RESEARCH ARTICLE

The interaction between Shroom3 and Rho-kinase is required for neural tube morphogenesis in mice

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ABSTRACT

Shroom3 is an actin-associated regulator of cell morphology that is required for neural tube closure, formation of the lens placode, and gut morphogenesis in mice and has been linked to chronic kidney disease and directional heart looping in humans. Numerous studies have shown that Shroom3 likely regulates these developmental processes by directly binding to Rho-kinase and facilitating the assembly of apically positioned contractile actomyosin networks. We have characterized the molecular basis for the neural tube defects caused by an ENU-induced mutation that results in an arginine-to-cysteine amino acid substitution at position 1838 of mouse Shroom3. We show that this substitution has no effect on Shroom3 expression or localization but ablates Rock binding and renders Shroom3 non-functional for the ability to regulate cell morphology. Our results indicate that Rock is the major downstream effector of Shroom3 in the process of neural tube morphogenesis. Based on sequence conservation and biochemical analysis, we predict that the Shroom-Rock interaction is highly conserved across animal evolution and represents a signaling module that is utilized in a variety of biological processes.

KEY WORDS: Shroom3, Rock, apical constriction, epithelial, neural tube

INTRODUCTION

The dynamic nature of the actin cytoskeleton is critical for regulating cellular processes and characteristics such as division, polarity, adhesion, migration, secretion and morphology (Babbin et al., 2009; Provenzano et al., 2009; Provenzano and Keely, 2009; Vicente-Manzano et al., 2009a; Vicente-Manzano et al., 2009b; Vicente-Manzano et al., 2009c; Zhou et al., 2009; Sawyer et al., 2010; Sawyer et al., 2011; Allard and Mogilner, 2013; Etienne-Manneville, 2013; Flynn, 2013; Lagrué et al., 2013; Luo et al., 2013; Ojelade et al., 2013; Shirao and González-Billault, 2013). Through the use of genetic models systems and the mapping of mutations that cause human diseases, it has been well-established that errors in these processes underlie a wide range of maladies, including birth defects, cancer, kidney disease, and neuronal degeneration. One embryonic tissue that seems particularly sensitive to errors in cytoskeletal dynamics or architecture is the neural tube, the precursor to the brain and spinal chord (Campbell et al., 1986; Copp et al., 1990; Greene and Copp, 2009). While less common in the developed world, neural tube closure defects (NTDs), including spina bifida and exencephaly are among the most common birth defects and complicate approximately 1 in 1,000 births worldwide (Campbell et al., 1986; Copp et al., 1990). Despite an extensive amount of research, a clear understanding of the etiology of human NTDs has remained elusive (Greene and Copp, 2009; Greene et al., 2009b; Greene et al., 2009a).

To date, over 200 genes have been implicated in neural tube morphogenesis in mice. One of these genes is Shroom3, a member of a unique family of F-actin associated proteins that regulate cellular morphology during a wide range of developmental processes (Hildebrand and Soriano, 1999; Dietz et al., 2006; Hagens et al., 2006a; Yoder and Hildebrand, 2007; Bolinger et al., 2010). In vertebrates, the Shroom family is comprised of four members, Shroom1–4, each of which have been implicated in the regulation of various morphogenic events during embryonic development, including neural tube closure (Hildebrand and Soriano, 1999), remodeling of the vasculature (Farber et al., 2011), eye development (Fairbank et al., 2006; Plageman et al., 2010), gut morphogenesis (Grosse et al., 2011; Plageman et al., 2011a), neuronal architecture and function (Hagens et al., 2006b; Taylor et al., 2008), ENaC channel regulation (Assaf et al., 2011), renal function (Köttgen et al., 2009), arterial hypertension (Sevilla-Pérez et al., 2008), and heterotaxy in humans (Tariq et al., 2011).

It is predicted that Shroom proteins function as adaptors that ultimately regulate the activity of contractile actomyosin networks. All Shroom proteins tested to date bind to both F-actin and Rho-associated kinase (Rock) via signature sequence motifs known as Shroom domain 1 (SD1) and 2 (SD2), respectively. In the case of Shroom3, this interaction with F-actin is required for its localization to the zonula adherens in polarized epithelial cells (Dietz et al., 2006). Shroom3 binds to Rock via its SD2 motif and recruits it to the zonula adherens (Nishimura and Takeichi, 2008). This results in localized activation of non-muscle myosin II (myosin II) via phosphorylation of myosin regulatory light chain (RLC) (Haigo et al., 2003; Hildebrand, 2005; Hagens et al., 2006b). As a result, the subcellular distribution of the actomyosin network within these cells is reorganized to form an apically positioned contractile ring. This ring exerts force to elicit apical constriction and facilitate the transition of columnar shaped cells into a wedge-shaped form (Hildebrand, 2005; Dietz et al., 2006). When this cell shape change occurs in a group of cells it can cause invagination or bending, leading to alterations in tissue morphology.

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A new allele of Shroom3, Shroom3<sup>m1Nisw</sup>, was recently identified in a forward genetic screen for ENU-induced mutations that cause neural tube defects (Zohn et al., 2005; Marean et al., 2011). Embryos homozygous for the Shroom3<sup>m1Nisw</sup> allele exhibit exencephaly and phenocopy the gene trap allele of Shroom3, Shroom3<sup>gt(ROSA)53sor</sup>, suggesting that it is a functional null allele. In this study, we investigate the molecular basis for the loss-of-function phenotype associated with this allele. We show that this mutation abrogates the ability of Shroom3 to bind to Rock. This renders Shroom3 incapable of eliciting apical constriction via the activity of myosin II. These data indicate that the Shroom3-Rock interaction is vital for neural tube morphogenesis and that the majority of Shroom3 activity in apical constriction is mediated by Rock.

**RESULTS**

**The Shroom3<sup>m1Nisw</sup> allele harbors a substitution mutation in the Shroom-domain 2 of Shroom3**

Shroom family proteins constitute a class of scaffolding protein that link the actin cytoskeleton to Rock localization via direct protein–protein interactions (Fig. 1A). One family member, Shroom3, has been shown to bind to Rock, recruit it to the zonula adherens, and facilitate the assembly of a circumapical contractile actomyosin network (Haigo et al., 2003; Hildebrand, 2005; Hagenes et al., 2006b). Mice homozygous for a null allele of Shroom3, Shroom3<sup>gt(ROSA)53sor</sup>, exhibit severe neural tube defects (Hildebrand and Soriano, 1999). Recent studies identified an ENU-induced allele of Shroom3 called Shroom3<sup>m1Nisw</sup> and embryos homozygous for this allele phenocopy homogous Shroom3<sup>gt(ROSA)53sor</sup> embryos suggesting that this is a functional null allele (Fig. 1B) (Marean et al., 2011). Analysis of the Shroom3<sup>m1Nisw</sup> allele indicates a C-to-T missense mutation at nucleotide position 5744 in the Shroom3 cDNA (accession number NM_015756), resulting in an arginine to cysteine amino acid substitution at position 1838 of Shroom3 (accession number NP_056571) (Fig. 1D). This would suggest that the mutant allele should still express full-length protein that is localized to the apical adhesion sites of cells. Consistent with this hypothesis, the staining of neural epithelium from wildtype or homozygous Shroom3<sup>m1Nisw</sup> embryos indicates that Shroom3 protein is expressed at approximately equal levels and exhibits similar subcellular distribution (Fig. 1C).

To quantify the level of Shroom3 expression in these different genetic backgrounds, we measured the fluorescent intensity of Shroom3 relative to that of

![Image of Shroom3 and Rock interactions](https://example.com/image.png)

**Legend:**

- **A**: Schematic of the Shroom3-Rock signaling module. Arrows denote known direct interactions. SD, Shroom domain; PDZ, Psd-95/DlgA/ZO1 domain; SBD, Shroom Binding domain; RBD, Rho binding domain; PH, pleckstrin homology domain. (B) Embryos homozygous for the Shroom3 null allele Shroom3<sup>gt(ROSA)53sor</sup> or the ENU allele Shroom3<sup>m1Nisw</sup> exhibit the same phenotype. (C) Expression of the Shroom3 R1838C protein. Wildtype or Shroom3<sup>m1Nisw</sup> homozygous e9.5 embryos were isolated, bisected sagittally to expose the neural epithelium, stained in wholemount to detect Shroom3 (green) and β-catenin (red), and visualized by confocal microscopy. Z-projections are shown beneath; scale bar, 10 μm. Graph represents quantification of Shroom3 expression. Fluorescent intensity (F.I.) of Shroom3, expressed as the ratio of the average Shroom3 fluorescent intensity relative to the fluorescent intensity of β-catenin, from wildtype or Shroom3<sup>m1Nisw</sup> homozygous mutants. Error bars represent ± s.d., values are not significantly different using an unpaired t-test, n=60 cells in two embryos per genotype; scale bar equals 10 μm. (D) The Shroom3<sup>m1Nisw</sup> mutation results in the substitution of a cysteine for a highly conserved arginine. Top panel shows the mutation while the bottom panel shows the sequence conservation of the SD2 in the vicinity of arginine 1838. Underlined amino acids constitute part of a conserved patch required for binding to Rock (Mohan et al., 2012). (E) Surface view of the Drosophila Shroom SD2 dimer as previously determined (Mohan et al., 2012) with the conserved arginine (R1474) residue in each monomer highlighted in green.
β-catenin. This analysis shows no significant difference between wildtype and mutant protein in either localization or expression. R1838 is located within the SD2 of Shroom3 and maps to a highly conserved patch of amino acids (Fig. 1D). We have previously shown that an SD2 variant harboring 5 substitutions within this patch (1834_{LSGRAL}^{1840} to 1834_{ALEADLE}^{1840}) abrogates the Shroom3-Rock interaction (Mohan et al., 2012). Importantly, an arginine is conserved at this position in all SD2 motifs identified to date, including the Drosophila Shroom protein, in which R1474 is analogous to R1838. We have recently solved the structure of the Drosophila Shroom SD2 motif (Mohan et al., 2012). In this structure, the SD2 forms a dimer and the two R1474 residues are surface exposed (Fig. 1E, R1474 residues are depicted in green). Based on this information, we predict that R1838 of Shroom3 is also surface exposed. As outlined in more detail below, this substitution mutation does not appear to affect the stability or folding of the protein. Therefore, these data indicate that the Shroom3 R1838C variant is defective for a specific activity or interaction that is required for neural tube closure in mice.

**Shroom3 R1838 is specifically required for binding to SD2 of Rock**

Based on the above results and previous studies from our groups, we predicted that the Shroom3 R1838C protein is unable to interact with Rock. To test this hypothesis, we generated substitution variants R1838A and R1838C of mouse Shroom3 SD2 and tested their ability to bind to the Shroom-Binding domain (SBD) of human (h) Rock1 using in vitro binding assays. We generated two different substitution variant(s to also address the idea that an arginine residue is important at this position and that any alteration in Shroom3 function is not the result of the chemistry associated with a cysteine residue at this position. Alanine was selected because it is a small, non-polar, uncharged amino acid. First, we performed pull-down assays by mixing GST-Shroom3 SD2 variants bound to beads with soluble, His-tagged Rock1-SBD spanning amino acids 707–946. In this assay, relative to the wild-type SD2, the R1838A and R1838C variants exhibit an approximate 45% and 95% reduction, respectively, in the ability to bind the Rock SBD (Fig. 2A). To verify the results from the pull-down assay and assess the stability of the interaction, we mixed GST-Shroom3 SD2 with His-tagged SBD in solution and resolved the proteins by native gel electrophoresis. In this assay, wild-type GST-Shroom3 SD2 and the SBD form a stable complex that has reduced mobility in the native gel (Fig. 2B). In agreement with the pulldown assay, we found that the R1838C variant is incapable of forming a stable complex and essentially all of the SD2 and SBD proteins remain in the unbound state. In contrast, the R1838A exhibits an intermediate level of binding, with 51% of the GST-Shroom3 SD2 protein remaining unbound. These data suggest that the R1838 position is important for binding. However, because the alanine substitution results in an intermediate level of binding, it suggests that the cysteine mutation is more severe and that there may be some tolerance for different amino acids at this position. To further investigate this interaction and to verify that the GST moiety, because it is a dimer, did not influence binding, we assessed the ability of untagged SD2 and SBD proteins to form stable complexes using native gel shift assays (Fig. 2C). In these experiments, we can readily detect a complex consisting of wild-type SD2 and the Rock SBD. In contrast, we were unable to detect complex formation for the R1838A and R1838C variants, suggesting a significant decrease in their relative affinity for the Rock SBD.

Structural data for the SD2 from Drosophila Shroom suggests that the relevant residue in mouse Shroom3 should be surface exposed, however it is possible that the substitution mutations perturb the intramolecular interactions required for dimerization or alter protein folding and stability. The R1838C and R1838A variants exhibit the same mobility on a native gel (Fig. 2B,C), suggesting this is not the case. In addition, we compared the protease sensitivity of wild-type, R1838A, and R1838C SD2 proteins (Fig. 2D) and found no significant changes, indicating that the R1838 substitutions do not alter overall protein folding or stability. Finally, size-exclusion chromatography performed on untagged Drosophila Shroom SD2 and each of the mouse Shroom3 variants yielded similar profiles, indicating that the substitutions do not grossly alter the overall tertiary structure or promote the formation of protein aggregates (Fig. 2E). Taken together, these data indicate that the R1838A and R1838C proteins are virtually indistinguishable from the wild-type SD2 in folding and stability, suggesting that R1838 is playing a prominent role in mediating the Shroom-Rock interaction and not altering other aspects of SD2 structure.

**Shroom3 R1838A and R1838C fail to colocalize with Rock**

Shroom proteins bind directly to and recruit Rock to specific subcellular locales to regulate cell morphology and behavior (Dietz et al., 2006; Nishimura and Takeichi, 2008; Farber et al., 2011; Mohan et al., 2012). Based on the above data showing that Shroom3 variants R1838A and R1838C fail to bind Rock, we predicted that these mutants would also fail to recruit Rock to specific subcellular locales in vivo. To test this hypothesis, we co-expressed the Rock SBD with either wild type or substitution variants of Shroom3 in MDCK and Cos7 cells and assayed their co-localization. Shroom3 localizes to cell–cell junctions in MDCK cells and cortical actin and actin stress fibers in Cos7 cells, while the Rock SBD is typically cytoplasmic in these cells. If Shroom3 is capable of binding Rock and recruiting it, Rock will then colocalize with Shroom3. As expected, wild type Shroom3 and Rock colocalize to cell–cell junctions and actin stress fibers in MDCK cells and Cos7 cells, respectively (Fig. 3A). As a control, a version of Shroom3 lacking the SD2 (ΔSD2) is incapable of recruiting the SBD (Fig. 3B). Similar to the SD2 deletion variant, Shroom3 R1838A and R1838C mutants fail to colocalize with Rock in either MDCK or Cos7 cells (Fig. 3C,D). We performed colocalization analysis to quantify the degree of co-distribution between Shroom3 and the Rock SBD in Cos7 cells using color scatter plots (Fig. 3E, left panels). In these experiments, we see significant co-distribution of wildtype Shroom3 and Rock, while this linear relationship is greatly diminished in cells expressing the ΔSD2, R1838A, and R1838C variants. To further quantify these data, we plotted the Pearson’s correlation (r value) for these scatter plots and we observe a significant difference between Shroom3 and the various SD2 variants (Fig. 3E, right panels). Thus, our data suggest that Shroom3-Rock binding is required for directing Rock to specific subcellular locales.

**Shroom3 R1838 is required for apical constriction and activation of the Rock-Myosin II pathway in polarized epithelial cells**

Previous work has shown that the Shroom3 SD2 is both necessary and sufficient to cause apical constriction in polarized MDCK
cells and that this activity is dependent on Rock catalytic activity (Hildebrand, 2005; Dietz et al., 2006). To address whether alterations at R1838 prevent apical constriction, we expressed either wildtype or R1838 variants of Shroom3 in polarized MDCK cells and tested their ability to elicit apical constriction. MDCK cells expressing wild-type Shroom3 show dramatic apical constriction, demonstrating an 89% decrease in apical area relative to non-transfected cells (Fig. 4A,D). In contrast, both the R1838A and R1838C variants are significantly impaired in the ability to induce apical constriction in comparison to wildtype Shroom3 (Fig. 4B–D). Consistent with some of the in vitro binding data, the R1838A variant induces a small but significant degree of apical constriction; a 22% decrease in apical area relative to non-transfected cells (Fig. 4B,D). Cells expressing the Shroom3 R1838C variant exhibit only a slight, 8% decrease in apical area that is not significantly different from control cells (Fig. 4C,D). These data suggest that the R1838C has a more severe effect on apical constriction in MDCK cells in accordance with its inability to bind Rock. These results are likely due to the inability of these proteins to bind Rock and not the degree of protein expression or stability as all are expressed at similar levels (Fig. 4E).

To further characterize the R1838 variants and their ability to regulate actomyosin contractility, we assessed activation of Myosin II by measuring the phosphorylation of the RLC (Fig. 4E). Consistent with the above phenotypes, only cells expressing wildtype Shroom3 exhibit increased phosphorylation of RLC at Thr18/Ser19 as detected by Western blotting (Fig. 4E). We detect no change in the overall levels of myosin II or the RLC in cells expressing any of the Shroom3 proteins. The above results are consistent with the hypothesis that the interaction of Shroom3 and Rock directly correlates with the ability of Shroom3 to induce apical constriction.

Under certain circumstances, the SD2 domain is both necessary and sufficient to induce changes in cytoskeletal organization and...
subsequent alterations in cell morphology (Hildebrand and Soriano, 1999; Hildebrand, 2005). SD2 elicits changes in cell morphology by altering the cellular distribution of contractile actomyosin networks (Hildebrand, 2005). This feat is accomplished via the direct association of the Shroom3 SD2 with Rock (Nishimura and Takeichi, 2008). We hypothesize that this interaction recruits Rock to specific subcellular compartments and activates it, resulting in the phosphorylation of Rock targets such as myosin RLC. This results in the activation of myosin II and the formation of a contractile actomyosin cable at zonula adherens which induces apical constriction (Hildebrand, 2005). Since Shroom3 R1838A and R1838C variants fail to bind Rock or cause apical constriction, we wanted to test if they are also incapable of activating Myosin II by assaying the phosphorylation status of RLC in polarized MDCK cells expressing these Shroom3 variants. MDCK cells were transfected with expression vectors for Shroom3 and Rock1, grown overnight on transwell filters to form polarized monolayers, and stained to detect Shroom3 and pRLC. Consistent with our previous findings, cells expressing Shroom3 R1838A or Shroom3 R1838C do not show enrichment of pRLC at cell junctions (Fig. 5B,C) compared to wild type Shroom3 (Fig. 5A). We quantified these data by plotting the fluorescence intensity of Shroom3 and pRLC. This was accomplished by drawing straight-line regions of interest (ROIs) that were 12 pixels in length and perpendicular to the zonula adherens of cells expressing the indicated Shroom3 protein. In cells expressing wildtype Shroom3, we see a significant increase in the fluorescence intensity of pRLC that co-distributes with Shroom3 at cell junctions relative to those expressing the R1838A or R1838C variant (Fig. 5A–C, right panels). Taken together, these results substantiate the role of this conserved arginine residue in mediating the Shroom3-Rock interaction and demonstrate that Rock binding, and subsequent localized activation of actomyosin, is required for proper neural tube morphogenesis.

To verify that R1838 is required for the assembly of apically located myosin II that causes apical contractility, MDCK cells expressing either wildtype Shroom3 or the R1838C variant were grown on transwell filters and stained to detect myosin IIb. Both the wildtype and R1838C proteins are localized to cell junctions (Fig. 5D,E). In cells expressing wildtype Shroom3 we observe a clear localization of myosin IIb to apical junctions, apically constricted cells, and straight cell junctions, consistent with cells under tension, as has been previously shown (Hildebrand, 2005).
In contrast, cells expressing the R1838C variant exhibit neither recruitment of myosin IIb to apical junctions nor any changes in cell shape, suggesting that it is incapable of activating the Rock-Myosin pathway (Fig. 5D–F). Together, these results indicate that the R1838C variant of Shroom3 localizes correctly in polarized epithelial cells but is incapable of binding to Rock and subsequently cannot activate apical actomyosin contractility to facilitate neural tube closure.

**DISCUSSION**

Neural tube closure is a complex morphogenetic event that has been intensely studied due to the severe birth defects associated with the failure of this process (Copp et al., 1990). Shroom3 plays an important role in neurulation in Xenopus, mouse, and chick embryos (Hildebrand and Soriano, 1999; Haigo et al., 2003; Nishimura and Takeichi, 2008). Shroom3 regulates tissue morphogenesis and cellular remodeling by modulating the architecture and dynamics of the actin cytoskeleton must be precisely regulated during neural tube closure. This is demonstrated by the fact that mutations in cytsokeletal regulators such as Nap1 (Rakeman and Anderson, 2006), Abll/2 (Koleske et al., 1998), p190RhoGap (Brouns et al., 2000), Mena profilin (Lanier et al., 1999), Vinculin (Xu et al., 1998), NF1 (Lakkis et al., 1999), paladin (Roffiers-Agarwal et al., 2012), Epb4.115 (Lee et al., 2007; Chu et al., 2013), and Marcks (Stumpo et al., 1995), all cause neural tube defects. We have previously shown that Shroom3 binds and bundles F-actin and may recruit Ena/Vasp proteins to the zonula adherens and that these activities are required for apical constriction of MDCK cells (Hildebrand and Soriano, 1999; Hildebrand, 2005; Plageman et al., 2010; Haigo et al., 2003; Hildebrand, 2005; Dietz et al., 2006; Fairbank et al., 2006; Hagens et al., 2006a; Yoder and Hildebrand, 2007; Farber et al., 2011). Thus, the Shroom-Rock pathway hints at a signaling module operating in a variety of cell types to regulate cellular behavior and morphology. However, the molecular mechanism of the Shroom-Rock interaction is still unknown. Our investigations into a substitution variant encoded by the Shroom3m1Nisw allele indicates that Rock binding is mediated by a defined part of the SD2 and subsequent apical recruitment is the vital step in Shroom3-mediated aspects of neural tube closure.

**R1838 is essential for Shroom3-induced changes in cell morphology**

Shroom3m1Nisw mutant mice exhibit severe exencephaly that is attributed to a point mutation in the Shroom3 SD2 that changes arginine 1838 to cysteine. We have shown that the SD2 domain from Drosophila Shroom forms a three-segmented anti-parallel coiled-coil dimer with highly conserved surfaces that mediate Shroom-Rock interactions. (Mohan et al., 2012). One of these surface patches, 1834SLSGLR1840, harbors R1838. We have shown that changing the positively charged arginine to either an uncharged polar amino acid (cysteine) or a nonpolar amino acid (alanine) disrupts binding to Rock. Interestingly, in some assays, these substitution variants have different severity, suggesting there is some tolerance at this position. However, both variants are significantly compromised in the ability to cause apical constriction, suggesting that an arginine at this position is essential. It will be interesting to solve the structure of the SD2-Rock complex to understand how this arginine residue participates in the interaction.
Therefore, in addition to regulating localized actomyosin contractility, Shroom3 could also control aspects of actin dynamics to facilitate neural tube closure. However, our previous mapping studies have shown that F-actin binding is mediated by the SD1 motif, bounded by amino acids 754–1108, and is clearly distinct from R1838. Our work here also shows that mutation of R1838 does not perturb Shroom3 protein localization. Additionally, the putative Ena/Vasp binding motif (FPPPP) is also distinct from the SD2. Additionally, we have previously shown that Drosophila Shroom, which lacks a clear Ena/Vasp binding motif, still causes apical constriction in Drosophila embryos (Bolinger et al., 2010). Therefore, based on the molecular nature of the Shroom3m1nisw allele and our analysis here, we propose that these activities are likely to be intact in the R1838C substitution variant. Therefore, while this does not rule out the requirement of these other activities in Shroom3 function in vivo, it is clear that they are not sufficient for neural tube morphogenesis, while Rock binding is essential.

The SD2-Rock interaction is evolutionarily conserved

In Drosophila, apically positioned contractile networks of actomyosin generate forces that are critical for germ band extension, ventral and dorsal closure, and various invaginations (Costa et al., 1994; Winter et al., 2001; Bertet et al., 2004; Nikolaidou and Barrett, 2004; Zallen and Wieschaus, 2004; Dawes-Hoang et al., 2005; Franke et al., 2005; Blankenship et al., 2006; Simões et al., 2006; Corrigall et al., 2007; Kolesnikov and Beckendorf, 2007; Mulinari et al., 2008; Xu et al., 2008). Our lab has shown that Drosophila Shroom, like Shroom3, binds to Rock.
and F-actin and induces robust apical constriction (Bolinger et al., 2010). We have solved the structure of the SD2 from dShroom and it is this analysis that allowed us to predict that R1838 of Shroom3 is surface exposed (Mohan et al., 2012). To verify that the Shroom-Rock interface is conserved, mutation of the analogous arginine in the dShroom SD2 (R1474 in dShroomA) renders the protein incapable of binding Rock (data not shown). Thus, we conclude that the Shroom-Rock-MyosinII pathway is evolutionarily conserved.

**Implications for understanding the Shroom-Rock interaction**

An unanswered aspect of the Shroom-Rock pathway is if Shroom proteins are capable of directly activating Rock. It is predicted that, in its inactive state, Rock adopts a folded, autoinhibited conformation in which the C-terminal tail interacts with the N-terminal catalytic domain. It is thought that binding of proteins such as RhoA (in the GTP bound state) or lipids or caspase cleavage of the C-terminus relieves this intramolecular inhibition and activates Rock (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996; Ishizaki et al., 1997; Amano et al., 1999; Feng et al., 1999; Araki et al., 2001; Coleman et al., 2001; Fukata et al., 2001; Sebbagh et al., 2001; Chen et al., 2002; Sebbagh et al., 2005; Yoneda et al., 2005). However, there is evidence for both Rho-dependent (Plageman et al., 2011b) and Rho-independent modes of Rock activation during Shroom3-induced apical constriction (Haigo et al., 2003; Hildebrand, 2005; Mohan et al., 2012). We predict that if Shroom binding is both necessary and sufficient for activation of Rock, then the Shroom3 R1838C substitution variant would be unable to perform either task.

Targeted Rock inhibition could serve as a potential therapeutic approach for many debilitating diseases, including cancer (Itoh et al., 2012). We have solved the structure of the Shroom3 SD2 spanning amino acids 1562–1986) bound to Glutathione beads, and mixed with GST pull-down assays were performed using either wild type GST- Shroom3 SD2 or R1844A and R1844C mutant versions (spanning amino acids 707–946 was mixed with increasing concentration of GST-fusion proteins; 20 nM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40) or Hi-sys buffer for His-fusion proteins; 20 nM Tris, pH 8.0, 0.5 M NaCl, 5% glycerol, 5 mM β-mercaptoethanol) supplemented with Protease inhibitor cocktail and soluble proteins were purified using either glutathione-sepharose resin or Ni-NTA beads. Beads were washed in lysis buffer and the proteins eluted with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 hours and collected by centrifugation. Cells were lysed by sonication in NETN buffer (for GST-fusion proteins; 20 nM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40) or His-lysis buffer (for His-fusion proteins; 20 nM Tris, pH 8.0, 0.5 M NaCl, 5% glycerol, 5 mM β-mercaptoethanol) and the flowrate of the column was 0.5 ml/min and the elution profile gathered by reading the absorbance at 280 nm.

**Protein expression and purification**

Large-scale protein expression of His-tagged SD2 and Rock SBD proteins was performed in BL21(DE3) E. coli cells using ZY autoinduction media as described (Farber et al., 2011; Mohan et al., 2012). The Shroom3 and Rock proteins were concentrated to 0.78 mg/ml (WT Shroom) and 1.43 mg/ml (R1838A) and 1.7 mg/ml (R1838C) and 1 mg/ml (WT Rock) in 20 mM Tris, pH 8.0, 0.5 M NaCl, 8% glycerol, and 5 mM dithiothreitol (DTT). For purification of GST-Shroom3 SD2 or small-scale (<50 ml) expression of His-tagged hRock SBD proteins, BL21 cells or RIPL cells harboring the relevant plasmids were incubated with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 hours and collected by centrifugation. Cells were lysed by sonication in NETN buffer (for GST-fusion proteins; 20 nM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40) or His-lysis buffer for His-fusion proteins; 20 nM Tris, pH 8.0, 0.5 M NaCl, 5% glycerol, 5 mM β-mercaptoethanol) supplemented with Protease inhibitor cocktail and soluble proteins were purified using either glutathione-sepharose resin or Ni-NTA beads. Beads were washed in lysis buffer and the proteins eluted with either free glutathione or imidazole in the respective lysis buffers. Drosophila Shroom SD2 (1393–1576) was purified as previously described (Mohan et al., 2012).

**Size exclusion chromatography**

Untagged Drosophila Shroom SD2 (1393–1576), mouse Shroom3 SD2 (1642–1951), or mouse Shroom3 SD2 containing a R1838A or R1838C substitution were purified as described above and analyzed by size exclusion chromatography using a Sephacryl S-200 column. All of these runs were performed in 20 mM Tris pH 8.0, 250 mM NaCl, 2% glycerol, and 1 mM betamercaptoethanol. The flowrate of the column was 0.5 ml/min and the elution profile gathered by reading the absorbance at 280 nm.

**In vitro analysis of protein structure and function**

GST pull-down assays were performed using either wild type GST-Shroom3 SD2 or R1844A and R1844C mutant versions (spanning amino acids 1562–1986) bound to Glutathione beads, and mixed with soluble, His tagged hRock SBD (residues 707–946). The binding reaction was incubated for 2 hours at room temperature. Complexes were washed with NETN, resuspended in SDS-PAGE sample buffer, resolved on 12% SDS-PAGE, and detected using Coomassie Blue. For native gel electrophoresis, a fixed concentration (5 μM) of hRock SBD spanning amino acids 707–946 was mixed with increasing concentration of purified Shroom3 SD2 spanning amino acids 1642–1951 (0.25–5 μM) and incubated for 2 hours at 4°C. Samples were then loaded on 8% PAGE gels, resolved by electrophoresis at 4°C and proteins detected with Coomassie blue. For limited proteolysis studies, 50 μM of wild-type and mutant Shroom3 SD2 proteins were treated with 40 μg of the protease Subtilisin A for the indicated times and samples taken at each time point were resolved via SDS-PAGE. Purified WT and mutant proteins were concentrated to 1 mg/mL in buffer containing 2% glycerol, 250 mM NaCl, 20 mM Tris pH 8.0, and 1 mM β-mercaptoethanol. 500 μl of were run over a Sephacryl S-200 gel.
filtration column, and traces were generated using Unicorn 6.3.2.89 Control Software.

**Cell culture and in vivo analysis**

T23 MDCK cells were grown in EMEM supplemented with 10% FBS, pen/strep, and L-Glutamine at 37°C and 5% CO2. Cos7 cells were grown in DMEM supplemented with 10% FBS, pen/strep, and L-Glutamine under similar conditions. Cells were removed from the plates using Trypsin/EDTA and passaged every 2–3 days. For transient transfection of cells on transwell filters, cells were plated at a density of 8 × 10^5 cells (MDCK) or 6 × 10^5 cells per well (Cos7) and grown for 24 hours. Cells were transfected with the DNA of interest (1 μg) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) and grown for 24 hours prior to processing. For immunofluorescent analysis, cells were fixed using either −20°C methanol for 5 minutes or 4% paraformaldehyde (PFA) in PBS for 15 minutes. Fixed cells were stained with primary antibody for 1 hour at RT, washed in PBT three times for 5 minutes at room temperature, stained with secondary antibody for 1 hour at room temperature, washed as above and mounted using VectaShield (Vector Labs, Burlingame, CA, USA) or Immunofluorescence Mounting medium (MP Biomedicals, Santa Ana, CA, USA).

Shroom3-induced apical constriction using expression plasmids pCS2-Shroom3, pCS2-Shroom3 R1838A, pCS2-Shroom3 R1838C was performed and imaged as described previously (Hildebrand, 2005). Transfected cells were stained with primary antibodies UPT132 and Rat anti-ZO1 and detected with Alexa-488 or 568 conjugated secondary antibodies. Apical constriction was quantified by measuring the apical area of either parental or transfected cells, as determined by ZO1 staining, in ImageJ. To determine colocalization of Shroom3, Rock SBD, and pRLC, MDCK cells or Cos7 cells expressing Shroom3 variants and/or myc-tagged, wild type hRock1 SBD (spaying amino acids 681–942) were plated on either transwell membranes or fibronectin (Sigma, St. Louis, MO, USA) coated coverslips, respectively, for 24 hours. To analyze role of Shroom3 on myosin IIb distribution, T23-MDCK cells were co-transfected with 20:1 ratio of linearized pCS2-Shroom3 or pCS2-Shroom3 R1838C with pTRE2-Hygro, selected in hygromycin, and surviving cells pooled and tested for Shroom3 expression. Cells were plated on transwell filters for 24 hrs and analyzed. Cells were stained to detect Shroom3, ZO1, myc-tag, pRLC, myosin IIb, or F-actin (tritc-phalloidin). Primary antibodies were detected using Alexa-488 or 568 conjugated secondary antibodies and imaged as described above. Images were acquired using a Biorad Radiance 2000 Laser Scanning System mounted on a Nikon E800 microscope or Olympus Fluoview FV1000 Confocal microscope (FV10-ASW) with 40× oil objectives and processed using either ImageJ or Photoshop. To determine the degree of colocalization in Cos7 cells, ImageJ plug-ins Colocalization Finder and Mander’s Coefficients were used to analyze individual channels from merged confocal images. To analyze the co-distribution of Shroom3 and pRLC, fluorescent intensity was measured along 12-pixel long line segments drawn perpendicular to the adherens junctions on individual channels. Data at each corresponding pixel from at least 30 measurements were averaged and plotted using Excel. To quantify fluorescent intensity of Myosin IIb at cells junctions using ImageJ, 1 pixel wide ROIs were drawn around individual cells using ZO1 as a guide. ROIs were copied to images of myosin IIb and fluorescent intensity was measured. To determine relative fluorescent intensity of Shroom3 proteins in neural epithelia, 1 pixel wide ROIs were drawn around individual cells based on Shroom3 or β-catenin staining. Fluorescent intensity was measured and the ratio of Shroom3 to β-catenin staining determined. Measures of statistical significance were determined by two-tailed, unpaired, Student’s t-Tests to distinguish significance between two data sets and a one-way ANOVA (Tukey’s post-hoc) for comparison of 3 or greater data sets. For all graphs, error bars represent standard deviation (± s.d.). Western blots were performed as described (Farber et al., 2011).

**Antibodies used**

Rabbit anti-Shroom3 UPT132 (1:100, Hildebrand, 2005), mouse anti-myc 9E10 (developed by J. M. Bishop, obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242), anti-RLC, p Ser19 RLC and ppThr18/Ser19 RLC (Cell Signaling, Danvers, MA, USA), anti-β-catenin (BD Transduction Lab), rabbit anti-nonnuscle Myosin Ia and Iib (Covance, Princeton, NJ, USA), and rat anti-ZO1 (R26.4C, Chemicon or Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA). Primary antibodies were detected using Alexa-488 or 568 conjugated secondary goat anti-rabbit or goat anti-mouse (1:400, Invitrogen) or HRP-conjugated Goat anti-Rabbit or anti-mouse (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

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**Competing interests**

The authors have no competing interests to declare.

**Author contributions**

DD, JKZ, SM, JDH, and TFP performed the experiments. DD, TFP, APV, and JDH analyzed the data and prepared figures. DD, JDH, and APV prepared the manuscript.

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