho probe. [O] Localization of total Rho protein (red) and the PKN Rho probe (green) at different z planes (0 µm is at the level of adherens junctions, and –3 µm is basal to the junctions). Anterior is left, and ventral is down. [P] Total Rho and the PKN Rho probe cortical levels normalized to the apical junctional intensity. PKN Rho probe signal at the basolateral membrane was reduced compared with total Rho (\(P < 0.002\) at –0.75 µm; \(P < 0.0001\) at –1.5, –2.75, and –3.0 µm). A single value was obtained for each image by averaging 100–200 edges/image; 4–16 images in 4–8 embryos were analyzed for each genotype at each z plane. Means ± SEM between images are shown. Bars, 10 µm.
Rho and Shroom direct myosin planar polarity

Simões et al.

We expressed Venus fused to the RB and PH domains of Rho-kinase (RB:PH). The Rho-kinase RB domain preferentially interacts with GTP-bound Rho in Drosophila and mammals (Fujisawa et al., 1996; Mizuno et al., 1999), and the PH domain is required for strong cortical localization (Fig. 1 F). The Rho-kinase RB:PH fusion displayed a small but reproducible asymmetry in the plane of the tissue (Fig. 1, D and K; and Fig. 2 N). These results indicate that Rho activity is increased at the apical cell cortex and that the active population of Rho that binds to Rho-kinase is at most subtly polarized in the plane of the tissue.

The Shroom actin-binding protein is planar polarized in intercalating cells.

Because the SB domain is required for Rho-kinase planar polarity, we hypothesized that Shroom enhances Rho-kinase planar polarity downstream of a subtle asymmetry in Rho signaling. To investigate this possibility, we analyzed the localization of ShrmA, the predominant Shroom isoform in the Drosophila embryo. Shroom localizes to adherens junctions at three-cell vertices before axis elongation (Fig. 3 A, stage 6), consistent with distribution along junctions. Conversely, activated RhoV14 frequently enhanced Rho-kinase and myosin localization and planar polarity ectopically in the basolateral domain (Fig. S3, E and F). Together, these results indicate that regulated Rho GTPase signaling is required for Rho-kinase planar polarity and adherens junction localization.

If Rho plays an instructive role in directing Rho-kinase planar polarity, Rho activity is predicted to be spatially localized. However, total Rho protein visualized with an antibody specific to Rho (Magie et al., 2002) was uniformly distributed along apical–basal and planar cell axes (Fig. 2, N–P). To determine whether an active subpopulation of Rho is spatially localized, we expressed a Venus fusion to the RB domains of protein kinase N (PKN). This fusion specifically interacts with active, GTP-bound Rho in vitro and in culture (Simões et al., 2006). Unlike total Rho protein, which is distributed throughout the lateral membrane, the PKN Rho probe was enriched at adherens junctions, in addition to cytoplasmic signal likely caused by unbound protein (Fig. 2, O and P). However, this fusion did not display obvious asymmetry in the plane of the tissue (Fig. 2 N).
ShrmA and ShrmB isoforms (ShrmAB RNAi) and strongly reduce Shroom protein levels (Fig. S4A) or a third dsRNA that specifically targets the ShrmA isoform (ShrmA RNAi). In addition, we generated two Shroom mutations, Shrm\textsuperscript{11} and Shrm\textsuperscript{13.6}, through $P$ element transposase-mediated recombination (Materials and methods). These mutations remove part of the Shroom open reading frame, including the Rho-kinase–binding domain, and show an absence of Shroom protein by immunostaining (Fig. S4, B–D). We characterized embryos lacking maternal and zygotic Shroom (referred to as Shroom mutants), which were the progeny of Shrm\textsuperscript{11} or Shrm\textsuperscript{13.6} females transheterozygous for the Df(2R)Exel7131 deficiency that removes the Shroom locus crossed to Shrm/Df or Shrm/+ males. Shrm/Df females were viable, but their progeny had increased embryonic lethality (Materials and methods).

In wild-type embryos, Rho-kinase is enriched at adherens junctions and is present at lower levels on the lateral membrane (Fig. 4, A and G). In contrast, in Shroom mutants, Rho-kinase accumulated less strongly at adherens junctions and was present with a previous study (Bolinger et al., 2010). In addition, we found that Shroom has a striking planar polarized distribution during axis elongation. During stages 7 and 8, when cells undergo active cell rearrangement, Shroom was strongly enriched in adherens junctions at AP interfaces, colocalizing with Rho-kinase and myosin II (Fig. 3, A–C). In contrast to the Baz/Par-3 junctional protein, which is enriched in the complementary domain in stage 6 before elongation (Blankenship et al., 2006), Shroom planar polarity was most evident during cell rearrangement in stage 7 (Fig. 3, C and D). This distribution is consistent with a potential role for Shroom in regulating planar polarity during axis elongation.

**Shroom increases Rho-kinase and myosin II planar polarity and junctional localization**

To determine whether Shroom regulates Rho-kinase localization and actomyosin contractility, we used two approaches to disrupt Shroom function. First, we injected embryos with two independent double-stranded RNAs (dsRNAs) that target both embryonic ShrmA and ShrmB isoforms (ShrmAB RNAi) and strongly reduce Shroom protein levels (Fig. S4 A) or a third dsRNA that specifically targets the ShrmA isoform (ShrmA RNAi). In addition, we generated two Shroom mutations, Shrm\textsuperscript{11} and Shrm\textsuperscript{13.6}, through $P$ element transposase-mediated recombination (Materials and methods). These mutations remove part of the Shroom open reading frame, including the Rho-kinase–binding domain, and show an absence of Shroom protein by immunostaining (Fig. S4, B–D). We characterized embryos lacking maternal and zygotic Shroom (referred to as Shroom mutants), which were the progeny of Shrm\textsuperscript{11} or Shrm\textsuperscript{13.6} females transheterozygous for the Df(2R)Exel7131 deficiency that removes the Shroom locus crossed to Shrm/Df or Shrm/+ males. Shrm/Df females were viable, but their progeny had increased embryonic lethality (Materials and methods).

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in ectopic aggregates in the apical–medial cortex and cytoplasm (Fig. 4, B, E, and I). In addition, Rho-kinase had significantly reduced planar polarity in Shroom mutants compared with wild type (Fig. 4 F). Consistent with the decreased Rho-kinase localization at adherens junctions, the apical enrichment of Rho-kinase was slightly reduced in Shroom mutants (Fig. 4, G–J). Similar defects were observed in ShrmAB RNAi embryos (Fig. 4 C). These results demonstrate that Shroom is required to promote Rho-kinase junctional localization and planar polarity during axis elongation.

To investigate the effect of Rho-kinase mislocalization on myosin activity, we analyzed relative contractile forces using laser ablation to sever single cell–cell interfaces. Laser ablation leads to a local cellular retraction that is proportional to the tension acting on the interface, assuming uniform viscoelastic properties (Hutson et al., 2003). This response is normally strongest at AP cell boundaries (Fernandez-Gonzalez et al., 2009). The response to laser ablation was significantly attenuated in ShrmAB RNAi embryos, and AP edges had lower retraction velocities compared with water-injected controls, with no difference in retraction velocities at DV edges (Fig. 4 K). Together, these results demonstrate that Shroom is required to recruit or stabilize Rho-kinase at adherens junctions and to enhance planar polarized force generation during axis elongation.

To analyze the effect of Shroom on myosin II localization and dynamics, we performed time-lapse imaging of embryos expressing GFP fused to the myosin regulatory light chain (Myo:GFP; Videos 1–6). Myosin is present in two populations in intercalating cells: a junctional population at cell–cell contacts and a medial population that spans the apical cell cortex (Rauzi et al., 2010; Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011). During axis elongation, myosin becomes
increasingly enriched at adherens junctions relative to the medial cortex in wild type (Fig. 5, A and E, 14–34 min). In contrast, myosin junctional enrichment did not increase during elongation in Shroom mutants (Fig. 5, B and E). In addition, the planar polarized enrichment of myosin at AP cell boundaries, which normally increases substantially throughout elongation, plateaued early in elongation and failed to reach wild-type levels in Shroom mutants and ShrmA RNAi embryos (Fig. 5, B–D, F, and G). Par-3 planar polarity occurred normally in Shroom mutants and ShrmA and ShrmAB RNAi embryos (unpublished data). These results demonstrate that Shroom is required to amplify myosin II planar polarity and junctional localization during axis elongation.

**Shroom is required for multicellular rosette formation and convergent extension**

The failure to maintain Rho-kinase and myosin II localization at specific planar junctions is predicted to impair the generation of sustained actomyosin contractility during cell rearrangement. To test whether Shroom is required for cell behavior, we performed time-lapse imaging of wild-type and Shroom mutant embryos expressing Spider:GFP (Videos 7 and 8). During axis elongation, the Drosophila germ band elongates along the AP axis and simultaneously narrows along the DV axis, producing a 4.00 ± 0.32-fold increase in the tissue aspect ratio (length to width ratio), a process known as convergent extension. Shroom mutants had reduced convergent extension, resulting in a smaller increase in the tissue length to width ratio compared with wild type (3.16 ± 0.15-fold, P = 0.02; Fig. 6 C). In contrast, tissue length along the AP axis was only slightly reduced in Shroom mutants (1.99 ± 0.04-fold) compared with wild type (2.13 ± 0.05-fold, P = 0.074). These results show that Shroom is required for convergent extension but does not strongly affect the final length of the tissue.
To investigate the cellular basis of the convergent extension defects in Shroom mutants, we analyzed cell rearrangement, the major mechanism driving convergent extension in Drosophila (Irvine and Wieschaus, 1994). Cell rearrangements occur through local neighbor exchange (resulting from single edge contraction events; Bertet et al., 2004) and multicellular rosette formation (resulting from the contraction of multiple consecutive edges; Blankenship et al., 2006). Myosin II mislocalization in Shroom mutants occurred partway through elongation in stage 8 (t = 15–30 min; Fig. 5, E–G), when multicellular rosette formation is the predominant form of cell rearrangement in the tissue (Fernandez-Gonzalez et al., 2009). Using computational cell-tracking methods, we found that Shroom mutants had a strong reduction in rosette behaviors (Fig. 6, A, B, and E), with little change in local neighbor exchange (Fig. 6 D). Similar defects were observed in ShrmAB RNAi embryos (Fig. S5, A–D). These results indicate that cell rearrangement is significantly decreased in Shroom mutants and can account for the reduction in convergent extension in the absence of Shroom activity.

The loss of Shroom is associated with increased apical cell area in the frog, chick, and mouse (Lee et al., 2007; Nishimura and Takeichi, 2008; Plageman et al., 2010). Consistent with these studies, we found that intercalating cells in Shroom mutants had slightly larger apical cell areas during elongation (6%, P = 0.03 at 20 min), with no difference between wild-type and mutant cells before elongation (P = 0.66 at 0 min). This is suggestive of reduced apical myosin contractility in Shroom mutants. In contrast, mesoderm invagination, which requires apical constriction, occurs with the proper developmental timing in Shroom mutants (Fig. S5 E), although we cannot rule out subtle defects in this tissue. The combined effect of decreased rosette formation and increased apical cell area in Shroom mutants is a significantly reduced tissue aspect ratio but nearly normal tissue length along the AP axis. Together, these results demonstrate that Shroom is required for multicellular rosette formation and convergent extension midway through elongation, coincident with the defects in myosin localization.

**Shroom planar polarity requires Rho activity and the actin cytoskeleton**

In one model, Shroom could act downstream of a subtle asymmetry in Rho GTPase activity to enhance Rho-kinase and myosin II planar polarity. Alternatively, Rho and Shroom could act independently to regulate planar polarity in Drosophila. Different relationships between Rho and Shroom have been reported in vertebrates, in which Rho and Shroom appear to act independently to regulate myosin localization and apical constriction in Xenopus (Haigo et al., 2003; Hildebrand, 2005; Nishimura and Takeichi, 2008; Nishimura et al., 2012), but Rho is required for Shroom3-mediated apical constriction in the lens placode of the chick and mouse (Plageman et al., 2011). To test whether Shroom acts downstream or independently of Rho signaling in Drosophila, we analyzed Shroom localization in embryos expressing dominant-negative RhoN19. Shroom planar polarity was abolished in RhoN19-expressing embryos (Fig. 7, A, B, and G), whereas depleting both isoforms of Shroom in ShrmAB RNAi embryos had no effect on Rho localization or activity (Fig. S5, F–I). Thus, Shroom localization requires Rho GTPase activity but not vice versa, consistent with a model in which Shroom acts downstream of Rho.

To investigate how Rho influences Shroom localization, we identified the sequences in the Shroom protein that are required for Shroom planar polarity. Shroom does not contain an RB domain, suggesting that the regulation of Shroom localization by Rho signaling occurs indirectly. The ShrmA isoform contains a central domain that associates with F-actin and a C-terminal domain that binds to Rho-kinase (Fig. 7 N; Bolinger et al., 2010). Ectopically expressed Venus-tagged ShrmA recapitulates the localization of the endogenous protein (Fig. 7 J). Shroom lacking the Rho-kinase–binding domain displayed substantial planar polarity, although slightly less than the full-length protein (Fig. 7, K and O). A similar distribution was observed for the F-actin–binding domain alone (Fig. 7, L and O). In contrast, deletion of the F-actin–binding domain completely eliminated Shroom planar polarity and strongly reduced its cortical localization (Fig. 7, M and O). These results suggest that Shroom localization requires a direct interaction with the actin cytoskeleton.

The finding that the Shroom actin-binding domain is required to generate Shroom planar polarity suggests that Rho could influence Shroom through its effects on actin. In wild-type embryos, F-actin is enriched at AP interfaces in a planar polarized fashion (Fig. 7, D and H; Blankenship et al., 2006). F-actin planar polarity was abolished in embryos expressing dominant-negative RhoN19 (Fig. 7, E and H), consistent with a model in which Rho generates Shroom planar polarity by modifying the actin cytoskeleton. Although the Rho-kinase–binding domain is largely dispensable for Shroom localization, Shroom planar polarity was reduced in Rho-kinase mutants (Fig. 7, C and G), suggesting that Shroom and Rho-kinase mutually reinforce each other’s localization. Rho-kinase regulates actin organization (Amano et al., 2001; Verdier et al., 2006), and consistent with this role, we found that Rho-kinase mutants display reduced F-actin planar polarity (Fig. 7, F and H), and Rho-kinase activity is necessary for its own planar polarized localization (Fig. S2, D and J). These results are consistent with a model in which Rho GTPase modulates the actin cytoskeleton to generate a planar polarized localization of Shroom, and Shroom in turn stabilizes Rho-kinase at planar polarized adherens junctions to enhance planar polarized actomyosin contractility during convergent extension (Fig. 7 I).

**Discussion**

Rho-kinase is an essential regulator of actomyosin contractility, but the mechanisms that generate Rho-kinase asymmetry to produce spatially regulated forces during development are not well understood. Here, we show that Rho GTPase signaling is required for the planar polarized localization of Rho-kinase and myosin II during Drosophila axis elongation. Direct interaction between Rho and Rho-kinase recruits Rho-kinase to adherens junctions but is not sufficient for full Rho-kinase planar polarity,
Rosette rearrangements are reduced in Shroom mutants, resulting in decreased convergent extension. These results support a role for Shroom in regulating planar polarized actomyosin contractility and junctional remodeling during convergent extension, expanding the morphogenetic functions of this highly conserved protein beyond its known role in apical constriction.

Our data support a model in which Rho GTPase and Shroom have distinct functions in regulating Rho-kinase localization and planar polarized myosin contractility during convergent extension. Rho GTPase recruits Rho-kinase to adherens junctions and initiates planar polarity, and Shroom plays a modulatory role in enhancing and maintaining planar polarized myosin contractility.
downstream of Rho signaling. Rho GTPase binds to Rho-kinase and could regulate its localization directly (Riento and Ridley, 2003; Jaffe and Hall, 2005). Rho does not bind to Shroom but may regulate Shroom planar polarity indirectly through its effect on the actin cytoskeleton. We show that Rho-kinase, usually viewed as a downstream effector of Shroom, feeds back to maintain Shroom planar polarity and its own planar polarized localization. Rho-kinase could directly phosphorylate Shroom to reinforce planar cell polarity. Alternatively, Rho-kinase could promote Shroom localization through remodeling of the actin cytoskeleton, as the Shroom actin-binding domain is necessary and sufficient for targeting to planar junctions, and Rho-kinase can phosphorylate known regulators of actin (Amano et al., 2001; Verdier et al., 2006).

These findings may be relevant to neural tube development in vertebrates, which involves a combination of apical constriction, polarized junctional remodeling, and cell shape changes (Nishimura and Takeichi, 2008; Nishimura et al., 2012). Shroom3 is required for neural tube closure in the mouse, frog, and chick (Hildebrand and Soriano, 1999; Haigo et al., 2003; Nishimura and Takeichi 2008), and disrupting the interaction between Shroom and Rho-kinase reduces the number of rosettes in the chick neural plate (Nishimura and Takeichi, 2008). Unlike mutants that have disrupted rosette-based movements caused by defects in cell adhesion (Tamada et al., 2012), the defects in Shroom mutants are likely a result of reduced myosin II activity. Rosette behaviors in Drosophila predominate midway through elongation at stage 8 (Fernandez-Gonzalez et al., 2009), coinciding with the stage when myosin becomes mislocalized in Shroom mutants. A failure to reinforce actomyosin contractility during elongation in Shroom mutants could selectively disrupt later-onset, higher-order cell rearrangements, with no effect on local neighbor exchange events that are more frequent at earlier stages. Alternatively, rosette formation may require more force, as rosettes form through the contraction of multicellular actomyosin cables that are under a higher level of tension and accumulate more myosin (Fernandez-Gonzalez et al., 2009). In Shroom mutants, defects in myosin junctional localization may prevent contractile forces from reaching the levels necessary to produce rosette-based convergent extension movements. It will be interesting to explore whether planar polarized Shroom activity plays a general role in promoting junctional remodeling and enhancing mechanical force generation in processes that require strong actomyosin contractility during development.

Rho GTPase signaling is an excellent candidate to break planar symmetry, as a small fraction of active Rho protein can trigger rapid and dramatic changes in the actin cytoskeleton (Jaffe and Hall, 2005). In one model, a subtle increase in Rho activity at AP cell boundaries could provide an instructive cue, guiding planar cell polarity by recruiting Rho-kinase, modifying the actin cytoskeleton, and facilitating the cortical association of the Rho-kinase regulator Shroom. Alternatively, Rho could regulate Rho-kinase planar polarity indirectly through its role in promoting Rho-kinase apical localization. Although it is challenging to visualize a small and highly dynamic population of active Rho protein in vivo, several findings support the idea that localized Rho activity could play an instructive role in planar polarity. First, myosin planar polarity and directional cell rearrangements occur normally at early stages in Shroom mutants, suggesting that other signals are able to generate localized myosin activity. The partial planar asymmetry of a fragment containing the RB domain of Rho-kinase, which is predicted to interact with the active pool of Rho GTPase, suggests that Rho could contribute to this asymmetry. Second, Rho is required for the planar polarized localization of Shroom, raising the possibility that Rho signaling could provide an essential source of Shroom asymmetry. Third, the upstream Rho activator Rho-GEF2 in Drosophila and PDZ-RhoGEF in the chick display a sub- planar asymmetry during epithelial bending and elongation (Levayer et al., 2011; Nishimura et al., 2012; Warrington et al., 2013). Multiple activators and inhibitors of Rho could act together to generate a spatially localized pattern of Rho activity, as is the case for apical constriction (Simões et al., 2006). Notably, although Rho GTPase activity is necessary to establish Rho-kinase and myosin planar polarity, it is not sufficient to maintain their activity at high enough levels to allow sustained force generation and rosette rearrangements in Shroom mutants. We propose that Rho promotes the recruitment of Shroom as part of a positive feed-forward mechanism that reinforces planar polarized actomyosin contractility during convergent extension.

Planar polarized cell rearrangements require the active maintenance of cell polarity in large populations of dynamically moving cells. We show that Shroom and Rho GTPase signaling play distinct roles in the establishment and maintenance of polarized actomyosin contractility during convergent extension. The upstream spatial cues that localize actomyosin contractility to specific planar cellular domains are not known. An asymmetry in the organization of the actin cytoskeleton is the earliest evidence of planar polarity in the Drosophila embryo (Blankenship et al., 2006). Distinct actin-binding domains in different Shroom isoforms have been proposed to target Shroom protein and its effectors to different regions of the cell (Hildebrand and Soriano, 1999; Dietz et al., 2006). Moreover, the actin-binding domain is critical for Shroom planar polarity. These findings support the idea that an asymmetry in the actin cytoskeleton is an essential spatial input that regulates the localization of Shroom, the contractile machinery, and ultimately the forces that control cell rearrangement and tissue structure. The upstream spatial cues that generate these asymmetries could involve an asymmetry in Rho signaling, perhaps through the local activation of upstream signaling proteins that regulate Rho GTPase activity. Alternatively, the critical event in the establishment of planar cell polarity could be a Rho-independent reorganization of the actin cytoskeleton that biases the activity of Shroom, Rho-kinase, and myosin, which in turn modify the cytoskeleton to allow robust and sustained cell polarization. Elucidation of the upstream spatial cues that regulate actomyosin localization and dynamics will provide insight into the mechanisms that direct polarized cell behavior.

### Materials and methods

**Fly stocks and genetics**

Embryos were generated at 25°C. Wild type was yw unless otherwise indicated. Alleles were Rok1, Rok2 (Winter et al., 2001), Shrm111, and Shrm13.6 (this work), and Df(2R)Exel7131 (Bloomington Drosophila Stock Center). The expression of Rho-kinase, Shroom, Rho1, PKN, and Dicer-2...
transgenes were driven by the upstream activating sequence (UAS) enhancer under the control of Ga4. Embryos expressing UAS-Rho–kinase transgenes, UASp-Shroom transgenes, UASp-GFP:Rho1, UASp-PKNG58A:Venus, or UAST-Dicer-2 were the F2 progeny of UAS males × matutb615 GfD females (gift of D. St Johnstone, University of Cambridge, Cambridge, England, UK), except UASp-Venus:ShrmA and UASp-Venus:RBD:PH, which were the F2 progeny of UAS males × matutb613 GfD females. Full K116A and ShrmA116 were generated by in vitro transposase-mediated male recombination (Chen et al., 1998). The P elements pSuport-pKG04646 (50F1) and p(EPg2)pCG8613 [EP06332] (50F6) were recombined with cn and sp, respectively. P-element–induced recombination occurred in males of the following genotype: w+/y; cn sp; P[P[RP1]1057] K116A > cn sp females (Bloomington Drosophila Stock Center), and the progeny were screened for deletions in Shroom by the presence of cn and sp and the loss of white, as both P Elements were excised in the deletions. Deletion breakpoints were molecularly mapped by PCR (Fig. S4 B).

Shroom mutants were the progeny of Shrm11/Df[2R] Exel7131 or Shrm11/Df[2R] Exel7131 females and males (Fig. 4, B, E, and F; Fig. 5, B and E–G; Fig. 6, B–F; and Fig. S5 E). Similar results were observed in the progeny of Shrm11/Df or Shrm11/Df females crossed to Shrm11/+ or Shrm11/+ males (Fig. 4, A and J; 6/8 Shrm11 and 8/11 Shrm11.6 maternal/zygotic mutants displayed aberrant Rho-kinase116/164 localization compared with 0/9 wild-type embryos, and 3/3 of Shrm11 maternal mutants displayed aberrant Myo:GFP localization compared with 0/3 of wild-type embryos. In crosses to Shrm/+ males, progeny that lack maternal and zygotic Shroom were identified by the absence of balancer markers. In crosses to Shrm/DF males, all progeny were maternally and zygotically mutant for Shroom and 25% are predicted to be homozygous for the small Df[2R]Exel7131 deficiency. The defects in the progeny of Shrm/DF females and males were fully penetrant, indicating that these defects are not a result of the zygotic loss of other genes uncovered by the deficiency. Moreover, crosses to Shrm/+ males and injection of three independent dsRNAs to Shroom gave similar results (Fig. 4 C and Fig. S5, A–D), suggesting that these defects are caused by the loss of Shroom.

Embryonic hatch rates were 10% of embryos from Shrm11/Df; GFP:Rok116A females and 23% of embryos from Shrm11/Df; GFP:Rok116A females crsomed to Shrm/+ males compared with 80% of embryos that lack control GFP:Rok116A females. Weaker but detectable lethality was also observed in Shroom mutants that do not express a GFP marker, and these could not be maintained as homozygous stocks. Rok and Rok2 germline clones were generated using the FLP/leaky-embryonic lethal dominant female sterility system (Chou and Perrimon, 1992) and ovoFRT19A (gift of N. Tolwinski, Yale-NUS College, Singapore) by heat shocking larvae of the following genotypes and crossing FLP recombinase–dominant female sterile system (Chou and Perrimon, 1992) into the FRT19A destination vector (gift of F. Wirtz-Peitz, Harvard University, Cambridge, MA) and were inserted in attp40 (gift of D. Strutt, University of Sheffield, Sheffield, England, UK) and Myo:GFP (sqh:GFP; Royou et al., 2004) embryos. Embryos were dechorionated 2 min in 50% bleach, washed in 0.5% Triton X-100 in 0.4% NaCl, cooled on ice, and devitellinized in heptane/methanol. Antibodies were rabbit GFP (1:100; Torrey Pines Biolabs, Inc.), rat HA (1:500; Roche), guinea pig Baz/Par-3 (1:500; Blankenship et al., 2006), mouse Rho1 (1:50; Developmental Studies Hybridoma Bank), rat Shrm2100 (1:100; Balanger et al., 2012; and mouse Arm/SP (1:50; Developmental Studies Hybridoma Bank). Rhodamine-conjugated phalloidin and secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Molecular Probes) were used at 1:500. PKNG48A:Venus was visualized after fixation without antibody. Embryos were mounted in Prolong gold (Molecular Probes) and imaged on a confocal microscope (LSM 700; Carl Zeiss) with a Plan Neofluor 40×/1.3 NA objective; 1.0-µm z slices were acquired at 0.5-µm steps. Maximum intensity projections of 2–3 µm in the apical junctional domain were analyzed.

Immunohistochemistry

To synthesize mRNA encoding HA:Rho506, HA:Rho514*, and HA:Rho114* coding sequences were PCR amplified from pGEX-Rho506 (gift of J. Settlement, Genentech, San Francisco, CA), pUASTRho514*, and pUASTRho114* (gifts of D. Strutt, University of Sheffield, Sheffield, England, UK). To synthesize mRNA encoding HA:Rok PH, a PCR fragment corresponding to Rok aa 1,084–1,391 was amplified from wild-type genomic DNA. Products were cloned into pcS2+ using BamHI and XhoI restriction sites. pcS2+HA:Rho506, pcS2+HA:Rho514*, pcS2+HA:Rho114*, and pcS2+HA: Rok PH were digested with NotI and used as templates for in vitro transcription to generate mRNAs encoding HA:Rho506, HA:Rho514*, HA:Rho114*, and HA:Rok PH, respectively, with the mMESSAGE mMACHINE SP6 kit (Ambion). Embryos were collected for 20 min, aged 2 h and 20 min at 25°C, injected ventrally during mid-to-late cellularization with 0.1 nl of 2-mg/ml mRNA, incubated 45 min at 25°C in a humidified chamber, and processed for immunostaining.

dsRNA injection

Templates to produce dsRNA against ShrmA or both ShrmA and ShrmB isoforms were generated by PCR from the LP13775 EST. PCR products were used as templates for the T7 transcription reactions with the 5x MEGAscript T7 kit (Ambion). dsRNA was injected ventrally (for immunostaining) or dorsally (for live imaging) with 0.1 nl of 2-mg/ml dsRNA into 0–1-h embryos that were the F2 progeny of UAS-Dicer-2 (X); UAS-Venus: Rok116A × matutb617;15 (Fig. 4, A and C), UAS–Dicer-2; sqh:GFP × matutb617;15 (Fig. 4, D and Fig. S5 C), UAS–Dicer-2; Spider:GFP × matutb67;15 (Fig. S5 E), and UAS–Dicer-2; UAS–Rho506 × PKNG58A: Venus × matutb67;15 (Fig. 2, O and P; and Fig. S5, F–I). All other injections were without Dicer-2. With Dicer-2, similar effects were observed at a greater distance from the site of injection. Embryos were incubated 2 h and 30 min at 25°C in a humidified chamber and processed for immunostaining or live imaging.

Planar polarity analysis

Using the SIESTA (Scientific Image Segmentation and Analysis) algorithm (Fernandez-Gonzalez and Zallen, 2011), user-drawn, 3-pixel-wide lines were obtained for each embryo, and error bars indicate the SEMs between all lines. Time-lapse imaging

Time-lapse imaging was performed with Spider:GFP (gift of A. Debec, INSERM Jacques Monod, Paris, France) and Myo:GFP (sqh:GFP, Royou et al., 2004). Embryos were dechorionated 2 min in 50% bleach, washed in 0.5% Triton X-100 in 0.4% NaCl, cooled on ice, and devitellinized in heptane/methanol. Antibodies were rabbit GFP (1:100; Torrey Pines Biolabs, Inc.), rat HA (1:500; Roche), guinea pig Baz/Par-3 (1:500; Blankenship et al., 2006), mouse Rho1 (1:50; Developmental Studies Hybridoma Bank), rat Shrm2100 (1:100; Balanger et al., 2012; and mouse Arm/SP (1:50; Developmental Studies Hybridoma Bank). Rhodamine-conjugated phalloidin and secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Molecular Probes) were used at 1:500. PKNG48A:Venus was visualized after fixation without antibody. Embryos were mounted in Prolong gold (Molecular Probes) and imaged on a confocal microscope (LSM 700; Carl Zeiss) with a Plan Neofluor 40×/1.3 NA objective; 1.0-µm z slices were acquired at 0.5-µm steps. Maximum intensity projections of 2–3 µm in the apical junctional domain were analyzed.
water, mounted on an oxygen-permeable membrane (YSI Incorporated) with halocarbon oil 27 (Sigma-Aldrich), and imaged on a spinning-disk confocal microscope (UltraView RS5, PerkinElmer) with Plan Neofluor 40x, 1.3 NA or 63x, 1.4 NA objectives (Carl Zeiss). Z stacks were acquired at 1-µm steps (40x objective) or 0.5-µm steps (63x objective) at 30-, 15-, or 5-µs intervals as indicated. Maximum intensity projections of 2–3 µm in the apical junctional domain were analyzed. Videos 7 and 8 were background noise corrected by multiplying pixel intensities in three consecutive z planes and normalizing to occupy the full intensity range. Cell behavior was analyzed as previously described (Simões et al., 2010; Tamada et al., 2012). Tissue aspect ratio was the ratio of the long axis to the short axis of an ellipse fit to a group of cells that were tracked ≥30 min after t = 0. Cumulative cell rearrangement events for cells tracked ≥30 min after t = 0 were counted as the number of neighbor exchanges in the entire area and topology (number of neighbors) were calculated for all cells at each time point. A single value was obtained for each embryo at each time point, and error bars indicate the SEMs between embryos. The p-values were calculated using the F test followed by the appropriate t test using the t = 30 min value as the test statistic.

**Laser ablation**

Embryos injected with dsRNAs targeting ShrmA, both ShrmA and ShrmB isoforms, or flip were mounted in halocarbon oil 27 and imaged on a spinning-disk confocal [UltraView RS5] with a Plan Neofluor 63x, 1.4 NA oil immersion lens that was also used to focus the MicroPoint laser. An N2 laser (MicroPoint; Photronics Instruments) tuned to 365 nm was used to ablate cell boundaries labeled with Myo:GFP. Z stacks of three optical slices at 0.5-µm steps were acquired at 2-s intervals. The two vertices attached to the cut edge were manually identified and analyzed in SIESTA (Fernandez-Gonzalez and Zallen, 2011).

**Molecular biology**

Full-length pENTR-Venus:Rok and pENTR-Venus:RokK116A plasmids were generated as previously described (Simões et al., 2010). For pENTR-Venus:Rokk3k116a (aa 1–1,576), a PCR fragment was amplified from the plasmids pENTR-Venus:Rok (aa 1–1,576) and cloned into pENTR-D-topo (Invitrogen). N-Terminal Venus was inserted into the Nost site (PCR amplified with the primers NostVenusF, 5’-ATGGACGATGTCATTTCAGCGATGAATTGGCTGGC-3’ and NostVenusR, 5’-ATGGCGGCCCGTACGGGTACGTCGGCTGCTTCGTCGTC-3’). For pENTR-Venus:RokA290f (aa 1,084–1,391), a stop codon was introduced at position 1,324 in pENTR-Venus:Rok (aa 1,084–1,391), and cloned into pENTR. N-Terminal Venus was inserted in the Nost site. For pENTR-Venus:ShrmA (aa 1–1,576), a PCR fragment was amplified from the plasmid pENTR-Venus:ShrmA (aa 1–1,576) and cloned into pENTR. N-Terminal Venus was inserted in the Nost site. For pENTR-Venus:ShrmA Factin (aa 445–920), a PCR product was amplified from the plasmid pENTR-Venus:ShrmA (aa 445–920) and cloned into pENTR. N-Terminal Venus was inserted in the Nost site.

**Shroom mutant breakpoint analysis**

Shrm111 and Shrm112 both delete exons within the Shroom open reading frame that encode the Rho-kinase domain, assessed by the failure to amplify PCR products with the following primer pairs: (a) ShrmF2A (aa 37–93): 5’-GGTGCCGCTCCGCAAGGAGG-3’ and ShrmR2A (aa 967–1,046): 5’-CCAC CACGAGGCCATGGAGGCGGCCCTAGCCCGTGCTGC-3’. For pENTR-Venus:ShrmA (aa 967–1,046), an internal deletion was created by PCR amplification from the LP13775 EST with the primers ShrmAf1, 5’-CGCCGTGGACCGGTGCTTGTACAG-3’ and ShrmAr1, 5’-TATTGGAATGACGACGATTCGCAAGAAATTGG-3’ and cloned into pENTR. The product was self-ligated. For pENTR-Venus:ShrmA Factin (aa 445–920), a PCR product was amplified from the plasmid pENTR-Venus:ShrmA (aa 445–920) and cloned into pENTR. N-Terminal Venus was inserted in the Ascl site (PCR amplified with the primers Ascl1, 5’-TGGGCCGCCGAGGAGG-3’ and Ascl2, 5’-TGCAATGGTGGACGCCAGCG-3’ and Ascl3, 5’-TGGGCCGCCGCTCCACGTTGCGCTTCGAC-3’).

**Primers for dsRNA injection, in vitro transcription, and mRNA injection**

Template DNA for the production of dsRNAs was produced by PCR from the plasmid pENTR-Venus:Rok using the following primer pairs that contain a 5’ phosphorylated primer: 5’-TCACATGGACTATCAGCTGCTGTCTAGCGAC-3’ and 3’-ATCTCGAGTTAGAGCAAAAGGCATCTGGTCTTC-3’. The template to produce control dsRNA against Rho-kinase domain, assessed by the failure to amplify PCR products with the following primer pairs: (a) CBG613f, 5’-GAGAAGAGTCTCTAGTACGAGG-3’ and CBG613r, 5’-CACACATACAGCAGAACGATAGG-3’; (b) seq12f, 5’-TGAGAATCCTAGCGCTGCTTGTACAG-3’ and seq12r, 5’-CCTTGACTAAGGCTGCTTGTACAG-3’; and (c) seq13f, 5’-CTATGTAATGCTCTCTATTGGCC-3’ and seq13r, 5’-CGACC GATGCGGAATGAGAGG-3’.

**Shroom mutants**

The product was self-ligated. For pENTR-Venus:ShrmA Factin (aa 445–920), a PCR product was amplified from the plasmid pENTR-Venus:ShrmA Factin (aa 445–920) and cloned into pENTR. N-Terminal Venus was inserted in the Ascl site (PCR amplified with the primers Ascl1, 5’-TGGGCCGCCGAGGAGG-3’ and Ascl2, 5’-TGCAATGGTGGACGCCAGCG-3’ and Ascl3, 5’-TGGGCCGCCGCTCCACGTTGCGCTTCGAC-3’).

**Shrm mutant breakpoint analysis**

Shrm111 and Shrm112 both delete exons within the Shroom open reading frame that encode the Rho-kinase domain, assessed by the failure to amplify PCR products with the following primer pairs: (a) ShrmF2A (aa 37–93): 5’-GGTGCCGCTCCGCAAGGAGG-3’ and ShrmR2A (aa 967–1,046): 5’-CCAC CACGAGGCCATGGAGGCGGCCCTAGCCCGTGCTGC-3’. For pENTR-Venus:ShrmA (aa 967–1,046), an internal deletion was created by PCR amplification from the LP13775 EST with the primers ShrmAf1, 5’-CGCCGTGGACCGGTGCTTGTACAG-3’ and ShrmAr1, 5’-TATTGGAATGACGACGATTCGCAAGAAATTGG-3’. The product was self-ligated. For pENTR-Venus:ShrmA Factin (aa 445–920), a PCR product was amplified from the plasmid pENTR-Venus:ShrmA Factin (aa 445–920) and cloned into pENTR. N-Terminal Venus was inserted in the Ascl site (PCR amplified with the primers Ascl1, 5’-TGGGCCGCCGAGGAGG-3’ and Ascl2, 5’-TGCAATGGTGGACGCCAGCG-3’ and Ascl3, 5’-TGGGCCGCCGCTCCACGTTGCGCTTCGAC-3’).
an analysis of activated Rho1 expression. Fig. S4 provides validation of Shroom mutants and RNAi treatment. Fig. S5 shows additional characterization of Shroom mutant and ShrmA RNAi embryos. Videos 1 and 2 show Myosin II junctional and medial populations in a wild-type embryo (Video 1) and Shroom mutant embryo (Video 2). Videos 3–6 show Myosin II dynamics in a wild-type embryo (Video 3), Shroom mutant embryo (Video 4), a control injected embryo (Video 5), and ShrmA RNAi embryo (Video 6). Videos 7 and 8 show cell behavior in wild-type (Video 7) and Shroom mutant (Video 8) embryos expressing GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201307070/DC1.

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