SHROOM2 REGULATES ENDOTHELIAL MORPHOGENESIS AND CENTROSOME DUPLICATION THROUGH THE SPECIFIC SUB-CELLULAR RECRUITMENT OF RHO-KINASE.

by

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The ability of epithelial cells to change shape is essential to the patterning of tissues and organs during development of the vertebrate embryo. Epithelial morphogenesis is mediated by the molecular regulation of cytoskeletal dynamics which underlies cellular adhesion, motility, polarity, and proliferation. The Shroom family of proteins regulates epithelial morphogenesis by promoting MyosinII-dependent changes in epithelial morphology through the ability to bind both F-actin and Rho kinase (Rock). Shroom3 is necessary to induce apical constriction of the neural epithelium and is required for proper neural tube closure during development. However, the roles of other family members are unknown. This work seeks to determine the role and mechanism of action for Shroom2 in epithelial cell biology.

Through RNAi, the loss of Shroom2 reduces contractility of endothelial cells. Shroom2 physically interacts with Rock and is necessary for its cortical localization. By impeding Rock localization and reducing contractility, Shroom2 knockdown alters cytoskeletal organization, adhesion, and motility which ultimately affects *in vitro* angiogenesis. During these studies, it also became clear that Shroom2 localizes to the centrosome where it is required to maintain efficient centrosome duplication in a Rock-dependent manner. The results described here expand a role for the Shroom proteins in the sub-cellular localization of Rock which mediates a subset of Rock functions within epithelial cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

It is imperative to first thank my advisor and mentor Jeff Hildebrand. Your passion for science, baseball, weird looking bugs, and The Police have made my graduate years enjoyable. And of course now I know 101 fun things to do with liquid nitrogen. Most of all, thank you giving me the freedom to explore and experiment on my own. With your occasional suggestions, tips, and guidance, I enjoyed the process of discovery in a way not possible in other labs. Through you, I became aware of my love of visual science and pretty pictures and became confident in my ability to design, implement, and communicate the scientific process. And thank you, thank you thank you, for allowing me to focus only on my centrosome manuscript and dissertation during my last semester.

My committee has also been very helpful in shaping my graduate career. At my first committee meeting it became clear my focus was a little off track; I was focused on too many things. With their guidance I learned to narrow my focus, and I learned how to develop a clear research plan. Thank you to Jeff Brodsky, Debbie Chapman, Lance Davidson, and Beth Roman for all of your time and help.

I could not have made it through graduate school without the baked goods of Debbie Chapman. Everything she makes is AMAZING! Even the lemon stuff. And she even manages to make it healthy! 100% good for you. And thank you too for all of your help and advice in handling mice and stem cells. Though I never got past chimeras and looking through the green

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1.0 INTRODUCTION

Epithelial morphogenesis is the process in which cells change shape in order to pattern diverse organ shape and function. Cellular functions such as motility, polarity, adhesion, and cytoskeletal dynamics are essential in specifying cell shape changes. Understanding the various mechanisms behind epithelial morphogenesis provides insight into animal development, congenital diseases, regeneration, and tissue engineering.

An epithelium is typically a polarized monolayer of cells where cell adhesion is essential for the formation of coherent sheets [1]. Subsequent changes in cell shape, intercalation, migration, proliferation, and apoptosis pattern more complex structures and forms the variety of tissues and organs of the body. A variety of mechanistic processes facilitate such shape changes. First, extracellular cues such as morphogens, the extracellular matrix, and physical forces initiate signal transduction to cells. Second, cells must possess the ability to transduce such signals through integrins and cellular adhesion structures. Third, as an outcome of signal transduction, cells must physically change their shape by altering the cytoskeleton and adhesive properties. And finally, cell morphogenesis can be specified by specific transcription factor profiles [2]. By varying the expression of signal transduction pathway components, the cell's ability to sense cues and execute morphogenesis can change. While this dissertation will discuss many of these processes, the focus will be on understanding cytoskeletal dynamics which influence epithelial morphogenesis through adhesion, migration, and contractility.

1.1 ACTIN DYNAMICS

The most basic element of cell shape change is the cytoskeleton. Just as a building cannot stand without a wooden, steel, or concrete infrastructure, a cell cannot take form without the cytoskeleton. The complex networks of actin filaments, microtubules (MT), and intermediate filaments (IF) make up the structural integrity of the cell and are responsible for a variety of cellular functions such as mitosis, migration, adhesion, and intracellular transportation. Microtubules are long, hollow cylinders with subunits of α and β tubulin heterodimers and are best known for their role in bi-polar spindle formation and chromosome segregation during mitosis [3]. They also play a structural role and provide intracellular networks on which to transport cargo. IFs consist of coiled-coil dimers which closely interact to form a rope-like filament [4]. IFs are best known for formation of a meshwork lining within the nuclear membrane. Within the cytoplasm, it is believed that IF networks impart physical strength to the cell. Actin is composed of a single monomer of globular (G) actin which polymerizes into two-stranded helices termed filamentous (F) actin [5]. Actin is closely associated with the plasma membrane, and as such, remodeling of the actin cytoskeleton greatly influences cell morphology. Changes in actin architecture underlie changes in cell migration, adhesion, and contractility. The molecular mechanisms of these processes are described in subsequent sections.

1.1.1 Actin binding proteins

Based upon the head-to-tail alignment of actin monomers within a filament, F-actin contains inherent polarity with a “pointed” and “barbed” end [6]. F-actin elongates when ATP-bound monomers are added to the barbed end. As the actin filament matures, ATP is hydrolysed and

the resulting ADP-actin monomers are released resulting in net depolymerization at the pointed end [7]. While actin can self polymerize, a variety of actin binding proteins are involved to expedite and control actin networks through regulation of the nucleation of new branches, the assembly and disassembly of existing fibers, and the organization of filaments into higher-order structures.

Two primary types of actin networks are prevalent throughout the cell: long cables and short, branched networks. The type of actin network depends upon the activity of nucleation promoting factors. For example, branched actin networks occur through the protein Arp2/3 which nucleates filaments from the side of existing fibers [8]. This activity is enhanced by interactions with members of the Wiskott-Aldrich syndrome protein family (ex: WASP, WAVE, and WASH) which are thought to provide actin monomers to new branches [9]. In addition, cortactin binds to cortical F-actin and recruits and stabilizes Arp2/3. Actin capping proteins such as gelsolin and capping protein, bind to the barbed end of F-actin and prevent the addition of new monomers [10]. By limiting the polymerization of exisiting filaments, capping protein also promotes Arp2/3 dependent branched network assembly [11]. Anti-capping proteins, such as the Ena/VASP family, can counter capping proteins by preferentially binding to the barbed end of F-actin while simultaneously binding to G-actin, promoting long, unbranched filament assembly [12]. The formin family of proteins also binds to the barbed end and enhances polymerization of long filaments [13].

Several actin binding proteins have been identified which either promote strand stability or depolymerization. The ADF/cofilin proteins remove ADP-actin from the pointed end thus promoting depolymerization [14]. In addition to capping function, gelsolin can act as a severing protein [15]. On the other hand, tropomyosin proteins bind along the filament length and

stabilize the filament from depolymerization [16]. While these proteins all interact with F-actin, certain proteins can interact with actin monomers. With an affinity for G-actin, profilin sequesters the available pool of monomers and can prevent spontaneous actin assembly [17]. Additionally, profilin promotes nucleotide exchange, converting less stable ADP-actin into filament friendly ATP-actin [18]. The addition of G-actin to the barbed end by Ena/VASP proteins is further enhanced by its ability to interact with profilin [19]. The molecular regulation of actin binding proteins and their roles in cell biology will be discussed in later sections.

Just as more force is required to tear a stack of papers than a single sheet, bundles of actin can withstand greater stress than single fibers. Some actin binding proteins organize F-actin into higher order networks by bundling or crosslinking actin fibers. Parallel actin bundles can be formed in one of two ways. First, certain actin binding proteins like α-actinin are homodimers with a single actin binding domain [20]. As they dimerize, they can bind to two separate F-actin fibers leaving about 30nm of space between the fibers [21]. Other proteins like fimbrin and villin are small in size and possess two actin binding motifs [22, 23]. Therefore they bundle F-actin into a tighter network (Figure 1).

While α-actinin and fimbrin form parallel actin networks, other proteins can form an actin web or gel. Spectrin is a long, flexible tetramer with two actin binding domains 200nm apart [24]. As spectrin can bind to peripheral membrane proteins, spectrin forms a gel-like network of actin fibers at the cell cortex. Filamin dimers position two actin binding domains at right angles and thus create a mesh-like network which is important for the formation of thin, flat lamellipodia during migration [25]. Depending on the organization of the actin network, different proteins gain access. For example, non-muscle Myosin II cannot fit between the small

spaces of fimbrin packed actin fibers but fits well in the space created by α-actinin. The relationship between actin and myosin will be discussed in the next section.

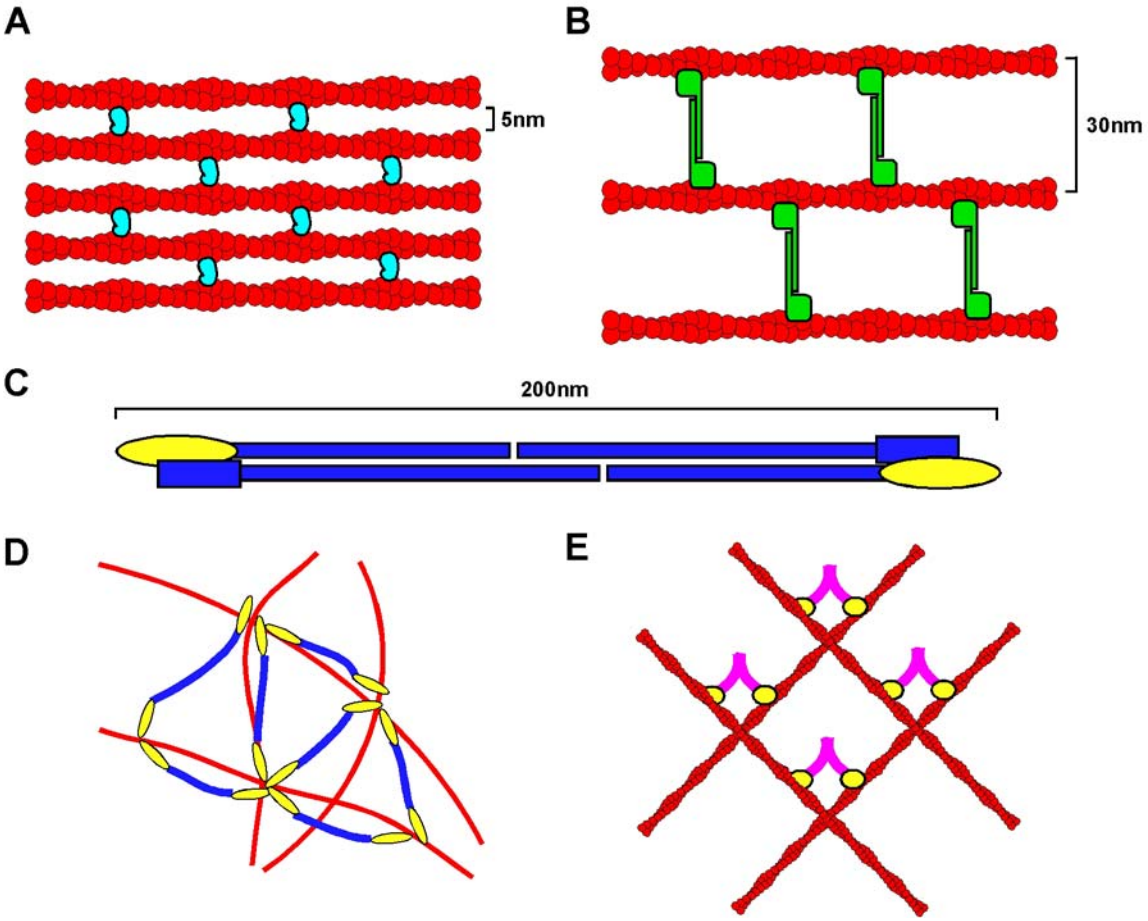

Figure 1: Actin binding proteins shape actin networks.

(A) Fimbrin is a small protein with two actin binding domains which forms tightly packed parallel actin fibers. **(B)** α-actinin has one actin binding domain but dimerizes, thus forming more loosely packed parallel actin fibers. **(C)** Spectrin is a long heterodimer with actin binding properties at either end. **(D)** Spectrin forms a mesh like network with actin at the cortex of red blood cells. **(E)** Filamin has one actin binding domain but dimerizes to bind two actin fibers at right angles. Figure adapted from [26].

1.1.2 Non-muscle Myosin II

Myosins contain a large family of motor proteins which are vital to cellular processes such as cytokinesis, intracellular transport, motility, and morphogenesis. Through ATPase and actin binding activity, myosin transfers energy into protein conformational change allowing the molecules to exert force upon F-actin, be it by walking along filaments or exerting tension between them [27]. Non-muscle myosin II (MyosinII) is the largest class of myosin and is responsible for most myosin-dependent processes in non-muscle cells. MyosinII contains three pairs of peptides: two myosin heavy chains (MHC), two myosin regulatory light chains (MRLC), and two myosin essential light chains (MELC). The MHCs constitute the bulk of MyosinII and contain two globular heads, a neck region, and a long coiled-coil; the two heads contain the ATPase and the actin binding domain. MRLC and MELC bind within the neck region, and the coiled-coil is responsible for dimerization and filament assembly [28]. While the globular head domain can bind to actin and exert force by itself, both ATPase activity and force exertion are greatly enhanced by phosphorylation of Ser19 in the MRLC [29]. In addition, it is thought that the MRLC is responsible for regulating actomyosin filament assembly. *In vitro*, unphosphorylated MyosinII folds into a looped conformation through an interaction between the head and tail; globular heads are inaccessible to actin and the coiled-coil is unable to form filaments [30]. Phosphorylation of MRLC may disrupt the head to tail interaction, inducing polarized filament assembly as demonstrated *in vitro* through smooth muscle myosin [31]. When MyosinII filaments bind to anti-parallel F-actin fibers, ATP hydrolysis induces a conformational change in the globular head which causes the F-actin fibers to contract. Contraction of actomyosin networks and their regulation are the fundamental basis of morphogenesis.

Many kinases have been linked to the phosphorylation of MRLC and activation of MyosinII such as Rho-kinase (Rock), Myosin light chain kinase (MLCK), citron kinase, Zipper interacting protein kinase (ZIPK), and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK) [32-34]. These kinases target Ser19, Thr18, or both to relieve the inhibition described above, but the activation of each kinase differs. For example, MLCK is activated by Ca^{2+} calmodulin signaling, whereas Rock and citron kinase are activated by RhoA. As another difference, MLCK only phosphorylates MLC, whereas Rock targets additional effectors which are described in section 1.1.4.3 [35]. Protein kinase C (PKC) phosphorylates MLC on Ser1, Ser2, and Thr9, which hinders the interaction between MLC and MLCK, thus decreasing MyosinII activity [36].

There are three different *MHC* genes in vertebrates which determine one of three MyosinII isoforms, A-C [37]. At least *in vitro*, it is apparent that the three differ in their kinetic properties. MyosinIIA has the highest rate of ATP hydrolysis and contracts filaments more quickly than the other two [38]. MyosinIIB remains bound to F-actin in a force generating state longer than MyosinIIA [39]. And MyosinIIB possesses a higher affinity for ADP, the release of which is slowed by backward strains exerted by actin filaments [40]. Thus MyosinIIA may function in more rapid, dynamic contraction events, while MyosinIIB is engaged with actin filaments for a longer period of time to maintain tension. Differential roles for MyosinII isoforms in cell function will be discussed below.

1.1.3 Rho Family of p21 Small GTPases

In order to elicit morphogenesis, the variety of actin networks within the cell must undergo remodeling through the molecular regulation of actin binding proteins and nucleation promoting

factors. The Rho-family of p21 small GTPases regulate a variety of cellular proteins, including actin binding proteins, to influence cell functions such as motility, adhesion, and proliferation. Rho family GTPases are known as molecular switches, interacting with downstream effectors to continue signal transduction pathways [41]. GTPases are in the "on" state when bound to GTP and in the "off" state following intrinsic phosphatase activity which dephosphorylates GTP into GDP. Hydrolysis of GTP by GTPases can be accelerated through interactions with GTPase activating proteins (GAPs), whereas the exchange of GDP for GTP is mediated by guanine-nucleotide exchange factors (GEFs) [42, 43]. The relative affinity of a GTPase for its effector in GTP versus GDP bound states can be as high as 100-fold, leading to very specific effector interactions in the "on" state only [44]. An additional means of GTPase regulation comes by way of guanine-nucleotide dissociation inhibitors (GDIs). By binding to GDP-bound GTPases, GDIs prevent nucleotide exchange and thus block activation of effectors [45].

The Rho family GTPases, RhoA, Rac1, and Cdc42, have been traditionally thought of as cytoskeletal regulators based on seminal work showing the effects of over expression in Swiss3T3 fibroblasts. RhoA increased stress fiber and adhesion formation, Rac1 caused flat lamellipodial extensions, and Cdc42 induced filopodial extensions [46-48]. In the years since, it has become apparent that these changes in actin structures are due to the regulation of actin binding proteins. For example, the mammalian formin Diaphanous (mDia) is an effector of Rho, and activation of mDia is sufficient to induce stress fibers [49]. As another example, Rac1 can activate WAVE proteins, a member of the WASP family [50]. WASP proteins contain an auto-inhibitory region which is repressed upon binding to Rac1. Following activation, WASP proteins can bind to Arp2/3 and induce branched actin networks as found in lamellipodia [51, 52].

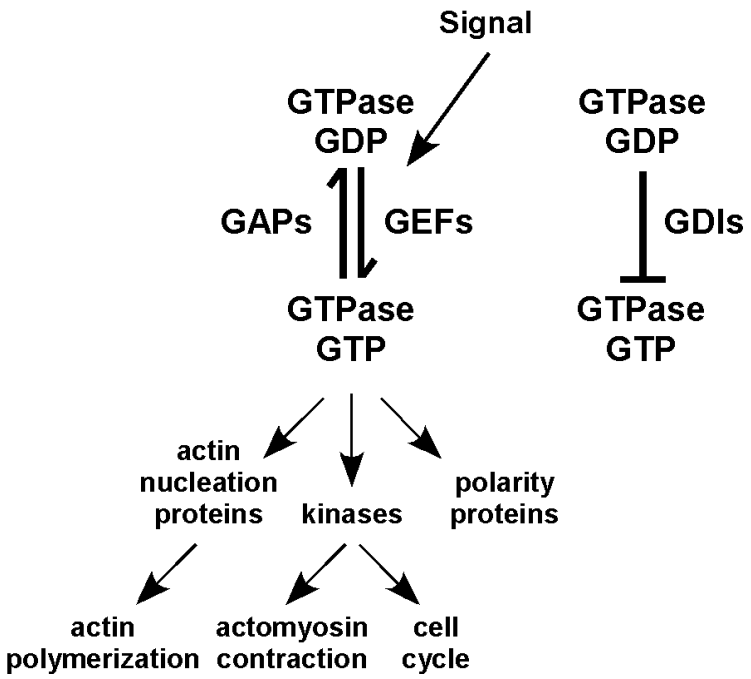

Figure 2: Overview of Rho GTPase signaling

Rho GTPases are molecular switches which contribute to signal transduction to influence cell functions. GEFs and GAPs can influence the activity of GTPases by promoting the exchange of GDP for GTP (GEFs) or accelerating the hydrolysis of GTP (GAPs). Additionally, GTPase activity can be sequestered through GDIs which bind GDP GTPases and prevent exchange of GTP. Rho GTPases target a variety of proteins including actin nucleation proteins which influence actin dynamics, kinases which induce actomyosin contractility, and polarity proteins such as PAR3 which mediate cell polarity.

1.1.4 Rho Kinase

Rho kinase (Rock) is a Rho effector involved in cytoskeletal dynamics [53]. Functioning as a serine/threonine kinase, Rock is composed of an N-terminal catalytic domain, a central coiled-coil domain, and a C-terminal Plekstrin homology (PH) domain. In crystallographic studies, the N- and C-terminal extensions around the catalytic domain facilitate formation of a head-to-head

homodimer [54]. Because Rock forms multimeric complexes and does not elute as a monomer, it is likely that Rock dimerizes in a parallel orientation [55].

1.1.4.1 Rock1 and Rock2

Two Rock isoforms exist in vertebrates, Rho-kinase β / $p160^{ROCK}$ / Rock1 and Rho-kinase α / Rock2, both sharing 65% identity between amino acids overall and 83% identity within the kinase domain. Rock1 and Rock2 are ubiquitously expressed in mouse tissues; however, Rock2 transcripts are more abundant in muscle and neural tissue, while higher levels of Rock1 are found in tissues such as the lung, liver, and testis [56]. There is also some evidence for differential regulation of Rock isoforms during apoptosis as Rock1 is cleaved by caspase-3, whereas Rock2 is cleaved by granzyme B [57, 58]. It has been speculated that Rock isoforms play different developmental roles, because mutant mice display different phenotypes. *Rock1-/-* mice display eyelid and ventral closure defects, while *Rock2-/-* mice display placental dysfunction [59, 60]. However, it was subsequently shown that altering the genetic background of *Rock2-/-* mice also resulted in eyelid closure defects and umbilical herniation. In support of functional redundancy between Rock1 and Rock2, heterozygosity for either *Rock1* or *Rock2* yields no phenotype, whereas *Rock1 +/- Rock2 +/-* compound heterzygotes lead to eyelid close defects [61]. Additionally, *Rock1 -/- Rock2 -/-* embryos die between embryonic day (e) 3.5 and e9.5, but *Rock1 -/- Rock2 +/-* or *Rock1 +/- Rock2 -/-* embryos show defects in the yolk sac vasculature [62]. Based on these results and the lack of evidence for unique substrates for either isoform, it is likely that Rock1 and Rock2 function redundantly and from henceforth will simply be referred to as Rock.

1.1.4.2 Activation of Rock

Interaction with Rho at the C-terminus of the coiled-coil domain moderately activates Rock activity [63]. Rock is subject to intramolecular inhibition by the C-terminal half of the protein, and Rho is thought to relieve this inhibition. Several lines of evidence support this hypothesis. First, deletion or proteolytic cleavage of the C-terminus leads to constitutive activation of Rock [53, 57, 58]. Second, expression of the C-terminal region is sufficient to inhibit the constitutive active form of Rock [64]. And finally, an antibody against the Rho binding region of Rock induces Rock activity [55]. While most Rock-dependent activities require Rho, some lipids, especially arachidonic acid, can activate Rock independent of Rho [65]. In addition, it has been shown that interaction of DynaminI with the PH domain of Rock is sufficient to induce catalytic activity [66]. If the alleviation of intramolecular inhibition within Rock is a key event to its activation, then it is highly likely that other protein interactions within the PH domain or coiled-coil region are also sufficient to induce Rock activity.

However, not all proteins that bind Rock activate its catalytic function. Gem and Rad, members of a small GTP binding family of proteins within the Ras family, bind near the Rho-binding motif but exert an inhibitory function. While Gem and Rad do not directly affect Rock catalytic activity, it is likely that they block accessibility of other interactions [67]. Another Rock inhibitor, RhoE, is a member of the Rnd subfamily of GTP binding proteins. RhoE binds near the kinase domain and interacts with Rock when it is activated by RhoA or cleaved by caspase. Because RhoE binds near the catalytic domain, it is likely that interaction with RhoE blocks the interaction with yet-to-be-determined Rock effectors [68].

1.1.4.3 Rock effectors and functions

One of the key molecular regulatory mechanisms responsible for induction of contractile actomyosin is the balance between kinases that phosphorylate MRLC and the activity of Myosin light chain phosphatase. Myosin phosphatase contains a catalytic subunit and two regulatory subunits, myosin phosphatase targeting subunit 1 (MYPT) and M20 [69]. As previously stated, phosphorylation of MRLC at Ser19 induces MyosinII contractility, whereas Myosin phosphatase acts to remove this phosphate and abrogate MyosinII activation. Rock is at the crux of this balance, as it directly phosphorylates MRLC to increase ATPase activity [70] and also phosphorylates and inactivates MYPT [71]. Rock can also phosphorylate and activate ZIPK [72]. Similar to Rock, ZIPK also phosphorylates MYPT and MRLC, however the net contribution of ZIPK to Rock-induced contractility is unknown [34].

LIM kinases (LIMK) are serine/threonine kinases which influence actin dynamics. Rock has been shown to phosphorylate both LIMK1 and LIMK2, which enhances LIMK activity and leads to phosphorylation of ADF/cofilin [73]. Phosphorylation of cofilin at this site inactivates the ability of cofilin to depolymerize actin [74]. Thus, Rock phosphorylation of LIMK inactivates cofilin and stabilizes F-actin. However, Cdc42 and Rac can also promote LIMK phosphorylation [75]. Given that only CA-Rock and not full-length Rock activates LIMK [76], it is likely that the other Rho GTPases are the predominant cofilin mediators.

Finally, Rock is important in the regulation of several proteins which link the actin cytoskeleton to the plasma membrane. Adducin is a filamentous protein which binds to F-actin, attenuates polymerization, and recruits spectrin. Phosphorylation of α-adducin by Rock enhances actin binding [77]. The ERM (Ezrin-Radixin-Moesin) proteins also act to crosslink actin and transmembrane proteins. Unphosphorylated ERM proteins retain a head to tail

conformation which masks actin and protein interaction domains. Phosphorylation of ERM proteins by Rock disrupts the head to tail conformation and permits protein interactions [78]. The molecular regulation of actomyosin contractility through Rho GTPases, Rock, and actin binding proteins is essential to drive morphogenesis of cells and tissues during development and will be discussed in more detail in subsequent sections.

1.2 EPITHELIAL ADHESION

Polarized epithelial cells are characterized by an apical surface which faces a lumen and a basolateral surface that contains adhesive structures which connect the lateral side with neighboring cells and the basal side with the underlying extracellular matrix (ECM) (Figure 3). Cellular adhesion is important for both transducing extracellular signals into morphogenetic responses and integrating cytoskeletal dynamics across a tissue. Such interactions must be dynamic and strong in order to resist and respond to stress. Especially during development, as cells and tissues undergo dramatic changes in morphology, cellular adhesion must persist to maintain the integrity of the tissue. Circumscribed along the apical-lateral region of epithelia are two distinct intercellular junctions, together referred to as the apical junctional complex (AJC). The AJC consists of lateral adherens junctions (AJ) which mediate cell-cell adhesion and apical tight junctions (TJ) which regulate the movement of molecules and cells through the epithelial monolayer. Basal adhesion is mediated by focal adhesions (FA) which connect the cell to the ECM.

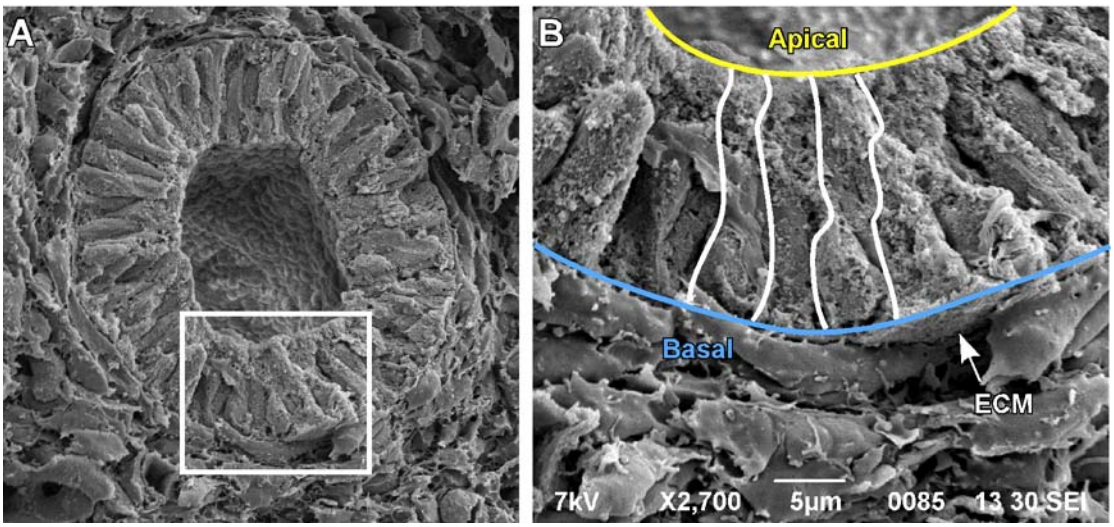

Figure 3: Basic Epithelial Organization

(A-B) Lungs from e11.5 mice were dissected and processed for SEM as described in section 5.17. The region of magnification in A is marked with a box and presented in B. The ECM can be seen as a meshwork of fibers which underlie the pulmonary epithelia.

1.2.1 Tight Junctions

The apical most adhesion structures within epithelia are TJs which are composed of the transmembrane proteins occludin [79], claudin [80], and junctional adhesion molecule (JAM) [81]. Tight junctions also contain many PSD/SAP90, Discs large, ZO-1 (PDZ) domain proteins which connect transmembrane proteins and the actin cytoskeleton. PDZ domains are characteristic of scaffolding proteins and facilitate protein-protein interactions [82]. Though this 100 amino acid domain occurs 785 times in 436 human proteins and shares structural similarity, PDZ domains greatly differ in their binding partners [83, 84].

Several PDZ-containing complexes are required for TJ assembly and apical-basal polarity. For example, apical localization of the PAR-3/aPKC/PAR-6 complex is mediated by interaction of PAR-3 and JAM-1 [85, 86] and is required for later stages of TJ assembly [87].

Another polarity complex, Crumbs/PALS1/PATJ localizes to TJs through an interaction between PALS and PAR-6 [88]. Localization of this complex to the TJ is further enhanced by an interaction between PATJ and claudin-1 [89]. Expression of a dominant negative (D/N) PATJ disrupts apical localization of the PAR-3/aPKC/PAR-6 and the Crumbs/PALS1/PATJ complexes implicating it in maintaining polarity and TJs [88].

Additional PDZ proteins important for TJ structure are ZO-1, ZO-2, and ZO-3 [90-92]. The ZO proteins are cytoplasmic proteins which can directly interact with each other, claudin, and occludin [93-95]. Because ZO proteins also interact with actin related proteins such as α-catenin, AF-6/afadin, and vinculin, it is thought that ZO proteins form scaffolding complexes which maintain connections between TJs and the actin cytoskeleton [95-97]. Additionally, the ZO proteins are essential for TJ formation as depletion of ZO-1 and ZO-2 in a model epithelial cell line completely abolishes TJ assembly [98]. However, disruption of ZO-1 or ZO-2 in mice leads to embryonic lethality due to failure in yolk sac angiogenesis or gastrulation, respectively [99, 100] suggesting these proteins may not function redundantly in every tissue. ZO-3 mutants have no discernable phenotype [99].

In addition to proteins which serve as links between integral TJ proteins and the actin cytoskeleton, a variety of non-PDZ, cytosolic, and nuclear proteins have been identified as TJ associated proteins which coordinate diverse functions such as paracellular permeability, proliferation, and tumor suppression (reviewed in [101]).

1.2.2 Adherens Junctions

AJs were first identified through electron microscopy as plasma membrane "organelles" found at cell-cell contacts [102]. The primary adhesion molecule found in AJs are the classical cadherins,

such as E-cadherin, which are transmembrane proteins that engage in homophilic interactions [103]. Classic cadherins contain a highly conserved cytoplasmic tail which interacts with p120catenin and β-catenin. β-catenin binds to α-catenin, an F-actin binding protein, thus connecting the cadherin-catenin complex to the actin cytoskeleton. Although α-catenin can directly bind to F-actin, it is also known to interact with several other actin binding proteins such as vinculin, formin, and ZO-1 [104, 105]. In *Drosophila* p120catenin and β-catenin may serve little regulatory function at AJs and only act to connect cadherin to α-catenin, because a cadherin-α-catenin fusion protein fully compensates for the loss of DE-cadherin or the β-catenin homologue, Armadillo [106]. However, in mammalian cells, β-catenin and p120catenin may regulate AJ stability, as loss of either protein increases cadherin turnover and endocytosis [107-109]. Additionally p120 catenin and β-catenin interact with kinesin and dynein respectively and may link cadherins to microtubules, facilitating the directed transport of AJ proteins [110, 111]. A second class of transmembrane proteins, nectins, also exists within AJs. Nectins bind to AF6/afadin which interacts with F-actin. Because afadin and α-catenin can physically interact, it is thought that cadherins and nectins perform similar function at AJs [112, 113].

Maintaining a connection between AJs and the actin cytoskeleton is critical to tissue morphogenesis. During *Drosophila* gastrulation, the ventral epithelium apically constricts to promote invagination in a process dependent upon apical actomyosin. Deletion of *canoe*, the Afadin homologue, leads to morphogenesis failure due to a disconnection of the cytoskeleton and AJs. *canoe* mutants show a striking accumulation of actin and MyosinII in the center of the cell as the contractile arrays separate from the plasma membrane and accumulate in the middle [114]. This study highlights the necessity of AJs during epithelial morphogenesis.

1.2.3 Focal Adhesions

FAs are vital protein complexes which link the cell to the extracellular matrix [115]. Integrins are the primary adhesive protein of FAs and consist of non-covalent heterodimers of α and β subunits. As transmembrane proteins, integrins have a large extracellular domain and a short cytoplasmic tail. As the extracellular domain binds to specific ECM proteins, the tail changes conformation and facilitates the accumulation of a large protein complex with estimates of as many as 156 proteins with 690 interactions [116]. Similar to AJs, a network of actin binding proteins connects integrins to the cytoskeleton. The best characterized are talin which initially binds and activates the cytoplasmic tail of integrins and vinculin which recruits another actin binding protein, α-actinin [117, 118]. FAs also contain many scaffolding proteins such as paxillin and signaling proteins such as focal adhesion kinase (FAK) [119, 120]. Effectors of FAK include GEFs and GAPs which control activation of Rho-family GTPases and subsequently regulate contractility and F-actin organization. An overview model of epithelial adhesion structures is presented in Figure 4.

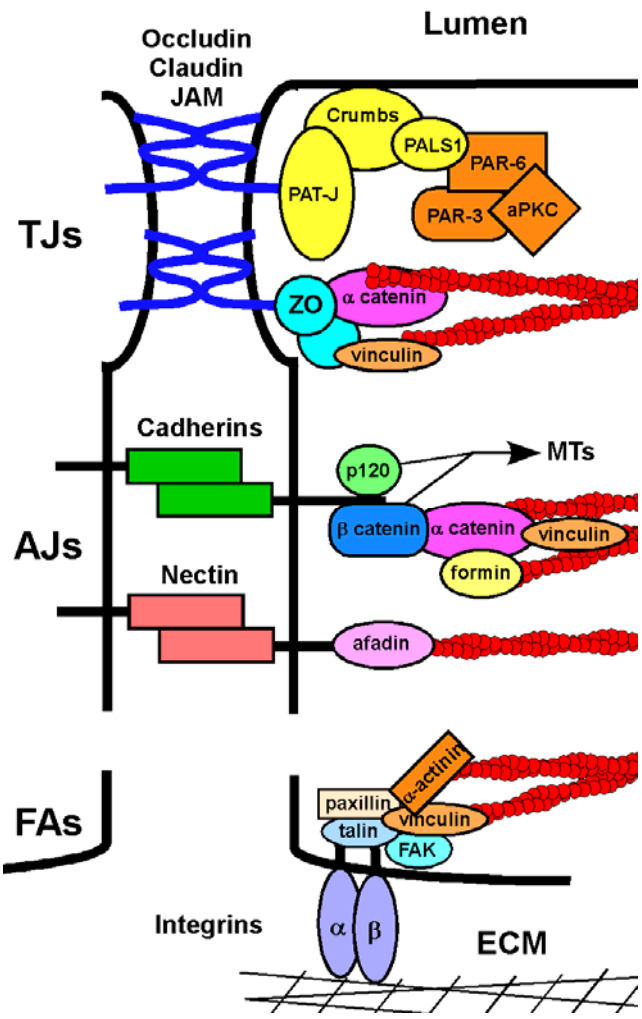

Figure 4: Model of Epithelial Adhesion Structures

The tight junction (TJ) is the apical-most junction in an epithelial cell whose position is defined by the polarity complexes PAT-J/Crumbs/PALS1 and PAR-3/aPKC/PAR-6. Through interactions with the transmembrane proteins occludin, claudin, and JAM, ZO proteins recruit the actin binding proteins α catenin and vinculin to maintain a connection with the cytoskeleton. Adherens junctions (AJs) along the lateral side involve cadherins which interact with p120 and β-catenin. β-catenin can recruit a number of actin binding proteins to connect AJs with the actin cytoskeleton. In addition, AJs serve as points of connection for MTs, since p120 and β-catenin can also interact with MT binding proteins. Along the basal surface, focal adhesions (FAs) connect the epithelial cell to the underlying ECM. Again through the recruitment of actin binding proteins, integrins maintain a connection with the cytoskeleton. Additionally, FAs recuit signaling molecules like FAK to induce further changes in the cytoskeleton.

1.3 CONTRACTILE ACTOMYOSIN AND MORPHOGENESIS

As observed by D'Arcy Wentworth in *On Growth and Form* in 1917,

> "An organism is so complex a thing, and growth so complex a phenomenon, that for growth to be so uniform and constant in all the parts as to keep the whole shape unchanged would indeed be an unlikely and an unusual circumstance. Rates vary, proportions change, and the whole configuration alters accordingly." [121]

Although early descriptions of developmental biology focused on the variety of gross morphological changes that shape the embryo, modern descriptions have focused on spatial and temporal gene expression which patterns the embryo. We now understand that it is a combination of these two ideas which lends a more precise understanding of cell and developmental biology where regulated gene expression influences cellular mechanics to shape cells, tissues, and organs. Some examples of the molecular regulation of actomyosin dynamics which may influence cell behavior during development are discussed below.

1.3.1 Actomyosin-dependent mechanisms in cell biology.

1.3.1.1 Stress fiber formation

Early studies described long, straight bundles of microfilaments which terminated in dense plaques at the base of the cell. Because these microfilaments were thought to arise due to tension on the cytoplasm, they were termed stress fibers [122]. Stress fibers are bundles of actin filaments held together by α-actinin, marked with intermittent MyosinII. The insertion of MyosinII between anti-parallel actin filaments allows contractility and shortening of the bundle. As stated previously, RhoA seems to be a major regulator of stress fiber formation which

functions through Rock to activate MyosinII and mDia. Inhibition of Rock signaling through overexpression of RhoE, Gem, Rad, or DN-RhoA blocks stress fiber formation [67, 68].

The activity of both Rock and mDia is required for stress fiber formation [49]. Alone, Rock activation induces disorganized stress fibers in the center of the cell, whereas mDia overexpression forms parallel actin filaments that are loosely bundled [49]. It is the combination of the two which leads to the organized, contractile bundles in stress fibers. While Rock is responsible for inducing MyosinII filament assembly, mDia localizes to focal adhesions where it is thought to nucleate actin filaments [123].

Stress fibers are critical for the mechanics of many cells. As an example, endothelial cells of the vasculature experience continual mechanical stress from hydrostatic pressure, cyclic stretch, and fluid shear. As blood flows across endothelial cells, actin stress fibers become enriched and aligned along the direction of flow [124]. Stress fiber formation from fluid shear has been connected to RhoA and Rock activation [125]. It is thought that within blood vessels, stress fibers help endothelial cells retain a flat and smooth luminal surface.

1.3.1.2 Cell Migration

As cells move, they must cycle between protrusive and contractile motions. In a polarized cell, a protrusion, such as a lamellipodium, extends from the cell and attaches to the substrate. Adhesion to the substrate then facilitates the contraction of the tail, allowing the cell to move in one direction.

The lamellipodium is formed by Arp2/3 and branched actin networks, and as such, MyosinII is not required for its formation [126]. Proximal to the lamellipodium is a structure known as the lamellum which is composed of thick actin bundles. Both regions undergo retrograde actin flow with lamellipodial actin coalescing into the bundles of the lamellum.

Following knockdown of MyosinII or inhibition by blebbistatin, the lamellum collapses and the net rate of protrusion is delayed [126]. It appears that MyosinII is responsible for slowing retrograde actin flow in the lamellum which counters actin polymerization at the lamellipodium. Adhesion to the substratum balances these two opposing forces and creates traction points to oppose retrograde actin flow, resulting in net protrusive activity [127]. Thus, focal adhesive contacts create a 'molecular clutch' which is essential for migration. Just as the loss of contractility can abolish migration, migration is also lost following excessive MyosinII activation [128]. There likely exists a precise amount of MyosinII activity which promotes cellular migration, as it has long been known that enhancing contractility also reduces migration.

Because the molecular clutch or FA is required for some forms of migration, its formation is essential for function. The lamellipodium contains nascent focal contacts to the substratum which begin to mature at the transition zone between the lamellipodium and the lamella. MyosinII is required for the maturation of these focal adhesions [129]. There are two possible roles for MyosinII in FA maturation. First, MyosinII bundles actin filaments. As a consequence of actin bundling, adhesive proteins at the ends of the fibers coalesce and can form larger complexes [130]. Another non-exclusive possibility is that MyosinII-generated tension induces conformational changes in FA components, exposing additional binding sites which lead to maturation. To support this hypothesis, it has been shown that when talin is mechanically stretched, it binds to vinculin which would serve to bind additional F-actin fibers and other FA proteins [131]. In this manner, the stiffness of the underlying substrate can affect the stiffness of the cytoskeleton. With decreased stiffness of the substrate, the clutch cannot generate sufficient force to remodel the actin cytoskeleton. For example, cells grown on softer substrates contain

smaller, dynamic adhesions compared to cells grown on stiffer substrates which contain large, stable adhesions [132].

Within motile fibroblasts, MyosinII isoforms have different roles. MyosinIIB localizes to non-dynamic actomyosin structures in the center and rear of the cell. MyosinIIA is dynamic and localizes to protrusions at the cell front [133]. Additionally, MyosinIIA activity is essential for tail retraction. General inhibition of MyosinII causes cells to elongate as they fail to retract the tail [134]. Differential localization of MyosinII isoforms establishes a front and rear to the cell and likely contributes to different actomyosin dynamics in these regions. In addition, the control of actomyosin in migrating cells creates stable FAs and highly bundled actin. These structures prevent protrusion and specify the sides of the cell [133].

1.3.1.3 Cell adhesion

The regulation of cell adhesion is critical in mediating the transition between mesenchymal and epithelial behavior. In addition, AJs must be dynamic in order to allow cell rearrangement and morphogenesis in the context of a tissue while still maintaining adhesion. Initial E-cadherin based cell-cell contacts are dependent upon local activation of Rac which drives actin-based filopodial protrusions enriched with E-cadherin [135, 136]. These filopodial protrusions extend into neighboring cells and establish nascent cell-cell contacts which recruit α-catenin, vinculin, Mena, VASP, and formin-1 [13, 136]. Additionally, Rac can activate phosphatidylinositol 3-kinase leading to Cdc42 and Arp2/3 activation [136]. The combined effects of activating actin binding proteins at early sites of cell adhesion are thought to expand the area of contact between cells and extend E-cadherin interactions.

The activities of Rac and Cdc42 can also activate Par6 and aPKC, leading to AJ maturation and apical-basal polarity [137]. Additionally, RhoA maintains E-cadherin based

cellular adhesion through control of Dia1 and Rock [138]. Actomyosin networks are critical to maintaining cellular adhesion as knock out of MyosinII leads to loss of E-cadherin at cell-cell junctions [139]. Inhibition of Rock phenocopies inhibition of MyosinII [140]. These results suggest that a balance of actomyosin networks and Rho family GTPases is required to promote and maintain adhesion. Recent evidence has suggested a differential role for MyosinII isoforms in maintaining AJs. Within MCF-7 cells the loss of MyosinIIA leads to the discontinuity of E-cadherin within the AJC, whereas the loss of MyosinIIB depletes the apical F-actin belt. In these cell lines, MyosinIIA is under the control of Rock, but MyosinIIB is regulated by Rap1 [141].

A mechanistic role for MyosinII in maintenance of AJs has recently been described. AJs are important for maintaining the integrity of cells within a tissue, but they can also be used to sense and respond to mechanical cues. Recent studies demonstrate that α-catenin binds to vinculin in a force dependent manner. Inhibition of MyosinII leads to the loss of vinculin at AJs, whereas localized MyosinII activation at AJs recruits vinculin to the areas subjected to force [142]. α-catenin contains a region within the protein that inhibits the interaction with vinculin [143]. Because the actin binding region of α-catenin, actin filaments, cadherin interactions, and MyosinII contractility are all required for α-catenin and vinculin binding, a model has been proposed where force exerted upon AJs causes stretching of α-catenin to expose the site for vinculin binding thereby promoting additional F-actin recruitment [143]. Such a mechanism ensures the localization of sufficient amounts of F-actin to maintain adhesion and balance within a cell population and provides insight into how cells can rapidly remodel AJs during development.

1.3.1.4 Wound closure

The process of wound closure involves the coordination of adhesion and migration as cells must maintain epithelial integrity while migrating into the open space. As thick actomyosin cables are present at the leading edge, it has long been hypothesized that a purse-string mechanism draws cells together as they migrate into the wound [144, 145]. Further analysis of actomyosin dynamics during wound closure of MDCK cells has revealed two roles for MyosinII. MyosinII is recruited to two distinct locations immediately after wounding: in a ring at the TJ and near lamellipodia at the base of the cell [146]. Inhibition of MyosinII blocks contraction of the apical ring and switches basal motility from a lamellipodia-based mechanism to a filopodia-based mechanism [146]. Rock predominately localizes to the TJ but is the primary effector of MyosinII activity at both locations [146]. ZO-1 co-localizes with apical MyosinII in this system and has been proposed to facilitate the connection between actomyosin and TJs [146]. Once the apical purse string has constricted, ZO-1 localizes to the leading edge and is required for localization of aPKC-Par3 and PATJ, likely mediating directed cell migration through activation of phosphatidylinositol 3 kinase [145, 147].

1.3.2 Actomyosin-dependent mechanisms in development.

Though vertebrate species show expansive diversity in adult form and function, there are striking gross morphological similarities between the early embryonic stages of vertebrates. As infamously drawn by Ernst Haeckel in his publication *Anthropogenie* in 1874, the early developmental stages of fish, salamanders, pigs, dogs, and humans are nearly indistinguishable. As all vertebrates must undergo similar developmental processes such as proliferation, differentiation, gastulation, elongation, neural tube closure, and organogenesis, it is through

conserved mechanisms of cellular morphogenesis that similar structures arise. A few roles for contractility during development are discussed below.

1.3.2.1 Germline ablation of MyosinII isoforms.

Given the significance of MyosinII in mediating cellular migration and adhesion, it is likely an important protein for normal development. The *in vivo* implications of MyosinII isoforms have been addressed through germline ablation and have revealed different phenotypes for each isoforms. MyosinIIA deficiency is lethal by e6.5 due to failure in cell adhesion and visceral endoderm formation [139]. MyosinIIB deficiency is lethal by e.14.5 due to cardiac and brain defects [148]. These differences suggest non-redundant functions for MyosinII isoforms, and in support of this notion, knock-in of *MHC IIB* into the *MHC IIA* locus in MyosinIIA deficient mice only rescues brain but not cardiac defects [149]. Similarly knock-in of *MHC IIA* into the *MHC IIB* locus in MyosinIIB deficient mice rescues cell adhesion defects but the embryos still die around e11.5 from angiogenesis and migration defects [150]. These results suggest that Myosin isoforms have overlapping but non-redundant functions. It is hypothesized that actomyosin-dependent functions during development which require only the actin cross-linking ability of MyosinII are not isoform specific, however those functions which require the specific kinetic properties of a particular MyosinII isoform cannot be rescued. While many studies to date utilize general MyosinII inhibition in the analysis of contractility, in the future it will be important to consider how different isoforms impart specific functions and how they are differentially regulated.

1.3.2.2 Embryo stiffness

During development the notochord is a well-known organizing center which produces morphogens to influence the differentiation of surrounding tissues. Though disruption of the notochord may impart loss of developmental signaling to surrounding tissues, some evidence suggests that mechanical cues to surrounding tissues are lost in several model systems including frog [151], newt [152], and zebrafish [153]. In a process known as convergent extension which is dependent upon RhoA and actomyosin, cells converge upon a midline within the plane of the cells in order to elongate the tissue [154]. Actomyosin-induced changes in shape influence the elongation of the overlying neural plate. The notochord also contains inherent tissue stiffness which prevents buckling during straightening of the *Xenopus* embryo [155]. It has also been demonstrated that the notochord, paraxial mesoderm, and endoderm exhibit actomyosin-dependent differences in stiffness compared to one another [156]. It is possible then that differences in adhesive strength and stiffness may function in boundary formation between tissues and may provide mechanical support between tissues during morphogenesis, however such a role has yet to be examined.

Stiffness of the underlying substrate has also been shown to be an important determinant of cell behavior and not just reactionary to cell signals. When the stiffness of the substratum is reduced, cell spreading, stress fiber formation, and FA maturation are reduced [157]. Mesenchymal stem cell (MSC) differentiation is dependent upon substrate stiffness. When MSCs are cultured on hard substrates which mimic the physiological stiffness of bone, cells differentiate along an osteogenic lineage. When MSCs are cultured upon soft substrates which mimic neural tissue, cells express neural markers [158]. The ability of MSCs to respond to their environment is dependent upon contractile actomyosin, as inhibition of MyosinII prevents

differentiation into all tested lineages [158]. It is likely that the ability of these cells to change shape underlies their ability to differentiate. In support of this notion, RhoA activation and Rock activity is essential for guiding MSCs along an osteogenic lineage. Upon ablation or inhibition of Rho or Rock activity, cells divert to an adipogenic lineage [159]. Rock-dependent cell shape changes during MSC differentiation mediate responsiveness to BMP and SMAD signaling which guides differentiation, however a molecular connection between the two has yet to be determined [160].

1.3.2.3 Apical constriction

While tissue stiffness may be associated with cortical contractility of the entire cortex, specific localization of actomyosin can mediate more specific morphogenetic changes. For example, apical constriction of the mesoderm during *Drosophila* gastrulation leads to invagination and generation of the primary germ layers. This process is dependent upon the transcription factors Snail and Twist which promote activation of RHOGEF2. RHOGEF2 then activates Rho and promotes the formation of apical contractile actomyosin through the stimulation of Rock [161, 162]. Constriction in this system is not uniform, but follows a cyclical pattern of brief contractility which correlates with MyosinII accumulation [163]. If such bursts were allowed to relax and revert to the original position, then a net change in the apical surface would not arise. Thus it is hypothesized and has been observed that a ratchet-like mechanism exists to maintain tension between contractions. The molecular identity of this system is unknown, but it also relies upon the expression of *twist* [163]. Pulsed apical contractility also plays a role in dorsal amnioserosa cells, essentially pulling the overlying epidermis towards the center [164]. Actomyosin also regulates apoptosis of amnioserosa cells and the leading edge

purse string in the epidermis. Together with apical constriction of the amnioserosa, these different functions of actomyosin are required for dorsal closure [164-166].

Twist transcription itself is likely mediated by changes to the cytoskeleton as physical compression of the stomodeal cells during germband extension increases *twist* expression, inducing apical constriction of the mesoderm which will pattern the gut [167]. Experimental manipulation of cellular tension through uniaxial stretching of the embryo also upregulates *twist* [168]. It is intriguing that a regulator of one contraction event can be regulated by another. These results likely reflect the dynamic nature of contractile actomyosin networks during mechanotransduction, demonstrating how actomyosin networks can remodel as needed in response to molecular signals or force.

1.3.2.4 Angiogenesis

As the embryo grows in size and cell number, tissues must be properly vascularized to supply oxygen as needed. The sprouting of new blood vessels from existing ones is not only important for development, but also for tissue engineering and vascularization of tumors. During angiogenesis, cells must sprout from formerly quiescent vessels, maintaining adhesion while migrating towards a stimulus. During this process, the cell at the forefront of migration is termed a "tip cell" while those that follow are termed "stalk cells." A primary activator of tip cell specification is VEGF, which upon binding to VEGFR2, stimulates a signal transduction cascade which activates Delta-like 4 (Dll4) [169]. Through lateral inhibition, Dll4 activates Notch signaling in neighboring cells which in turn, downregulates VEGFR2 expression, ensuring that only tip cells respond to VEGF [170].

Early studies of capillary morphogenesis identified filopodial extensions within the tip cells which guide migration [171]. Increasing evidence suggests that VEGF activates Rho

GTPases to influence cell migration within the tip cell. Both Cdc42 and Rac1 are activated by VEGF which induces filopodia and lamellipodia respectively [172, 173]. VEGF also activates RhoA in endothelial cells. Expression of D/N RhoA or inhibition of Rock abolishes VEGF-induced changes in the actin cytoskeleton and prevents angiogenesis [174, 175]. Indeed, stimulation of individual endothelial cells with VEGF increases their contractility in a Rock-dependent manner [176]. However conflicting reports on the role of Rock in angiogenesis exist such that Rock inhibition or knockdown in a retinal neovascularization model actually enhances angiogenesis [177]. Consistent with these results, MyosinII can be visualized at the endothelial cell cortex and is lost prior to sprouting activity [178]. Localized inhibition of Rock abolishes cortical MyosinII localization and promotes sprouting, suggesting that Rock induced contractility negatively regulates branching [178]. It was recently demonstrated that an ideal level of cellular adhesion to the substrate promotes angiogenesis; too few or too many adhesions can be detrimental to angiogenesis [179]. While it is difficult to compare the role of Rock in different endothelial cell lines and angiogenesis models, it may be that differential responses to Rock inhibition create differences in cellular adhesion thus altering the angiogenic response. Determining how angiogenic signals induce sprouting in relation to contractile actomyosin *in vivo* will be an important task in understanding developmental angiogenesis and the development of anti-tumor drugs.

1.4 THE SHROOM FAMILY OF PROTEINS

The first Shroom protein was serendipitously identified in 1992 while trying to generate a cDNA against the α subunit of a Na^+, K^+ –ATPase in *Bufo marinus*. Named after the characterization

of its localization, Apical Protein from *Xenopus leavis* (APX) was identified as a protein essential for amiloride-sensitive sodium channel activity but was not a physical pore component [180]. Subsequent experiments confirmed a role for Apx in epithelial sodium channel activity (ENaC) [181] and suggested apical localization was due to the formation of a macromolecular complex containing α-spectrin [182]. Additional studies found related proteins in mammals, named APX-like (APXL) [183], Shroom [184], and KIAA1202 [185]. Identified as distinct family members with conserved domains rather than as homologues to the *Xenopus* APX, the proteins were renamed in the chronological order in which they were identified: Shroom1 (Apx), Shroom2 (Apxl), Shroom3 (Shroom), and Shroom4 (KIAA1202) [186].

The Shroom family of proteins is characterized by possession of several conserved domains: an N-terminal PDZ domain, a central Shroom Domain 1 (SD1), and a C-terminal Shroom Domain 2 (SD2) [187, 188]. At present, the function of the PDZ domain remains unclear, as it is not required for Shroom3 function nor for Shroom4 localization and only mildly affects Shroom2 localization [188, 189]. The SD1 lies near an actin binding region, yet while not all Shroom proteins contain an SD1, they all bind F-actin. [188, 190, 191]. The only domain common to all family members is the SD2 (Figure 5 and Table 1). The SD2 is required for the formation of Shroom-dependent actomyosin networks and has recently been shown to physically bind to Rock 1/2 [189, 192]. Thus far, only Shroom3 and dShroom have been shown to physically interact with Rock, however due to the high degree of conservation amongst the SD2 of all family members and the ability of all members to induce morphological changes, Rock binding is likely a shared function of the Shroom family.

Table 1: Overview of Shroom family of proteins

Previous name	Current name	Domains Present	Actin Binding?
APX	Shroom1	SD1 SD2	Y
APXL	Shroom2	PDZ SD1 SD2	Y
Shroom L	Shroom3	PDZ SD1 SD2	Y
KIAA1202	Shroom4	PDZ SD2	Y
CG8603	dShroom A dShroom B	SD2 SD2	Y N

1.4.1 Shroom3 and implications for conserved Shroom family functions.

The ability of Shroom family members to induce cell shape change in a variety of animal model systems and tissues will be described in the following sections. The first example of a morphogenetic role for Shroom proteins was identified for Shroom3 in which mutant mice were generated through a series of gene trap mutagenesis experiments in embryonic stem (ES) cells [184]. Shroom3 mutant embryos show open neural tubes at embryonic day (e) 9.5 and extensive neural tube defects by e14.5 including exencephaly (100%), facial clefting (87%), spina bifida (23%), and ventral closure defects which result in organ herniation (12%) [184]. Though ventral closure defects occur infrequently, 100% of Shroom3 mutant ventral neural tubes are malformed with a collapsed lumen, apparent loss of rigidity, and aberrant roof plate morphogenesis [184].

Within murine neural epithelia and MDCK cells, Shroom3 localizes to tight junctions and induces apical constriction and apical-basal epithelial lengthening [187, 189, 193]. It is believed that Shroom3-induced morphogenesis is sufficient to induce wedge-shaped cells which promote neural plate bending and subsequent NT closure [187]. The necessity of Shroom3 for apical constriction and NT closure is consistent in other model systems as depletion of Shroom3 in

Xenopus and chick embryos also leads to aberrant epithelial morphology and NT closure defects [187, 192].

In addition to expression within the neural epithelium, Shroom3 is expressed within most developing epithelial tissues particularly the optic pit, hindgut, foregut, lungs, and somites [184]. Shroom3 likely contributes to morphogenesis of these tissues, as Shroom3 mutant embryos also display defects in lens placode invagination and foregut looping, each of which are attributed to the loss of apical constriction and epithelial lengthening [194-196].

Shroom-induced epithelial lengthening is thought to occur through γ tubulin recruitment to the apical surface, orienting MTs along the apico-basal axis [194, 197]. As Shroom proteins and apical γ tubulin have not been shown to interact and do not precisely co-localize, apical γ tubulin localization could be a secondary effect. A direct role for Shroom3 and apical constriction is better understood. The ability of Shroom3 to induce apical constriction is dependent upon its ability to bind to actin and establish a contractile MyosinII-dependent network [189, 195]. Interestingly, Shroom3 is the only family member which induces apical constriction of MDCK cells when over expressed, yet all Shroom proteins retain the ability to do so when properly targeted. When a chimeric protein is generated in which the SD2 of Shroom3 is replaced with another family member's SD2, apical constriction is rescued [188]. Because apical constriction is dependent upon the SD2 through physical recruitment of Rock and establishment of actomyosin networks, this suggests that all Shroom proteins have the ability to elicit morphogenetic changes through actomyosin recruitment.

Why then can not all Shroom proteins elicit apical constriction in MDCK cells? There are seemingly two possibilities. First, while all Shroom proteins can bind to actin, they may do so in different ways. This is best demonstrated in Rat1 fibroblasts as Shroom2, Shroom3, and

Shroom4 all display differential actin-based localization. In Rat1 cells, Shroom3 can bind and bundle F-actin stress fibers; Shroom2 cannot bundle actin and preferentially localizes to cortical actin; and Shroom4 uniquely recruits actin into unknown structures which perpendicularly span stress fibers [188, 190]. Specifically how these proteins interact with different populations of F-actin may influence apical constriction. It might also be possible that Shroom proteins bind actin with different affinities. Second, unique protein binding partners have been identified for several Shroom proteins (Figure 5) which will be elaborated upon in the following sections. In regards to Shroom3, Shroom3 is the only family member to possess an EVH1 domain. Deletion of this region or transfection of the Mena EVH1 domain which acts as a D/N blocks apical constriction [195]. Because the EVH1 domain of Shroom3 is required for apical constriction, it is likely that other interacting proteins, the identity of which remains to be determined, are necessary for morphogenesis. Plenty of SH3s (POSH), a multi-domain scaffolding protein which assembles an active JNK / MAPK module also interacts with Shroom3 [198]. Such an interaction is required for negative regulation of axon length [199]. From these observations it is clear that differential actin binding and protein interactions are important for mediating apical constriction. Specifically how unique proteins influence localization and whether or not they influence actin binding remain to be determined.

Ultimately, precise sub-cellular localization of each Shroom protein is also important for its function. In support of this notion, the SD2 of any Shroom protein can be targeted to the apical membrane with an Endolyn tag. Within MDCK cells, Endolyn sorts to the apical plasma membrane and can be over expressed without affecting morphology [200]. Apical localization of the SD2 with this tag induces apical constriction [189]. Without a localization signal, the SD2 is cytoplasmic and fails to induce morphogenetic change. As the SD2 is not required for

localization of Shroom proteins, Shroom proteins likely localize through unique protein interactions outside of the SD2. Determining additional protein interactions which may mediate localization of the Shroom proteins will be useful to elucidate specific Shroom protein functions.

While Shroom3 influences Rock localization, precisely how Rock is activated after recruitment remains unclear. Overexpression of D/N RhoA has no effect on alleviating the high frequency of apically constricted MDCK cells after Shroom3 expression [187]. While D/N Rap1 can abolish apical constriction, Rap1 has not been shown to regulate Rock activity [187]. Rap1 was recently shown to mediate the connection of the actin cytoskeleton with AJs in *Drosophila* through regulation of the Afadin homologue Canoe. Loss of either Rap1 or Canoe leads to a striking accumulation of actomyosin balls which have detached from AJs, preventing morphological change [114]. These results may explain the effect of D/N Rap1 on eliminating Shroom3-dependent apical constriction as the cytoskeleton may have lost association with AJs. Alternatively, MyosinIIB is regulated by Rap1 in MCF-7 cells [141], so it might be that Shroom3 functions primarily through MyosinIIB. Because D/N RhoA fails to abolish apical constriction, it remains a possibility that physical interaction of Rock with Shroom proteins is sufficient for activation. Interestingly, the region with which Rock binds to Shroom lies just N-terminal to the RhoA binding site [192]. Because intermolecular inhibition of Rock is relieved by an interaction with RhoA, it is an appealing idea that Shroom activates Rock in a similar manner. There is no current evidence for post-translational modification of Shroom proteins.

Transcriptional regulation can influence cell morphogenesis by controlling the presence or absence of key regulators within a cell population. Two examples of transcriptional regulation have been identified for Shroom3. First, Pax6 is a transcription factor present in the lens placode which is essential for *shroom3* expression [195]. A direct or indirect effect for Pax6

remains to be determined. Second, Pitx1 is required for s*hroom3* expression within the *Xenopus* gut. The promoter of *shroom3* contains several Pitx1 consensus sequences. When this promoter is attached to a luciferase reporter, Pitx1 increases expression 18-fold, implicating a direct role for Pitx1 and *shroom3* expression in the gut [194].

1.4.2 Shroom2

Shroom2 was first identified by sequencing genes within a large X-chromosomal deletion thought to underlie Ocular albinism type 1 (OA1) [183]. OA1 is an X-linked disorder which causes impairment of visual acuity, involuntary eye movement, misalignment of the eye, and retinal hypopigmentation. Shroom2 lies upstream of the *OA1* gene, yet its role in OA1 remains unclear [183].

The best support for the involvement of Shroom2 in ocular albinism comes from studies in *Xenopus*. When expressed within naïve epithelial blastomeres, Shroom2 ectopically recruits pigment to the apical surface [197]. Shroom3 exhibits similar activity, yet consistent with previous studies in MDCK cells, Shroom3 induces apical constriction while Shroom2 does not. Shroom2 is expressed within the developing eye and when knocked down by morpholino, loss of Shroom2 leads to hypopigmentation and disruption of Retinal Pigment Epithelium (RPE) morphology [197]. The exact role for Shroom2 in this process is unclear, but morphological change brought about by Shroom2 activity may help establish apical accumulation of γ tubulin and apical / basal oriented MTs for pigment transport [197]. A second hypopigmentation disorder, ocular albinism with sensorineural deafness (OASD), also maps to the same region of the X-chromosome as OA1 [201, 202]. Because Shroom2 is expressed within epithelial cells of the retina and the inner ear in mice [188, 203], Shroom2 is a likely candidate for mediating

proper development of such organs, disruption of which may lead to sensory diseases such as OA1 and OASD. There is currently no *Shroom2* mutant mouse to address such questions. As Shroom2 is also expressed within the embryonic vasculature, gut, neural tube, and kidney [188] and a variety of adult epithelial tissues [203], additional studies are necessary to understand Shroom2 function in these tissues.

MyosinVIIa is an unconventional myosin which is abundant at cell-cell junctions and binds to vezatin, a transmembrane protein incorporated into the cadherin-catenin complex [204]. Mutations in MyosinVIIa lead to Usher syndrome type I, identified by congenital deafness, vestibular dysfunction, and progressive retinitis pigmentosa [205, 206]. Through a yeast two hybrid screen, Shroom2 was identified as an interacting protein, binding to MyoVIIa through a region N-terminal to the SD1 (a.a. 350-721) [203]. The smallest MyoVIIa construct which binds to Shroom2 contains the MyTh4 and FERM domain. However, interaction with MyoVIIa does not likely mediate Shroom2 localization, as Shroom2 still localizes to TJs of hair cells in MyoVIIa defective *shaker-1* mice [203, 207]. Both MyoVIIa and Shroom2 are important for proper melanosome biogenesis and apical localization [197, 208]. In addition, Shroom2 and MyoVIIa are localized to inner hair cells of the ear [209, 210]. Therefore, it will be interesting to examine the relationship between the two for insight into the development of certain auditory-visual disorders.

Consistent with the observation that MyoVIIa is not sufficient for Shroom2 localization, the PDZ and Serine/Proline Rich region (SPR) (a.a. 1-513) of Shroom2 localize efficiently to TJs in MDCK cells [188]. In order to elucidate the molecular target of Shroom2 at the TJ, Etournay *et al.* performed a yeast two hybrid with Shroom2 PDZ/SPR bait against an inner ear cDNA library. They identified and confirmed ZO-1 as an interacting partner with an interaction

between the SH3/GuK domains of ZO-1 and the SPR region of Shroom2 [203]. Interestingly, the PDZ domain of Shroom2 is not essential for the interaction with ZO-1. However, the relationship between ZO-1 and Shroom2 in the context of a cell is not as straightforward. While TJ formation has been shown to precede Shroom2 recruitment, Shroom2 localizes to cortical actin and nascent adherens junctions devoid of ZO-1 [188, 203]. More importantly, in fibroblasts which express E-cadherin and form junctions which contain ZO-1 but lack true TJs, Shroom2 fails to localize [203]. These observations suggest that either a multi-step process or multi-protein complex at mature TJs is responsible for Shroom2 localization. Additionally, it may be that Shroom2 can interact with other ZO proteins, as murine ZO-1 and ZO-2 share 66% identity within the SH3/GuK region. ZO-1 and ZO-3 share 49% identity within the same region. The mechanism of Shroom2 localization is confounded further because the PDZ domain, a common domain of scaffolding proteins and TJ-associated proteins, is not required for Shroom3 localization and only mildly affects Shroom2 localization *in vitro* yet contains 64% identity between Shroom2 and Shroom3 [184]. As Shroom3 also localizes to TJs and shares loose similarity to Shroom2 within the SPR, Shroom3 may also interact with ZO-1.

1.4.3 Shroom4

Gross defects in morphogenesis can be severely detrimental, i.e. lethal, to an organism. More subtle defects in morphogenesis may allow survival yet severely disable the organism. As a consequence of subtle neural defects, mental retardation affects 1-3% of the population. Over 10% of these cases are due to mutations or gross chromosomal abnormalities within the X chromosome, termed X-Linked Mental Retardation (XLMR) [211]. A number of genes responsible for XLMR involve actin dynamics including several Rho effectors and actin binding

proteins [212]. Two XLMR patients have been identified with causative X chromosome breakpoints within Shroom4 [185]. As Shroom4 is an actin binding protein which can induce MyosinII dependent changes in the cytoskeleton, it is likely that defects in neural morphogenesis underlie the development of XLMR in Shroom4 defective patients [190]. As Shroom4 is expressed within the epithelium of many adult and embryonic structures, additional studies will be essential to elucidating Shroom4 interactions and function [190].

1.4.4 Shroom1

hShroom1 was recently identified in a yeast two hybrid screen as an interacting protein with melanoma cell adhesion molecule (MCAM) [213]. Within which region of Shroom1 MCAM interacts remains unclear. hShroom1 contains the characteristic SD1 and SD2 of the Shroom family and may bind to F-actin, however the SD2 of *Xenopus* Shroom1 is more similar to hShroom2, 3, and 4, suggesting that hShroom1 is not the homologue of *Xenopus* Shroom1 [213]. hShroom1 is expressed in brain, heart, skeletal muscle, colon, small intestine, kidney, placenta, lung, and melanoma cells lines. In addition, it is strongly expressed in a variety of tumor tissues, yet its role in such cells has yet to be determined [213].

1.4.5 dShroom and evolutionary implications for the Shroom family

Based on homology to the SD2, the invertebrate *Drosophila* ortholog, dShroom, was identified [188]. While the SD2 is the only region of conservation between dShroom and vertebrate Shroom proteins, dShroom still retains the ability to bind to F-actin. Two isoforms, dShroomA and dShroomB are detectable with dShroomA as the most abundant isoform. dShroomA, the

longer isoform, localizes to AJs which are in a synonymous location as vertebrate TJs. dShroomB is shorter, lacks the actin binding motif, and localizes to the surface of epithelial cells. dShroomA and dShroomB both induce different actomyosin networks based on their localization. When overexpressed in the dorsal ectoderm, dShroomA induces robust apical constriction from AJs while dShroomB assembles a disorganized actin network at the apical surface. Consistent with vertebrate Shroom proteins, dShroom-induced actomyosin networks are dependent upon an interaction between the SD2 and dRok [191].

Despite a lack of sequence conservation in the N-terminus between dShroomA and vertebrate Shroom3, they share localization to a particular region which is conducive to apical constriction. As dShroom does not contain a PDZ, SD1, or any other region of similarity with vertebrate Shroom proteins outside of the SD2, it appears selective pressure was exerted upon Shroom3 to maintain the appropriate localization conducive for apical constriction [191]. It is then quite possible that other Shroom proteins were adapted for additional Rock-dependent functions as more complex mechanisms evolved.

The SD2 appeared very early in the animal lineage as a distantly related open reading frame is identified in the cnidarian *Hydra magnipapillata* and potential orthologs are found in the ascidian *Ciona intestinalis* and the echinoderm *Strongylocentrotus purpuatus* [188]. Unlike dShroom, the predicted proteins from the sea squirt and sea urchin contain a PDZ domain. As yeast and *Arabidopsis thaliana* genomes do not contain any Shroom-like proteins, it is likely that Shroom is an animal specific protein; however not all animals possess s*hroom* as the nematode worm *Caenorhabditis elegans* and the flatworm *Schmidtea mediterranea* contain no clear orthologs. Phylogenetic analysis of SD2 sequences in vertebrates suggests that a duplication of

the ancestral *Shroom* gene gave rise to two homologues, one that became *Shroom4* and one that duplicated again to form *Shroom2* and *Shroom3* [188].

1.4.6 Summary

The Shroom proteins are regulators of epithelial morphogenesis, best characterized by their ability to organize actomyosin networks. All Shroom proteins are characterized by their ability to bind F-actin and by high conservation of the C-terminal SD2 which binds to Rock. Establishment of actomyosin networks relies upon two modules within the Shroom proteins. First the protein must properly localize through actin binding and interaction with additional proteins. Second, from specific sub-cellular locations within the cell, Shroom recruits Rock through an interaction with the SD2, activating contractile actomyosin. Due to high conservation within the SD2 and the ability for all Shroom proteins to bind to actin and cause apical constriction when properly targeted, the establishment of actomyosin networks through recruitment of Rock is the key mechanism of Shroom function. Unique sequences within the N-terminus of the Shroom proteins convey unique protein interactions and differential protein localization. Thus each Shroom protein may act in similar yet unique ways. A summary of known domains and protein interactions is provided in Figure 5. While Shroom3 has been well characterized, the roles for other Shroom proteins in cell and developmental biology remain to be determined.

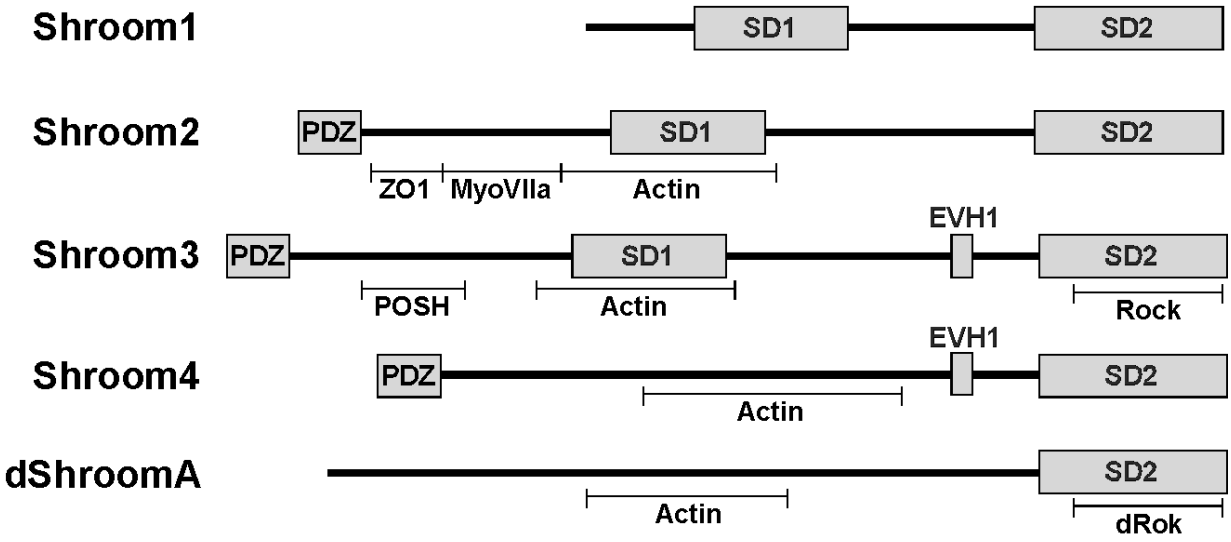

Figure 5: Shroom Family of Proteins Schematic

An overview of known protein domains (grey boxes) and known protein interactions (underlying lines).

1.5 DISSERTATION AIMS

To date only Shroom3 has been well defined as a regulator of epithelial morphogenesis during development. While Shroom2 binds to actin and mediates apical constriction of MDCK cells when properly targeted, no study has yet identified a role for Shroom2 in morphogenesis. Given that certain endothelial cells endogenously express Shroom2 which localizes to cortical actin, and cortical actomyosin networks are essential for angiogenesis, we thought endothelial morphogenesis provides an ideal model to address Shroom2 function. Thus, the first aim of this dissertation is to elucidate and characterize the function of Shroom2 in endothelial cells.

In *Xenopus* embryos, Shroom2 recruits γ tubulin to establish apically oriented microtubules for apical/basal elongation and directed transport of cargo [197]. While this phenomenon has not been observed in mammalian systems, I have observed Shroom2

localization at the centrosome, a γ tubulin rich structure important for ciliogenesis and mitotic spindle orientation during mitosis [214, 215]. It is currently unknown how Shroom2 plays a role in the regulation of the centrosome. Thus the second aim of this dissertation is to establish Shroom2 as a bona fide centrosomal protein, to determine how Shroom2 localizes to the centrosome, and to identify its role in centrosome function.

2.0 SHROOM2 REGULATES CONTRACTILITY TO CONTROL ENDOTHELIAL MORPHOGENESIS

The intrinsic contractile, migratory, and adhesive properties of endothelial cells are central determinants in the formation of vascular networks seen in vertebrate organisms. Because Shroom2 is expressed within the endothelium, is localized to cortical actin and cell-cell adhesions, and contains a conserved Rho kinase (Rock) binding domain, we hypothesized that Shroom2 may participate in the regulation of endothelial cell behavior during vascular morphogenesis. Consistent with this hypothesis, depletion of Shroom2 results in elevated branching and sprouting angiogenic behavior of endothelial cells. This is recapitulated in HUVECs and in a vasculogenesis assay where differentiated embryonic stem (ES) cells depleted for Shroom2 form a more highly branched endothelial network. Further analyses indicate that the altered behavior observed following Shroom2 depletion is due to aberrant cell contractility, as evidenced by decreased stress fiber organization and collagen contraction with an increase in cellular migration. Because Shroom2 directly interacts with Rock and Shroom2 knockdown results in the loss of Rock and activated MyosinII from sites of cell-cell adhesion, I conclude that Shroom2 facilitates the formation of a contractile network within endothelial cells, the loss of which leads to an increase in endothelial sprouting, migration, and angiogenesis.

2.1 INTRODUCTION

Shroom3 has been shown to be a critical regulator of cell morphology in several cellular contexts and animal model systems [184, 187, 189, 192, 194, 199, 216]. Shroom3-mediated morphogenesis is dependent on its ability to bind both F-actin and Rock [189, 192]. It is predicted that actin binding targets Shroom3 to the tight junction in polarized epithelia. Shroom3 can then recruit Rock to the tight junction, resulting in the localized activation of MyosinII and subsequent apical constriction [189, 192]. In addition to the ability to regulate actomyosin networks, Shroom3 has been implicated in regulating the apical positioning of γ tubulin and subsequent microtubule organization in *Xenopus* epithelial cells [216]. It is unclear if these two activities of Shroom3 are directly related or occur independently. In vertebrates, the Shroom proteins contain another family member, Shroom2, which shares several structural and functional characteristics with Shroom3. Like Shroom3, Shroom2 contains an N-terminal PDZ of unknown function, a centrally located, conserved actin binding module centered around the SD1, and a C-terminally located SD2, which, in the case of Shroom3, directly binds to Rock [188, 192]. Unlike Shroom3, Shroom2 does not induce apical constriction in either *Xenopus* ectodermal epithelium [197] or cultured MDCK epithelial cells [192]. However, Shroom2 has been shown to control other aspects of morphogenesis in *Xenopus* embryos, such as epithelial thickening and pigment accumulation [193, 197].

In mice, Shroom2 is highly expressed in various populations of polarized epithelial cells, including the neural epithelium, gut, eye, lung, and kidney. Additionally, Shroom2 is highly expressed in the endothelium of the developing vasculature [188]. This is consistent with the other cell types that express Shroom2, as the endothelium itself is a polarized population of epithelia. Endothelial cells exhibit the remarkable capacity to undergo dramatic changes in

morphology and migration in order to form the network of tubes that are seen in the embryo during and after vasculogenesis. Initial formation and subsequent remodeling of the vascular network are dependant on both the ability of endothelial cells to sprout new branches via the formation of filopodia and migration to new positions in the network [217]. One of the critical determinants of the migratory behavior of endothelial cells is MyosinII contractility downstream of Rock. It has been demonstrated that inhibition of MyosinII or Rock results in increased endothelial sprouting, suggesting that this pathway is a critical regulator of vascular architecture [177, 178, 218, 219]. In this context the function of Rock seems to negatively regulate membrane protrusion and cell migration at the level of cortical contractility. Specifically, a cortical Rock-MyosinII network inhibits the ability of cells to form membrane protrusions and migrate. This is supported by the observation that localized addition of pharmacological inhibitors of this pathway results in the rapid formation of endothelial filopodial outgrowth followed by cell migration [178].

In this study I have investigated the function of Shroom2 as a potential regulator of the cellular and angiogenic behavior of endothelial cells. Based on previous work, I hypothesized that Shroom2 might regulate these biological processes via Rock localization, impacting subsequent contractility. Using siRNA and shRNA approaches in both established endothelial cells and in primary endothelia derived from mouse ES cells, I show that depletion of Shroom2 results in increased angiogenesis due to decreased cellular contractility. This decrease in contractility appears to result from diminished Rock and MyosinII activity at the cell cortex and the disorganization of the actin cytoskeleton. These alterations in cellular architecture cause increases in cell protrusions and alter cellular migration. Together, these data indicate that Shroom2 is a vital regulator of endothelial cell behavior during vascular morphogenesis.

Additionally, my findings expand the inclusion of the Shroom-Rock complex in multiple cellular processes and suggest that this conserved signaling complex may be utilized in a variety of biological events that require Rock activity.

2.2 RESULTS

2.2.1 Knockdown of Shroom2 increases sprouting in angiogenesis and vasculogenesis assays

Previous studies from our lab have described the expression and localization of Shroom2 in the developing vasculature of mouse embryos and the C166 endothelial cell line [188]. Because C166 cells are derived from the murine yolk sac [220], I sought to confirm endogenous expression and localization of Shroom2 in the yolk sac *in vivo*. Staining of yolk sacs of e9.5 embryos to detect both Shroom2 and PECAM-1, an endothelial specific adhesion protein, shows that Shroom2 is expressed throughout the yolk sac vasculature, in both large vessels (Figure 6A) and the capillary plexus (Figure 6B). In addition, the Shroom2 protein is enriched at sites of cell-cell adhesion, similar to what is seen in the embryo proper and in C166 cells. Based on these data and that from other published works [220, 221], C166 cells are a viable cell type to explore the function of Shroom2 in endothelial cell behavior. Therefore, I utilized siRNA to knockdown the expression of Shroom2 in these cells. C166 cells treated with a control non-targeting siRNA (siControl) form a confluent monolayer and exhibit Shroom2 and ZO1 distribution at cell-cell junctions (Figure 6C). Cells treated with Shroom2 specific siRNA (siShroom2) also form confluent monolayers with no appreciable change in adherens junctions or tight junctions, but

Shroom2 staining is virtually eliminated from the majority of these cells (Figure 6D, Figure 7A, B). Consistent with the immunostaining results, Western blotting shows that Shroom2 protein is reduced by approximately 70% using two different siRNAs, one targeting the 3' UTR (siShroom2-7) and the other targeting the coding sequence (siShroom2-8) of the Shroom2 mRNA (Figure 6E). Treatment of cells with siRNA did not alter the rates of proliferation (data not shown) and all results have been verified using both Shroom2 siRNAs, but data are typically shown from experiments using siShroom2-8. Because I obtain similar results using two different siRNAs that target unique regions of the *Shroom2* transcript, the observed phenotypes likely result from specific depletion of Shroom2 protein.

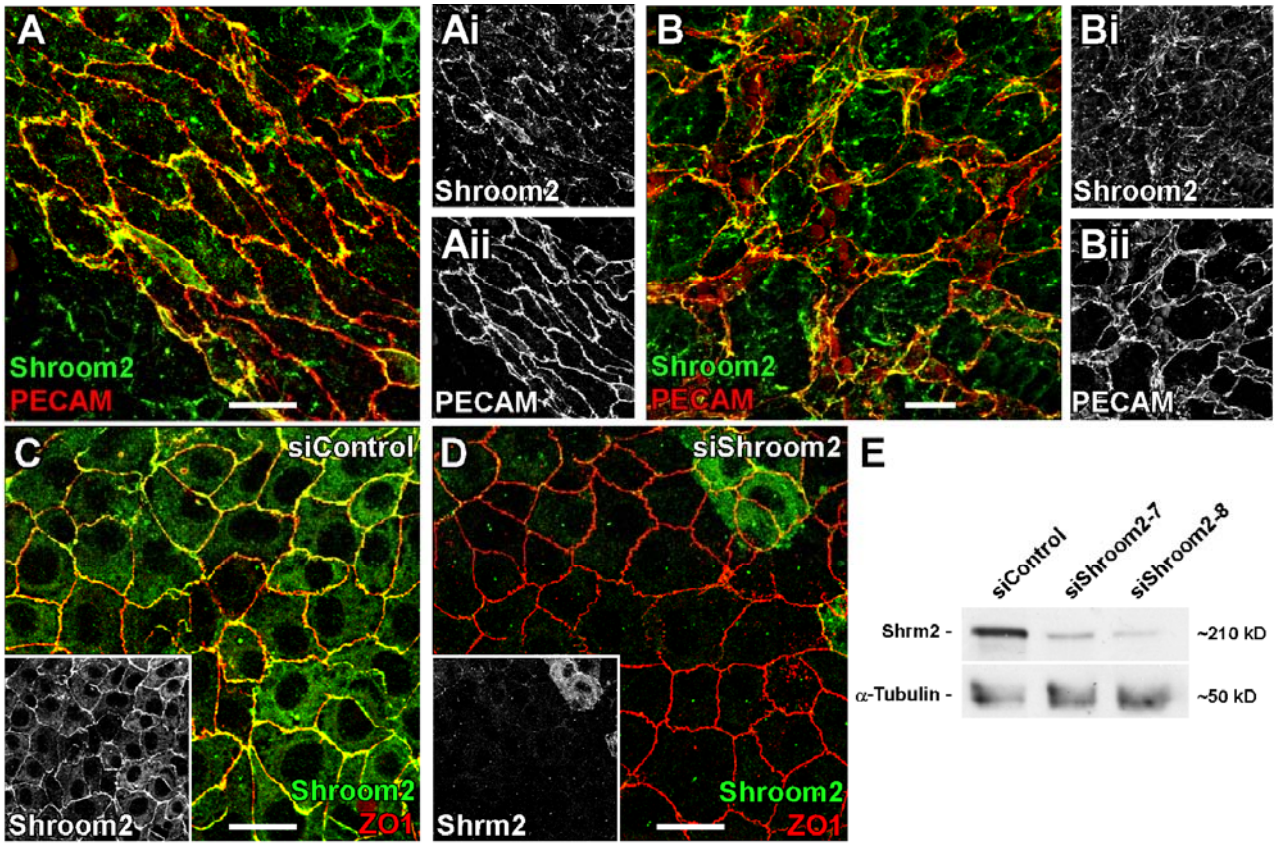

Figure 6: Expression and knockdown of Shroom2 in yolk sac endothelial cells.

(A-B) Murine yolk sacs at E9.5 stained with PECAM and Shroom2 antibodies show Shroom2 localization at cellular junctions in both large vessels (A) and the capillary plexus (B). **(C-D)** C166 endothelial cells were treated with a

nontargeting siRNA (siControl) (C) or a Shroom2-specific siRNA (siShroom2) (D) and were stained with Shroom2 (inset) and ZO1. **(E)** Shroom2 knockdown from two different siRNAs was confirmed via Western blot. α-Tubulin was used as a loading control. Scale bars = 25 μm.

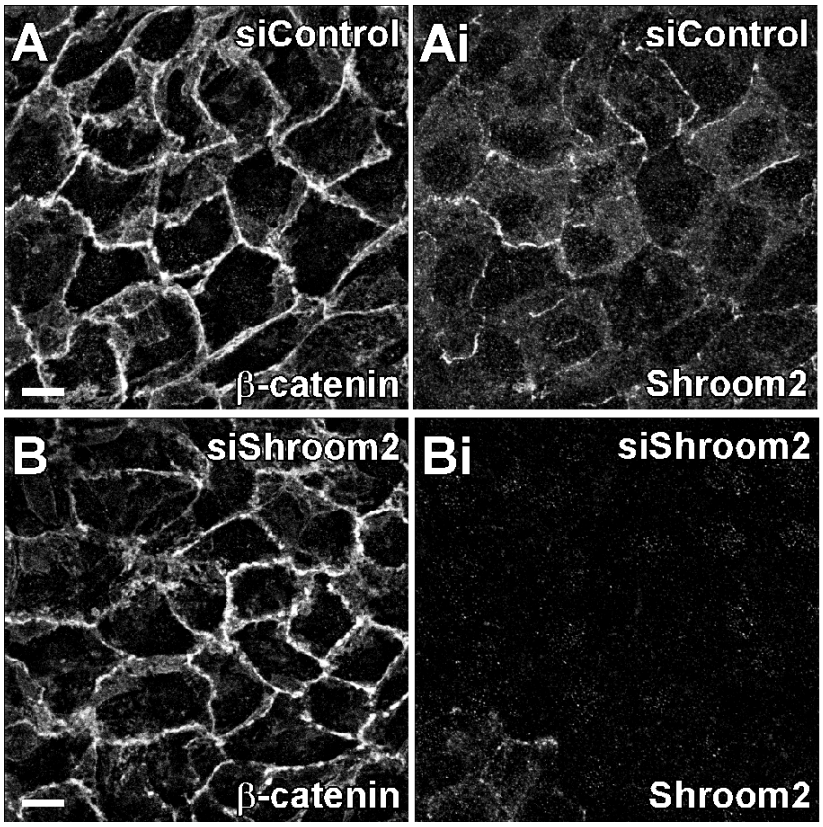

Figure 7: Shroom2 knockdown does not impact cellular adhesion in a monolayer.

(A-B) siControl (A,Ai) or siShroom2 (B,Bi) C166 cells were stained with β-catenin (A,B) and Shroom2 (Ai,Bi) antibodies 72 hours after transfection. There is no apparent difference in β-catenin staining. Scale bars = 25μm.

One hallmark of endothelial cells is their ability to form a capillary network when cultured on matrigel. Control C166 cells form a multi-cellular vascular network when grown under these conditions (Figure 8A). To test the role of Shroom2 in this endothelial behavior, siShroom2 C166 cells were plated on matrigel and allowed to undergo angiogenesis. Under these conditions, not only do siShroom2 C166 cells retain the ability to form vascular networks,

but they actually show elevated branching capacity (Figure 8B, C). Shroom2 remains depleted throughout the matrigel angiogenesis experiments (Figure 9D-E). When fewer numbers of C166 cells are plated ($1.2x10^6$), short cords emerge but fail to form an interconnected network, whereas Shroom2 siRNA increases network formation (Figure 9A-B).

While treatment with both siShroom2-7 and siShroom2-8 yields a more highly branched network, C166 cells with the greatest degree of knockdown (siShroom2-8), show areas which have failed to undergo cord formation (arrow, Figure 8C). Similar outcomes have been seen with a broad Rock inhibitor, Y27632, such that angiogenesis is inhibited in both C166 cells (data not shown) and bovine retinal endothelial cells [222]. Since Shroom2-deficient endothelial cells form a more branched network on matrigel, I wanted to further investigate and validate the effect of Shroom2 loss using an in-gel sprouting angiogenesis assay. Control siRNA and siShroom2 spheroids of equal cell number were embedded in collagen gels, cultured for 48 hours, and then analyzed for the extent of sprouting. Consistent with the matrigel angiogenesis assay, siShroom2 cells demonstrated significantly elevated sprouting compared to control cells (Figure 8D-F). In order to verify these results in a primary cell line, I confirmed expression of hShroom2 in primary human umbilical vein endothelial cells (HUVEC) (Figure 8G). After transfection with hShroom2 siRNA, Shroom2 protein is reduced by approximately 75% (Figure 8G). Similar to C166 cells, Shroom2 knockdown in HUVECs increases branching during matrigel angiogenesis (Figure 8 H, I).

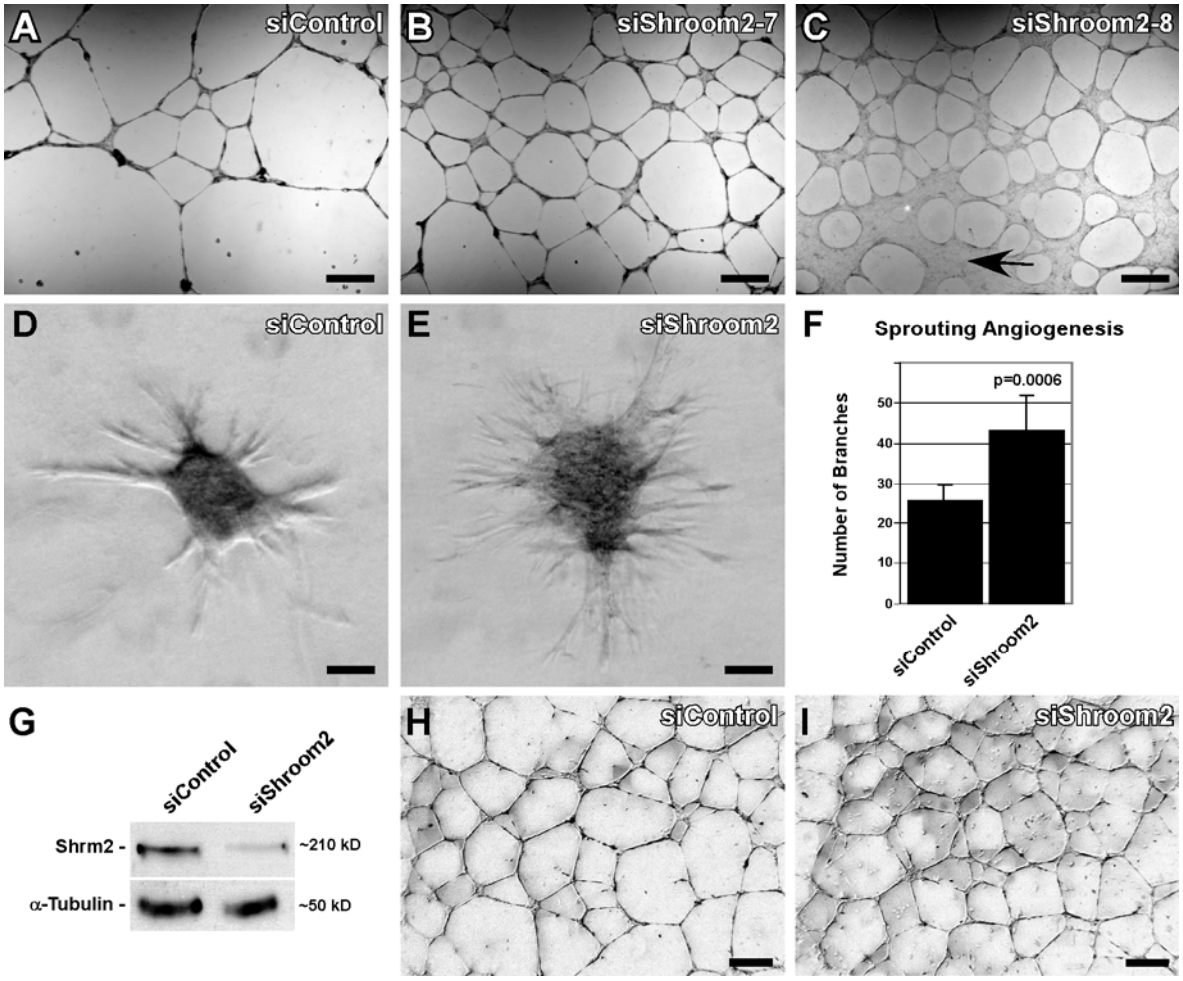

Figure 8: Transient Shroom2 knockdown stimulates angiogenesis.

(A-C) siControl (A), siShroom2–7 (B), or siShroom2–8 (C) –treated C166 cells were plated on matrigel to examine angiogenic potential. Arrow indicates an area that has failed to undergo angiogenesis. **(D-F)** siControl (D) and siShroom2 (E) C166 cells were grown as spheroids for use in a collagen sprouting angiogenesis assay. Quantification of collagen sprouting angiogenesis is shown in (F). The numbers of branch tips are represented as the mean ± SD (n = 7 spheroids). **(G)** Western blot of Shroom2 knockdown in HUVECs. **(H-I)** Matrigel angiogenesis assay for siControl (H) and siShroom2 (I)–treated HUVECs. Scale bars = 1 mm in A-C; 125 μm in D,E,H, and I.

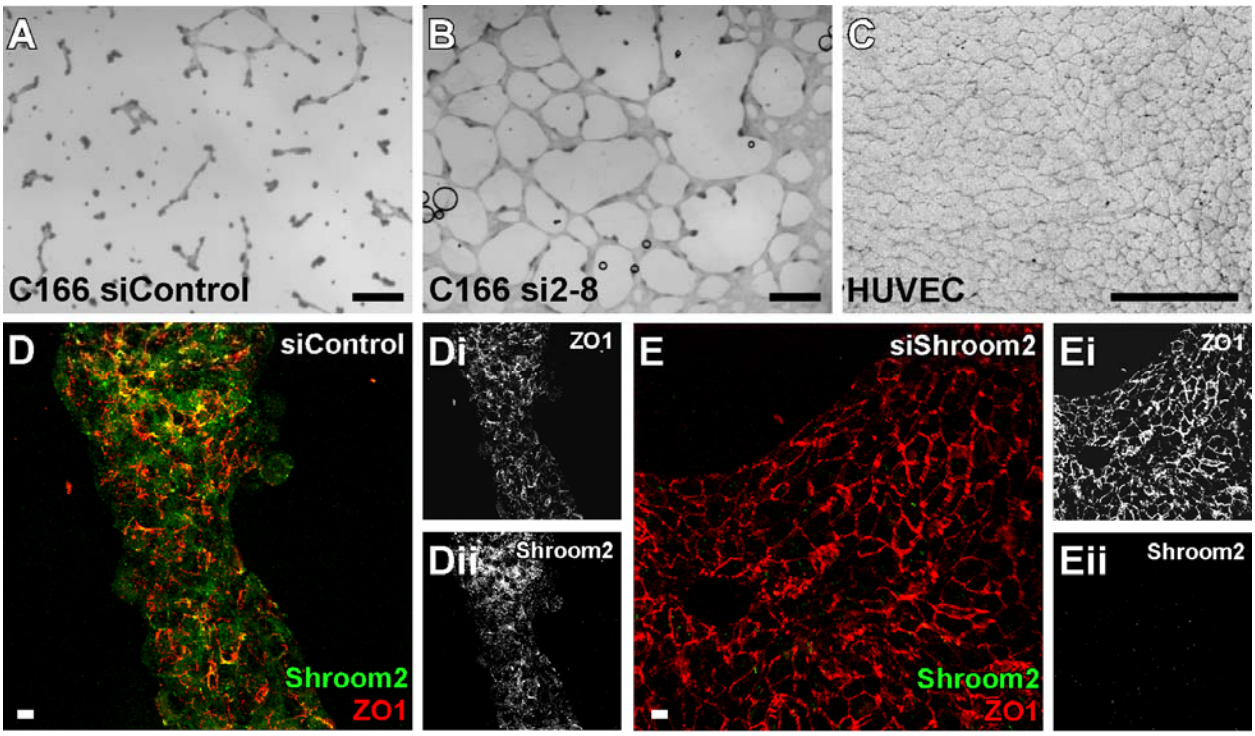

Figure 9: Shroom2 knockdown persists during angiogenesis assays.

(A-B) 72 hours after siRNA transfection, $1.2x10^6$ C166 cells were plated on a matrigel-coated coverslip in a 6 well plate and photographed after 24 hours. Lower numbers of cells fail to form an interconnected network of multicellular cords, however there is still a difference in branching between control (A) and Shroom2-deficient cells (B). **(C)** $1.2x10^6$ HUVEC cells form much smaller, single cellular, capillary networks on matrigel. **(D-E)** After 24 hours on matrigel, control (D) or siShroom2 (E) C166 cords were stained directly on coverslips with ZO1 (Di,Ei) and Shroom2 (Dii,Eii) antibodies. No Shroom2 is detected in siShroom2-treated cells. Scale bars = 1mm in A-C; 25μm in D-E.

While Shroom2 knockdown in both C166 cells and HUVECs leads to an increase in branching, these cell types form morphologically distinct networks on matrigel. C166 cells form large multicellular cords, while HUVECs form a single cellular capillary-like network (Figure 9 A-C). Thus, I sought to determine the effect of Shroom2 knockdown in a model more similar to C166 cells and the vascular network observed in the yolk sac and embryo during development. Because C166 cells are derived from the yolk sac where initial vasculogenesis occurs, I

employed a vasculogenesis assay in which ES cells can be differentiated into a multicellular branching endothelium [223]. It should be noted that ES cells express Shroom2 endogenously (Figure 10A). In order to address the role of Shroom2 in endothelial cells derived from ES cells, I generated cell lines that stably express Shroom2-specific shRNAs. Shroom2 knockdown was confirmed via both immunofluorescence staining (Figure 10Ai, Bi) and Western blot (Figure 10C). Stable ES cells were then grown in suspension to form embryoid bodies, allowed to reattach to tissue culture dishes, and grown for 9-11 days in differentiation media. Differentiated cultures were fixed and immunostained against PECAM to visualize the vasculature. Consistent with the results obtained using C166 cells and HUVECs, shShroom2 ES cells generate hyper-branched endothelial networks when compared to vector control ES cells (Fig 10D, E). When observed at higher magnification, the control vasculature exhibits uniform cell borders and few filopodial extensions (Figure 10Di). In stark contrast, the Shroom2 deficient vasculature exhibits a plethora of filopodia-like extensions (Figure 10Ei). Shroom2 remains efficiently knocked down after differentiation (Figure 10F-G). Together, these experiments suggest that within the endothelium, Shroom2 is involved in negatively regulating vessel branching, and that this may be controlled at the level of the cytoskeleton.

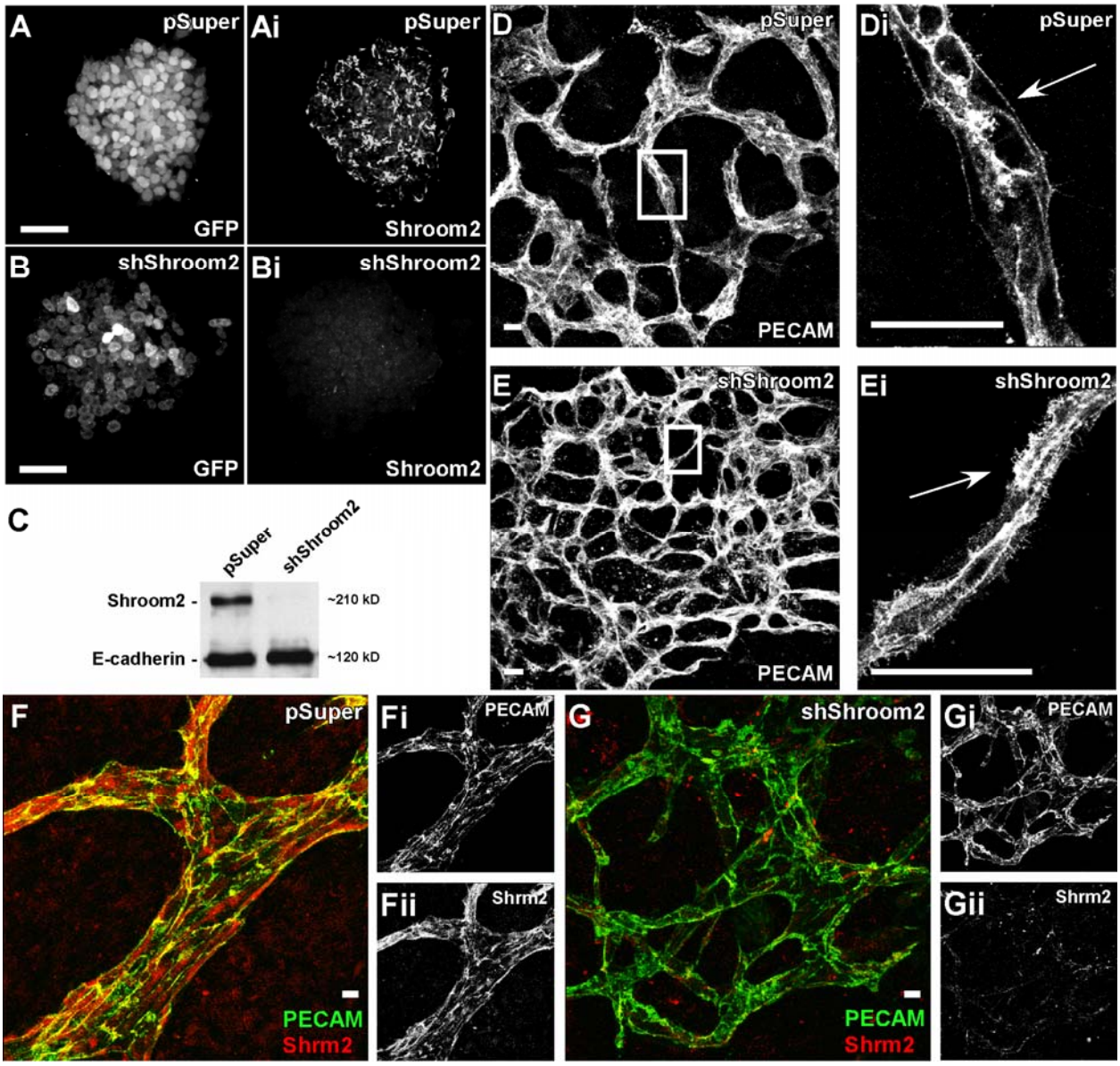

Figure 10: Stable knockdown of Shroom2 in murine ES cells enhances vasculogenesis.

(A–C) ES cells stably transfected with the parental vector pSuper-GFPneo^r (pSuper) (A) or pSuper-shShroom2 (shShroom2) (B) were stained to detect GFP and Shroom2. Knockdown was confirmed by Western blotting (C). **(D-E)** After differentiation, pSuper (D) and shShroom2 (E) cells were stained with PECAM. Boxed regions in D and E are enlarged in Di and Ei. While distinct boundaries exist between control endothelial and surrounding cells (Di), numerous filopodia-like extensions are found throughout the Shroom2-deficient endothelium (Ei) (compare arrows). **(F-G)** After endothelial differentiation of pSuper (F) or shShroom2 (G) stable ES cells, cells were stained to detect PECAM (Fi,Gi) and Shroom2 (Fii,Gii). Scale bars = 25 μm in A-B and F-G; 100 μm in D and E.

2.2.2 Shroom2 regulates endothelial contraction through an interaction with Rock.

Several studies have previously shown that reduced Rock and MyosinII activity correlate with both increased angiogenesis and increased filopodia formation in endothelial cells [177, 178, 219]. Along these lines, Shroom2 contains the conserved SD2 motif that has been shown to mediate a direct interaction between Shroom3 and Rock and between *Drosophila* Shroom and dRok to facilitate apical constriction [188, 191, 192]. Based on these observations, I hypothesized that the increase in sprouting observed from Shroom2 knockdown is due to loss of Rock-mediated contractility. To test this hypothesis, I first verified an interaction between Rock and Shroom2. In GST pull down experiments, the Shroom Binding Domain (SBD) of hRock1 interacts with Shroom2 (Figure 11A). This interaction does not appear to be isoform specific as the Shroom2 SD2 interacts with endogenous Rock1 and Rock2 (Figure 11B). In addition, the Shroom2 SD2 and the hRock1 SBD domain can be co-purified when co-expressed in bacteria (Figure 11C). This is a specific interaction, as the actin binding domain (SD1) of Shroom2 does not pull-down the Rock SBD (data not shown). In C166 cells grown on coverslips, Rock intermittently localizes to cell-cell junctions as indicated by co-localization with ZO1 (Figure 12A). Importantly, this localization appears to be dependent on Shroom2, as this population of Rock is lost in siShroom2 C166 cells (Figure 12B). Knockdown of Shroom2 has no effect on Rock expression as indicated by Western blot (Figure 11D). Shroom2 recruitment of Rock is also observed in MDCK cells engineered to express Shroom2 following withdrawal of doxycyclin. In the presence of doxycycline, no Shroom2 is detected and no Rock is observed at tight junctions (Figure 12C). After Shroom2 induction, both Shroom2 and Rock co-localize at tight junctions (Figure 12D). These data indicate that Shroom2 can directly bind Rock *in vitro* and regulate its sub-cellular distribution *in vivo*.

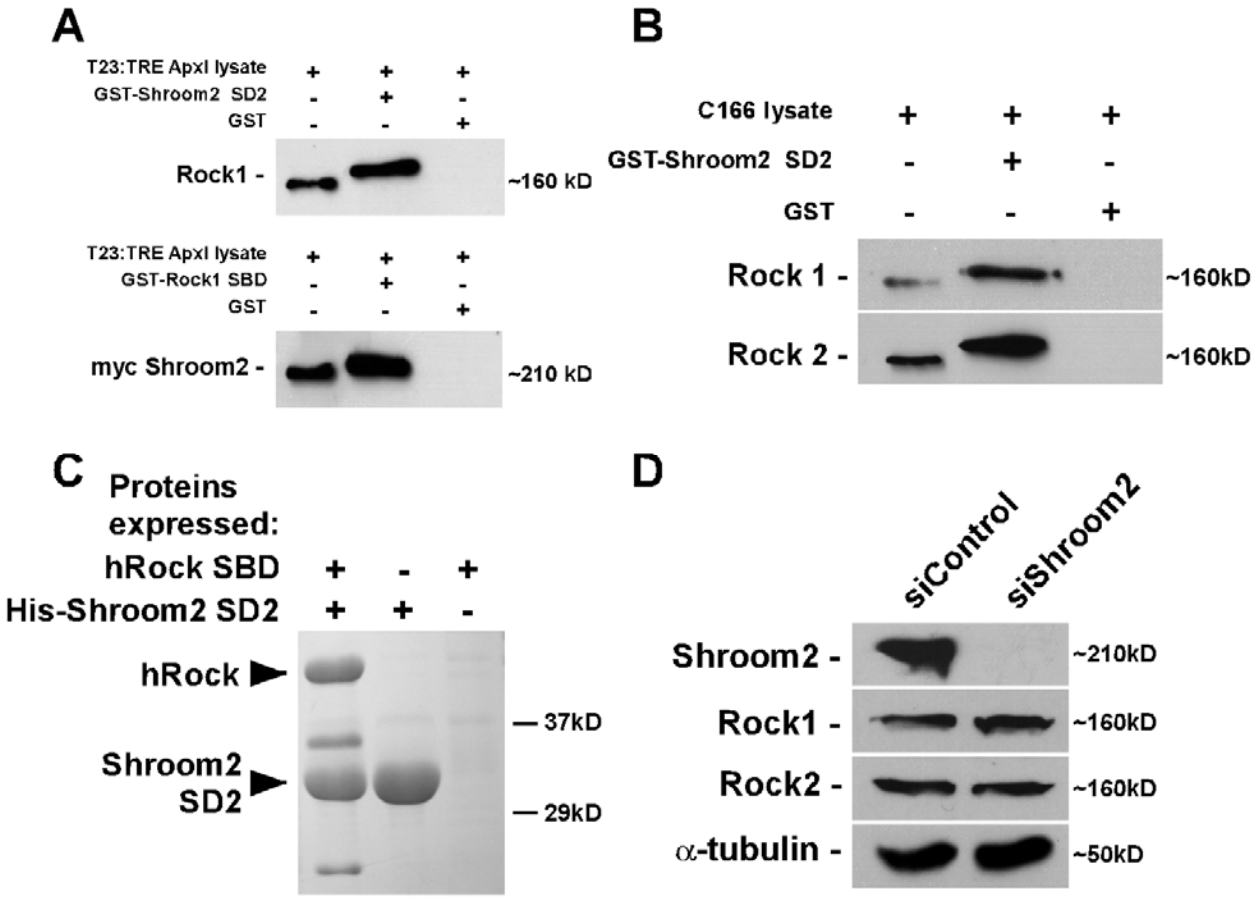

Figure 11: Shroom2 physically interacts with Rock.

(A) A GST-tagged mShroom2 SD2 or a GST-tagged Shroom binding domain (SBD) of hRock1 were incubated with total cell lysate from T23 cells engineered to express myc-Shroom2 under the tetracycline response element promoter (T23:TRE Apxl). Following GST pull down, the Shroom2 SD2 interacts with endogenous Rock1, and the Rock1 SBD interacts with full-length myc-Shroom2. **(B)** The GST-tagged Shroom2 SD2 was subjected to a pull down assay with C166 total cell lysate. The Shroom2 SD2 interacts with endogenous Rock1 and Rock2. **(C)** His-tagged mShroom2 SD2 and the untagged Shroom binding domain (SBD) of hRock1 were individually or co-expressed in bacteria. Shroom2 SD2 was purified with Ni-NTA resin and bound proteins eluted and visualized by SDS-PAGE and Coomassie staining. **(D)** There is no change in Rock1 or Rock2 protein levels 72 hours after Shroom2 knockdown as indicated by Western blot.

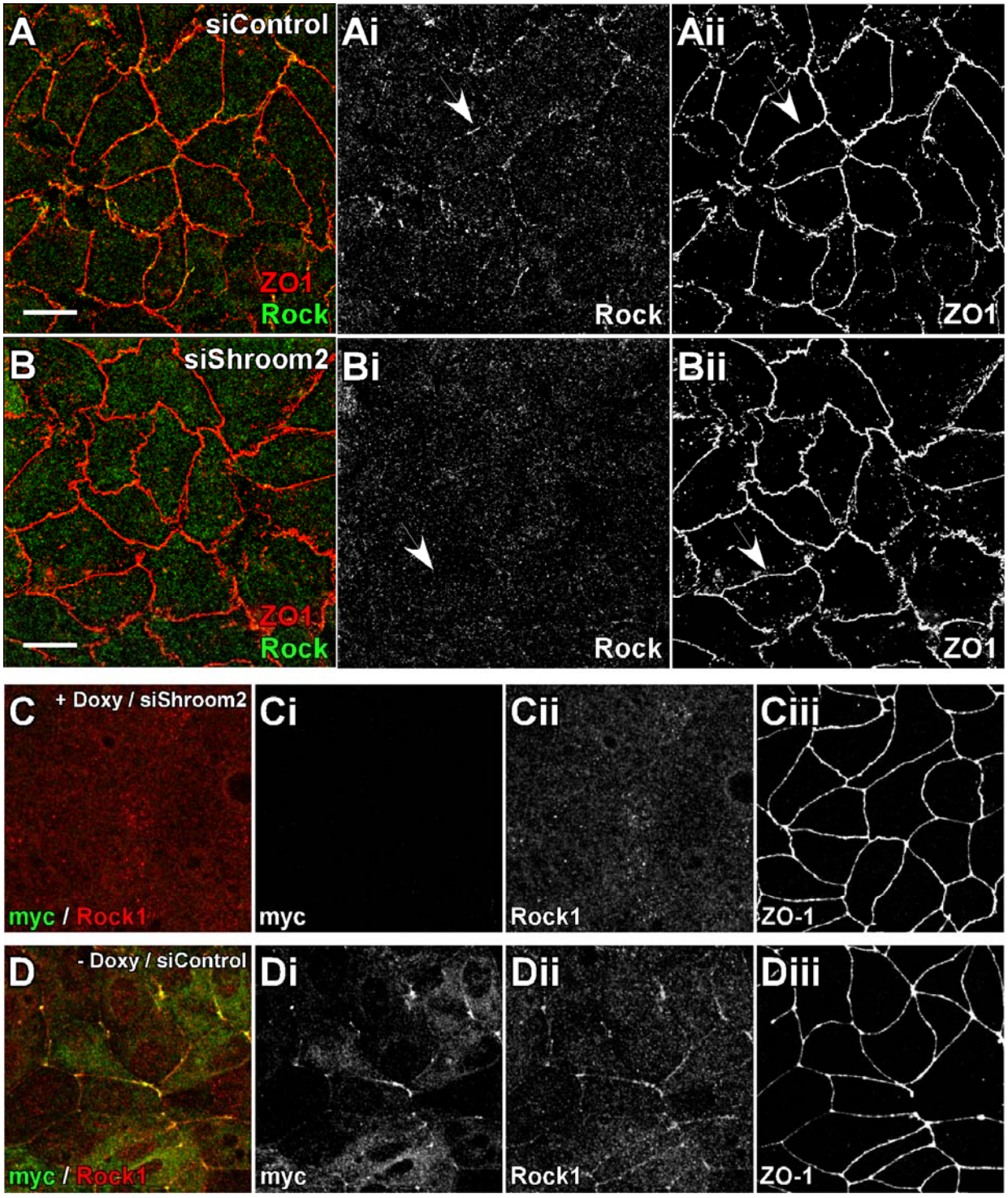

Figure 12: Shroom2 mediates the sub-cellular localization of Rock.

(A-B) siControl (A) or siShroom2 (B) C166 cells were stained with Rock1 (Ai and Bi) and ZO1 (Aii and Bii) antibodies. Arrows indicate loss of Rock localization to tight junctions after Shroom2 knockdown. **(C-D)** T23 MDCK cells with inducible myc-Shroom2 expression show no Rock1 immunostaining (Cii) at tight junctions (Ciii)

when Shroom2 expression is inhibited (Ci). Upon expression of Shroom2 (Di), Rock1 (Dii) is localized to tight junctions (Diii). As Shroom2 expression was leaky, cells were also treated with Shroom2 (C) or control (D) siRNA for 72 hours prior to immunostaining. Scale bars = 25μm.

It has been speculated that Shroom proteins can regulate cell and tissue morphology by controlling MyosinII activity. Based on this notion and the above results, I hypothesized that if Shroom2 is involved in localizing Rock to cell-cell junctions, then it may play a role in establishing endothelial contractility. To test this, I analyzed the ability of Shroom2-deficient cells to contract collagen gels. Control and siShroom2 C166 cells were plated on a bed of collagen attached to the well and allowed to form a confluent monolayer. Following monolayer formation, the mechanically loaded gels were detached from the well and allowed to contract for 6 hours. Quantification shows that Shroom2 deficient cells are not able to contract the collagen gel to the same extent as control cells (Figure 13A). Collagen gel contraction is also dependent upon Rock, as treatment of control cells with Rock inhibitor contracted gels to a lesser extent. Interestingly, Shroom2-deficient endothelial cells are more sensitive to Rock inhibition and contract the gel less than the inhibitor or siShroom2 alone. This sensitivity may be due to an interaction between Shroom2 and Rock, which helps establish endothelial contractility. These results document for the first time that Shroom proteins can indeed control the contractile properties of a population of polarized cells.

Activated Rock is thought to cause MyosinII contraction by directly phosphorylating the Regulatory Myosin Light Chain 2 (MLC2) [70] and inhibiting the targeting subunit of myosin phosphatase–1 (MYPT) [71]. Rock may also influence contractility by regulating actin dynamics through activation of LIM kinase and subsequent cofilin phosphorylation [73]. To examine whether or not Shroom2 knockdown affects Rock activity, I examined the

phosphorylation state of several downstream Rock effectors. Interestingly, while mono-phosphorylated MLC2 (pMLC) is unaffected, di-phosphorylated MLC2 (ppMLC) levels are reduced 50% in siShroom2 C166 cells (Figure 13B). These results suggest that loss of Shroom2 leads to a loss of Rock activity. A similar role for Rock in the regulation of ppMLC2 but not pMLC2 has been observed in MDCK cells [224]. Additional Rock effectors, MYPT and FAK [225] show reduced phosphorylation by approximately 25% while p-cofilin levels remain unchanged (Figure 13B). These observations suggest that the Shroom2-Rock complex could be working at the level of MyosinII activity to control actin organization. To address this issue, control and siShroom2 cells were stained to detect ppMLC2 at cell-cell junctions (apical) and stress fibers (basal) (Figure 13C-F). In control cells ppMLC can be detected at both of these subcellular locations (Figure 13Ci, Ei). In contrast, the staining in cell-cell junctions is largely lost and the stress fiber staining is both reduced and disorganized in the siShroom2 cells (Figure 13Di, Fi). Based on these results, Shroom2 may control endothelial contractility via the localized activation of actomyosin.

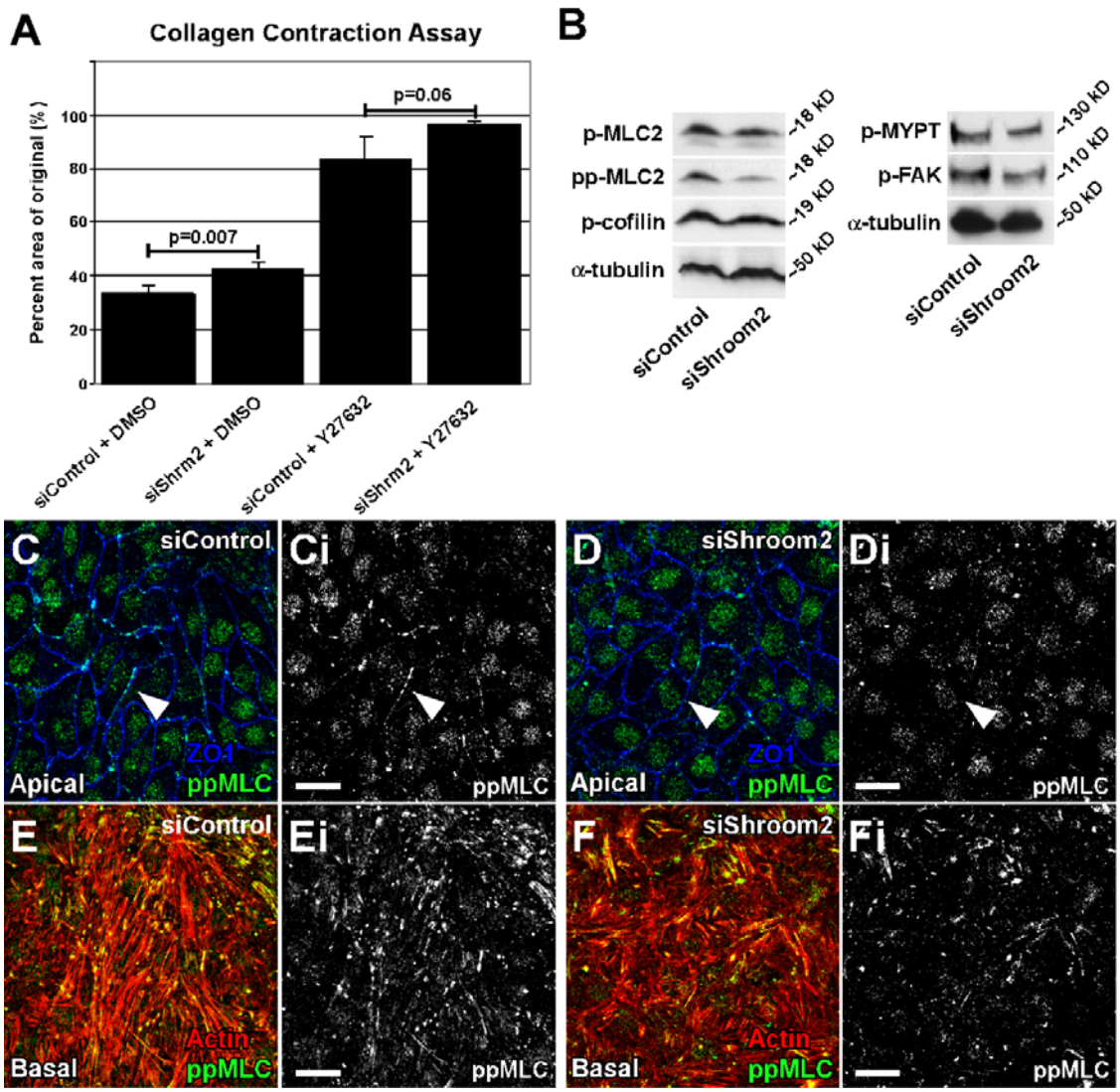

Figure 13: Shroom2 regulates endothelial contractility.

(A) Contractility of siControl or siShroom2 C166 cells with or without the Rock inhibitor Y27632 was assessed through the ability of a monolayer to contract a collagen gel. Quantification is graphed as the percentage of area of the original after 6 h, represented by the mean ± SD (n = 3). **(B)** Phosphorylation of Rock effectors was visualized by Western blotting. α-Tubulin was used as a loading control. Representative blots from three independent experiments are shown. p-MLC2, phospho-myosin light chain 2 (Ser-19); pp-MLC2, diphospho-myosin light chain 2 (Thr-18/Ser-19); p-MYPT, phospho-myosin phosphatase binding subunit 1 (Thr-696); pFAK, phospho-focal adhesion kinase (Tyr-397). **(C-F)** Control (C and E) and Shroom2 knockdown (D and F) C166 cells were stained for ppMLC (Ci-Fi) and either ZO1 (C and D) or actin (E and F). Loss of Shroom2 leads to loss of pp-MLC2 at both stress fibers and cell–cell junctions (compare arrowheads). Scale bars = 25 μm.

Because Rock activity and MyosinII contractility have been shown to play a significant role in the formation and organization of actin stress fibers [226, 227], I examined stress fibers in siShroom2 C166 cells. In control cells, stress fibers are typically arranged in thick bundles that are aligned parallel to each other within the cell and appear to be contiguous with bundles in adjacent cells (inset, Figure 14A); a similar organization has been observed in HUVEC endothelial cells [228]. This organization is lost in siShroom2 cells, as stress fibers appear randomly oriented (Figure 14B). A similar change in stress fiber organization can be found in control C166 cells treated with a low concentration of Rock inhibitor (1 μM) (Figure 14C). Because Rock activity is required for stress fiber formation and high concentrations of Rock inhibitors abolish stress fiber formation, a low concentration was selected to slightly reduce but not eliminate Rock activity. Consistent with the idea that Shroom2 and Rock work together to control cell morphology, stress fibers are greatly diminished and further disorganized in siShroom2 cells that are treated with 1 μM Rock inhibitor (Figure 14D). Because Shroom2 is not detected at focal adhesions, a major regulator of stress fibers, yet changes occur in stress fiber organization and pFAK levels are reduced after Shroom2 knockdown, these changes may occur indirectly through the reduction of cortical contractility.

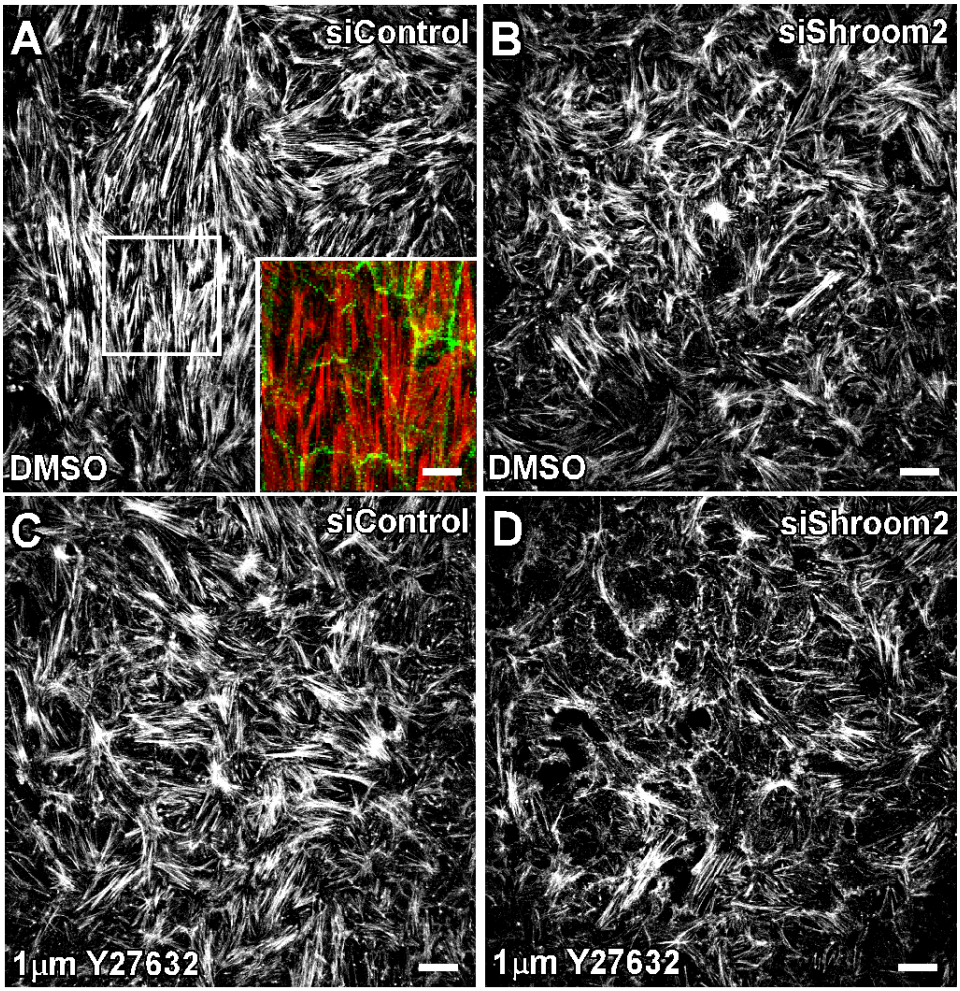

Figure 14: Shroom2 influences stress fiber organization.

(A-D) Stress fiber organization was examined by immunostaining for actin in C166 cells treated with siControl (A), siShroom2 (B), siControl and Y27632 (C), and siShroom2 and Y27632 (D). Inset is a merge for apical Shroom2 (green) and basal stress fibers (red). Scale bars = 25 μm.

2.2.3 The loss of Shroom2 influences endothelial migration

Rock activity can enhance or inhibit cellular migration depending on the particular cell type [reviewed in [229]]. In order to observe the effect of Shroom2 knockdown and concomitant loss of endothelial contractility on C166 migration, I subjected siShroom2 C166 cells to a scratch wound assay. Loss of Shroom2 significantly increases migration of C166 cells into the wound relative to control cells (Figure 15A). siShroom2 cells completely closed the wounds by 24 hours while control wounds were about 90% closed at this time (Figure 15B). To confirm these results, migration was also assessed using a Boyden chamber. Consistent with the above wound closing assay, siShroom2 cells demonstrate a significant increase in migration in comparison to control cells (Figure 15C). I next examined the effects of Rock inhibition on C166 migration. Rock inhibition enhances migration in both a scratch wound (Figure 15A) and Boyden chamber assay (Figure 15C). After incubation with a low concentration of Rock inhibitor, Shroom2 deficient cells migrate more quickly than with Rock inhibition or siShroom2 alone. The opposite has been observed in HUVEC cells, where Rock inhibition attenuates VEGF stimulated migration [174]. Similarly, Shroom2 knockdown in HUVEC cells results in significant reduction of migration as assayed in a Boyden chamber (Figure 15D).

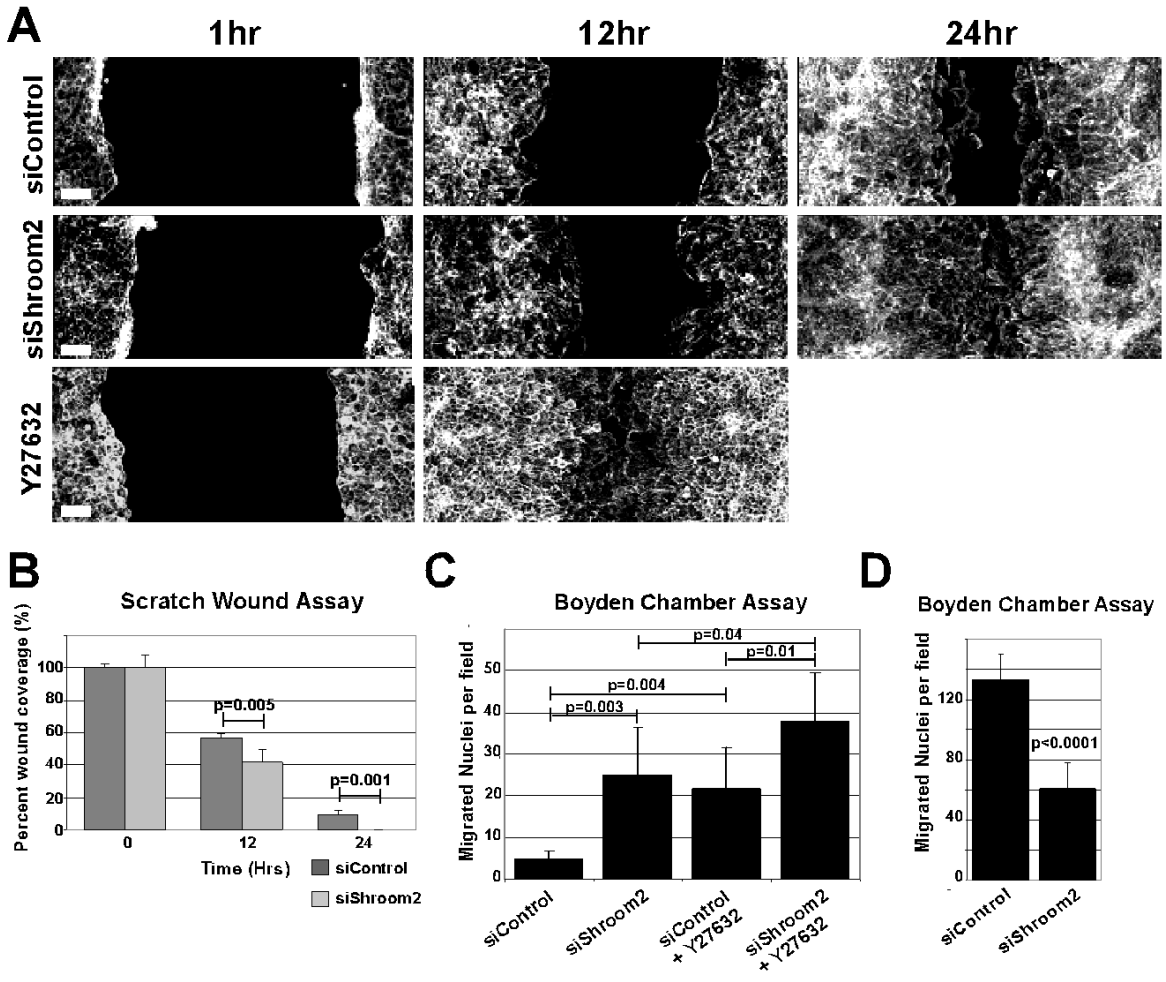

Figure 15: Shroom2 knockdown affects cell migration.

(A) siControl, siShroom2, or untreated cells with Y27632 were subjected to a scratch wound assay and were stained with phalloidin at 1, 12, and 24 h postscratch. **(B)** Quantification of a scratch wound assay from live C166 cells treated with siControl or siShroom2, represented by the mean percentage of wound closure ± SD (n = 5). **(C)** Migration of siControl and siShroom2 C166 cells in the presence or absence of Y27632 was assessed with a Boyden chamber. **(D)** Migration of siControl and siShroom2-treated HUVEC cells in a Boyden chamber. The number of migrated nuclei is represented by the mean ± SEM (n = 6) in C and D. Scale bar = 100 μm.

To better understand how Shroom2 may regulate migration, I evaluated the localization of Shroom2 during wounding. Wounded monolayers of C166 cells were fixed and stained to detect Shroom2 and actin (Figure 16A). Two hours after wounding, Shroom2 can be found on

thick F-actin cables at the leading edge. This structure is hypothesized to resemble a purse string, drawing cells into the wound [146]. At this time, Rock is also found at the leading edge. Again, this localization is dependent upon Shroom2, as Shroom2 knockdown abolishes Rock localization at the leading edge (Figure 16B, C). 12 hours after wounding, Shroom2 is diffusely localized at the leading edge and by 24 hours is ultimately lost from the leading edge as cells completely detach from the monolayer. Because these detached cells have little Shroom2 protein, it seems logical that reduction of Shroom2 promotes migration into the wound.

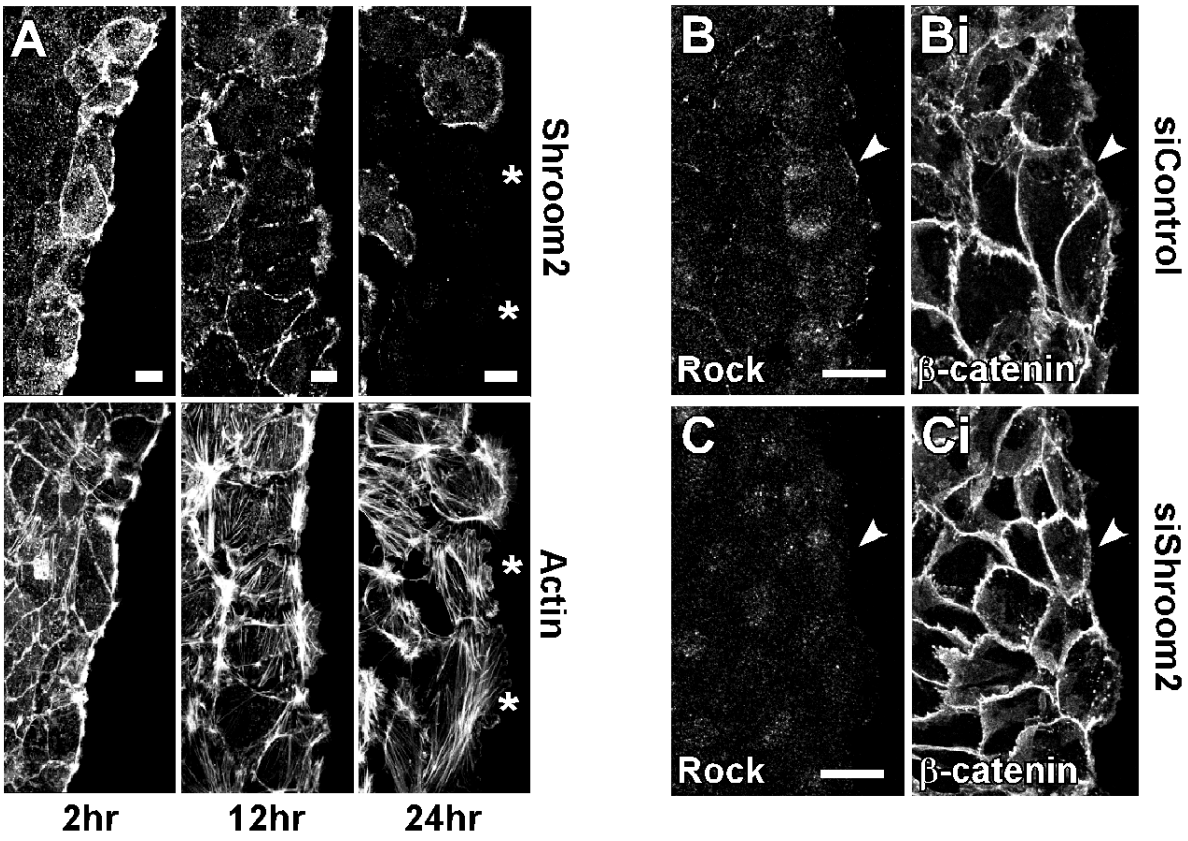

Figure 16: Transient knockdown of Shroom2 regulates endothelial migration.

(A) Untreated C166 cells were scratch wounded and stained for Shroom2 and actin at 2, 12, and 24 h post scratch. Asterisks indicate cells that have detached from the epithelial sheet and have lost Shroom2 expression. **(B-C)** 2 h after scratch wounding C166 cells, Rock1 localizes to the leading edge of siControl (B) but not siShroom2 (C) C166 cells. Immunostaining for β-catenin (Bi and Ci). Scale bars = 25 μm.

To better understand the consequence of long-term loss of Shroom2 function in endothelial cells, I generated a stable C166 cell line expressing a Shroom2 specific shRNA from the pSuper-GFPneo^r vector. Stable expression of the shRNA leads to apparent 100% knockdown as Shroom2 protein is undetectable via Western blot (Figure 17A). Analysis of the effect of stable Shroom2 knockdown indicates a change in morphology: a loss of dense peripheral actin bundles observed in control cells and an increase in filopodial extensions (Figure 17C, D). Control cells are often found clustered and adherent to one another, while shShroom2 cells fail to cluster or form stable cell-cell contacts. shShroom2 C166 cells contain fewer central focal adhesions (FAs) but larger peripheral FAs (Figure 17E-H). The loss of central FAs has also been observed in MEFs treated with Y27632, highlighting the importance of Rock-mediated contractility in FA regulation [230]. As expected, expression of shShroom2 further enhances migration in a Boyden chamber compared to control or transient siRNA knockdown (Figure 17B). Because shShroom2 cells fail to adhere to one another and form a monolayer, wound healing was assessed by mixing vector control (pSuper) or stable shShroom2 cells (both GFP positive) with wildtype C166 cells. Monolayers were then wounded and the migration of control or shShroom2 cells into the wound was assayed 30 minutes later. In controls, the GFP positive cells are still integrated into the monolayer (arrow) and possess robust actin belts at the leading edge (arrowhead) (Figure 17I). In contrast shShroom2 cells fail to adhere to the monolayer and after only 30 minutes, have started to migrate into the wound, past the actin belt (Figure 17J). Presumably due to the increase in migration and loss of cellular adhesion, these cells fail to undergo angiogenesis on matrigel (data not shown). Based on these results and those presented above, I predict that loss of signaling via the Shroom2-Rock complex reduces that degree of cortical actomyosin contractility, which in turn promotes the migration of the C166 cells.

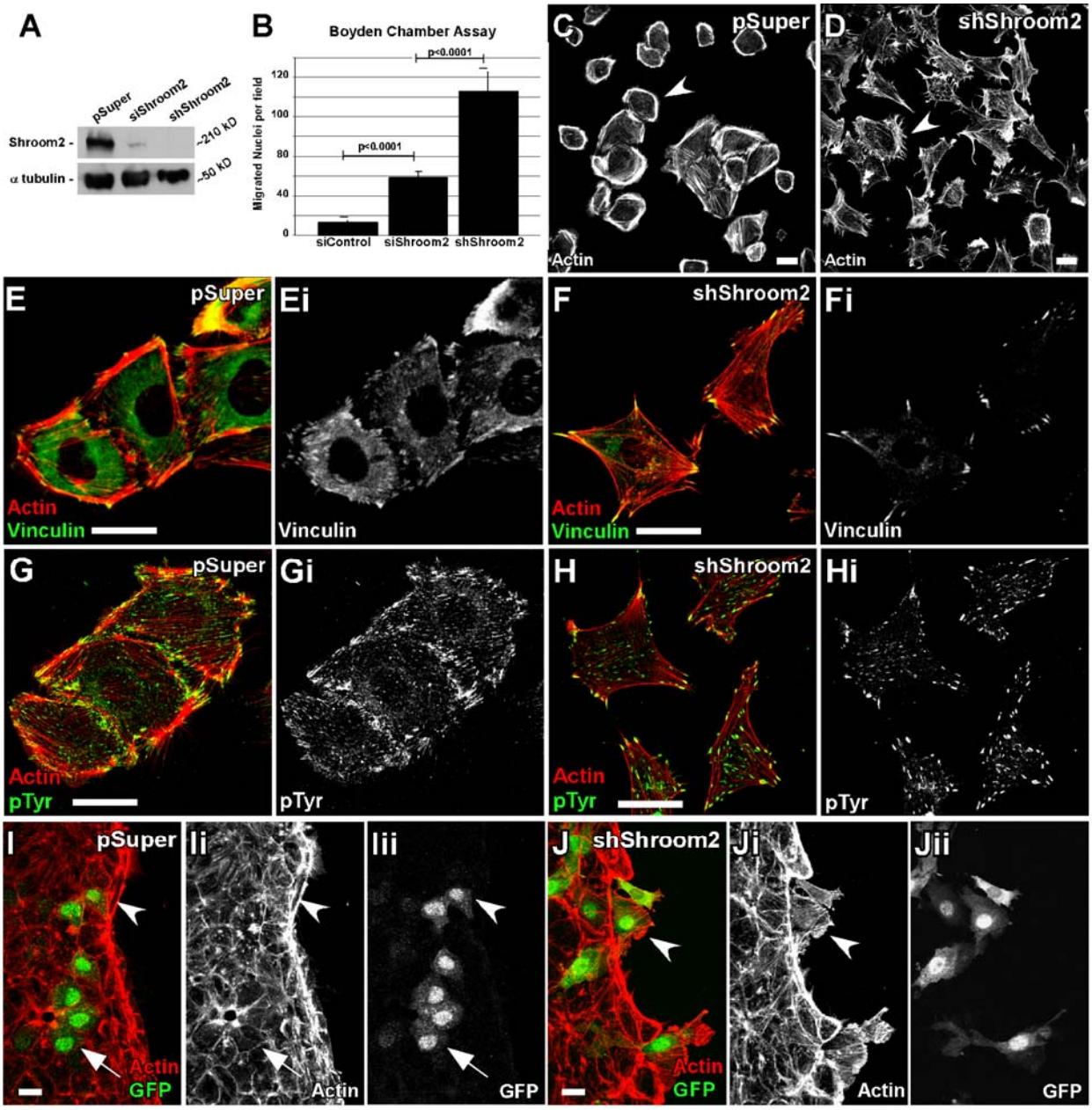

Figure 17: Stable expression of shShroom2 changes endothelial morphology and enhances migration.

(A) Western blot of transient siShroom2, stable shShroom2, or stable vector control (pSuper) C166 cells for Shroom2. α-Tubulin was used as a loading control. **(B)** Quantification of Boyden chamber migration for siControl, siShroom2, or shShroom2 C166 cells. The number of migrated nuclei is represented by the mean ± SEM (n = 6). **(C-D)** pSuper (C) or shShroom2 (D) C166 cells were allowed to spread on fibronectin-coated coverslips for 4 h and were immunostained for actin. Arrowheads indicate differences in cortical actin. **(E–H)** pSuper (E and G) or shShroom2 (F and H) C166 cells were immunostained for vinculin (E and F) or phospho-tyrosine (G and H) to

visualize focal adhesions. **(I-J)** pSuper (I) or shShroom2 (J) C166 cells (indicated by GFP) were mixed with parent C166 cells and wounded. Cells were allowed to migrate for 30 min and were then stained for GFP and actin. Arrows indicate stable junctions formed between pSuper and parent C166 cells. Arrowheads indicate pSuper cells that contribute to the actin belt or shShroom2 cells that migrate quickly into the wound past the actin belt. Scale bars = 25 μm.

2.3 DISCUSSION

The actin and Rho kinase binding protein, Shroom2, is expressed within the mouse vasculature during development. Here, I demonstrate a role for Shroom2 in the regulation of endothelial morphology, as knockdown of Shroom2 in C166 endothelial cells, HUVECs, and differentiated mES cells results in increased endothelial branching. I propose the following role for Shroom2 in the regulation of angiogenesis during embryonic development (Figure 18). Through the actin binding SD1, and perhaps through the Serine/Proline rich region (SPR) responsible for interaction with ZO1, Shroom2 localizes to cortical actin [188, 203]. Through the SD2, Shroom2 recruits Rock, which is predicted to phosphorylate the MRLC and activate MyosinII, thereby establishing a cortical, contractile network. As indicated by changes in stress fiber organization, pFAK levels, and FA architecture in Shroom2 knockdown cells, Shroom2-dependent contractility can influence additional cellular processes including actin organization, cellular migration, and endothelial branching, ultimately affecting the morphology of the vascular network.

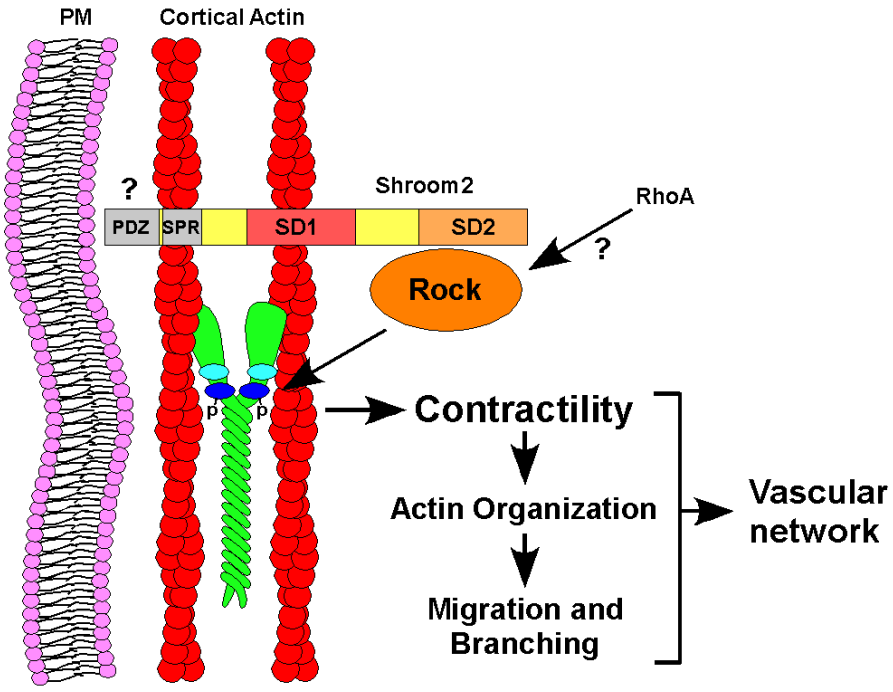

Figure 18: Model of Shroom2 function in endothelial cells.

Shroom2 localization to cortical actin is mediated, in part, by the actin binding domain SD1. The Shroom2 SD2 recruits Rock to cortical actin, where it activates the myosin regulatory light chain of MyosinII, establishing cellular contractility. Changes in contractility are thought to influence cellular migration and branching through changes in actin organization, which ultimately impacts vascular morphology.

It is currently unknown how the Shroom2-Rock complex might be regulated. While many processes that require Rock also require the activity of Rho, previous studies suggest that the ability of Shroom proteins to control contraction is independent of RhoA [187, 189]. Therefore it is possible that Shroom2 binding to Rock serves to both localize and activate the kinase. It is possible that one critical step in regulation is correct sub-cellular distribution. This is supported by previous observations that Shroom2, Shroom3, and Shroom4 exhibit different sub-cellular localization and cause different phenotypes when expressed in cells despite the fact that they all have the capacity to trigger MyosinII-dependent changes in cell shape [188]. While

actin binding is a critical aspect of the correct localization of Shroom proteins, other sequences in the N-terminal region, including the PDZ and SPR domains, likely contribute to their functions. The SPR domains of Shroom2 and Shroom3 appear to participate in direct interactions with ZO1 and POSH, respectively, and their SH3 domains are required for these interactions [199, 203]. Through an interaction with POSH, Shroom3 negatively regulates neurite outgrowth in a Rock-dependent manner [199]. It is intriguing that both Shroom2 and Shroom3 can negatively regulate two physiologically different branching processes. While the Shroom family of proteins have the capacity to alter morphology through an interaction with Rock, Shroom2 is not able to reproduce the apical constriction of MDCK cells caused by Shrm3 expression [188]. It is therefore apparent that through tissue-specific expression and unique localization mechanisms, the Shroom family can alter morphology in similar yet unique ways. It has been proposed that the ZO1/Shroom2 interaction helps facilitate tight junction stability [203]. However, I see no changes in ZO1 localization following Shroom2 knockdown, and siShroom2 treated cells can readily form tight junctions after passage to a new plate (data not shown). Reciprocally, ZO1 knockdown has no effect on Shroom2 localization (Figure 19), suggesting that Shroom2 does not localize via ZO1 alone. Additional experiments will be necessary to elucidate the relationship between ZO1, Shroom2, and the establishment of cortical contractility.

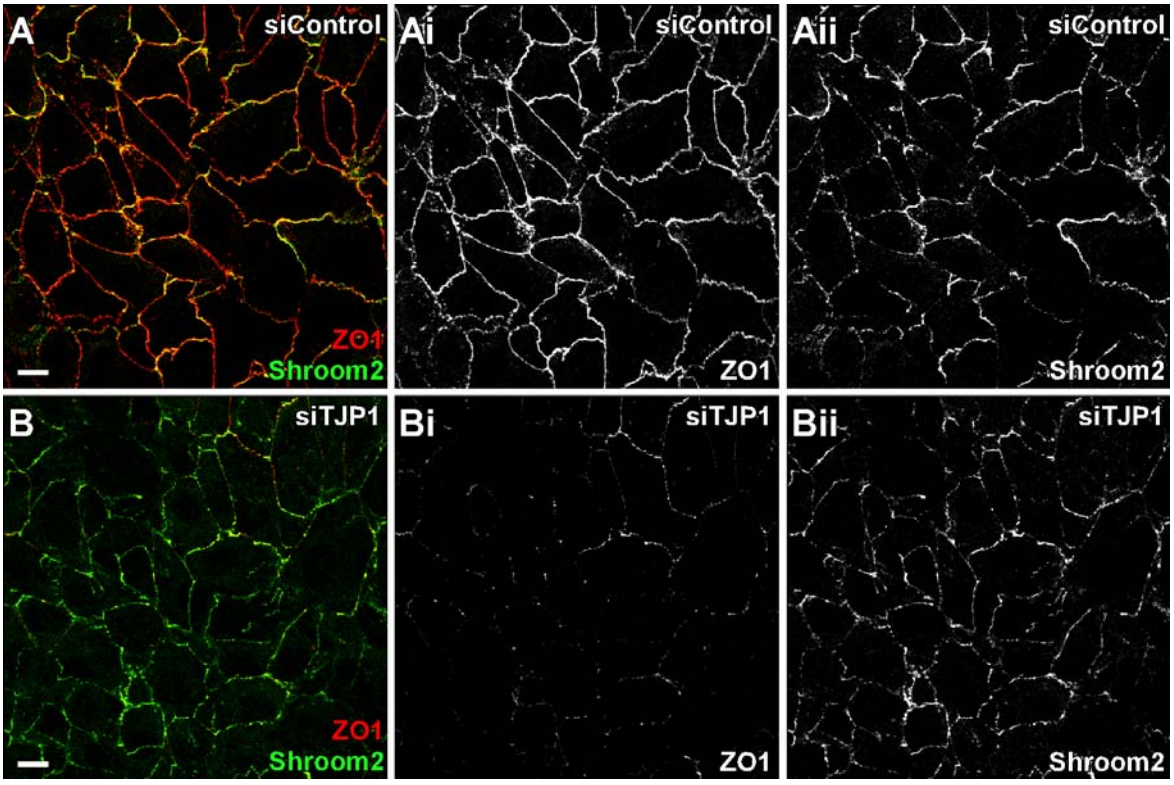

Figure 19: ZO1 knockdown does not impact Shroom2 localization.

(A-B) siControl (A) or siTJP1 (ZO1) (B) treated C166 cells were stained with ZO1 (Ai, Bi) and Shroom2 (Aii,Bii) antibodies 72 hours after transfection. Following ZO1 knockdown, there is no appreciable change in Shroom2 localization. Scale bars = 50μm.

The Shroom proteins are characterized by their evolutionarily conserved Rock binding domain, SD2 [188, 192]. These results demonstrate an interaction between the SD2 of Shroom2 and Rock. I also show that Shroom2 is required for localization of Rock at endothelial tight junctions, and that Shroom2 deficient C166 cells are more sensitive to Rock inhibition, decreasing contractility and increasing migration. These data support a role for Shroom2 in the localization of Rock and establishment of endothelial contractility, the loss of which results in increased angiogenesis. Interestingly, near complete reduction of Shroom2 through stable expression of shRNA or incubation with a Rock inhibitor completely abolishes matrigel

angiogenesis of C166 cells (data not shown). Because transient knockdown with Shroom2 siRNA leads to an increase in branching, I propose that a certain level of Shroom2 / Rock-dependent contractility threshold exists such that slight reductions in contractility increase branching, whereas significant reduction abolishes angiogenesis. It is also likely that Shroom2 mediates only a subset of Rock activity essential for angiogenesis, as Rock activation and localization can be regulated by other factors such as RhoA, lipids, and Dynamin I [66]. And while Shroom2 can interact with both Rock1 and Rock2, it remains to be determined whether or not the different Rock isoforms impact angiogenesis

Conflicting reports for the role of Rock in angiogenesis have been well documented (reviewed in [231]). Many of these studies utilize general Rock inhibitors which present the possibility of off-target effects. Here, I suggest that Shroom2 is involved in a specific aspect of Rock activity during angiogenesis, specifically the cortical recruitment of Rock and activation of downstream MyosinII activity. The unique ability of Shroom2 to recruit Rock to a specific sub-cellular location, suggests that Shroom2 may provide a novel way to target specific Rock-dependent processes while leaving others unaffected. Several studies of the role of Rock in the vasculature support our findings and predictions. Rock RNAi increases endothelial sprouting in both HUVEC spheroid culture and murine retinas [177]. It has also been demonstrated that local loss of MyosinII cortical contractility results in filopodia-like extensions in endothelial cells [178]. Additional studies suggest that Rock-dependent actomyosin contractility can lead to VE-cadherin accumulation at endothelial cell-cell junctions promoting cell adhesion, inhibition of VEGFR2, and vessel quiescence. Perturbation of this system through Rock inhibition or knockdown of VE-cadherin leads to an increase in vessel sprouting [218]. The phenotypic outcome of Shroom2 knockdown is reminiscent of that observed following VE-cadherin

knockdown such that both lead to increased cord formation and decreased MLC2 phosphorylation [218]. Interestingly, following transient knockdown of Shroom2 in C166 cells, I do not see changes in cell-cell adhesion, as measured by β-catenin and ZO1 staining. Therefore, it appears that the ability of Shroom2 to control contractility and subsequent endothelial morphogenesis may be independent of cadherin-mediated adhesion. However, it should also be noted that C166 cells are devoid of VE-cadherin (data not shown) and thus may use another cadherin for cell-cell interactions that does not function in an analogous manner to VE-cadherin. Another distinction between these experimental systems is that C166 cells do not require the addition of VEGF, suggesting that the relationship between VEGF, VE-cadherin, and morphogenesis has been uncoupled in these cells. This suggests that there may be different, independent pathways working to promote the formation of peripheral actomyosin networks which control the angiogenic behavior of endothelial cells. Alternatively, it could be that these two pathways may intersect at the level of the cortical actin network in adherent cells in order to reinforce one another and that both are required to maintain cortical contractility and restrict the migratory and branching potential of endothelial cells. The link between cadherin signaling and Shroom2 will be an interesting and valuable avenue of investigation.

In conclusion, I show that Shroom2 is expressed in the developing vasculature and is required for proper angiogenesis. Reducing Shroom2 levels with Shroom2 RNAi decreases endothelial contractility and leads to increased endothelial sprouting. Because Shroom2 physically interacts with Rock and Shroom2-deficient cells are more sensitive to Rock inhibition, I propose that Shroom2 and Rock interact to regulate cellular contractility which in turn controls cytoskeletal architecture, motility, and ultimately, endothelial angiogenesis.

3.0 SHROOM2 IS A CENTROSOME-ASSOCIATED PROTEIN IMPORTANT FOR CENTROSOME DUPLICATION

The Shroom proteins are regulators of epithelial morphogenesis and are characterized by their ability to bind both F-actin and Rho-kinase (Rock), an activator of contractile, nonmuscle MyosinII. In this section, I describe the characterization of a novel function for Shroom2 in the regulation of centrosome duplication. The centrosome consists of two orthogonal microtubule structures termed centrioles which duplicate precisely once during the cell cycle. Disruptions to the temporal, spatial, or numerical control of centrosome duplication can lead to extra centrosomes which have been linked to chromosomal instability and cancer. Through immunostaining, biochemical isolation, and exogenous protein localization, I demonstrate that Shroom2 is a centrosome-associated protein. Chronic depletion of Shroom2 leads to inhibited proliferation, increased multi-nucleation, and ectopic centrosomes. Results from deletion and rescue analysis suggest that Rock activity is required for Shroom2 function at the centrosome. From these observations I propose a novel role for Shroom2 in the regulation of centrosome duplication.

3.1 INTRODUCTION

Shroom-family proteins are regulators of epithelial morphogenesis and are essential for the development of vertebrate tissues such as the neural tube [184, 187, 192], gut [194, 196], lens placode [195], and vasculature [232]. In vertebrates, the Shroom2-4 proteins have been characterized by two distinct activities. First, all contain a highly conserved C-terminal SD2 that binds to Rock1/2 [184, 188, 192, 232] and leads to subsequent activation of Myosin II. Second, these proteins directly bind F-actin. Actin dependent localization of Shroom proteins and their recruitment of Rock to specific sub-cellular locales is important in the establishment of contractile actomyosin networks required for apical constriction and subsequent neural tube closure, bending of lens epithelium, looping of the gut, and branching morphogenesis of endothelial cells [184, 187, 189, 192, 232].

While the ability to bind both Rock and actin is conserved amongst the Shroom proteins, Shroom family members may not function redundantly. For example, only Shroom3 can elicit apical constriction of either MDCK cells or ectodermal cells in *Xenopus* embryos [188]. However, in such a model of apical constriction, when chimeric proteins are generated in which the Shroom3 SD2 is replaced with a Shroom2 or Shroom4 SD2, apical constriction is rescued [188]. This confirms the importance of the SD2 / Rock interaction in mediating morphogenesis, but also suggests the importance of the N-terminus in defining localization and/or function. Consistent with this notion, I have recently identified the importance of Shroom2 and Rock in controlling endothelial cell morphology as described above. These results suggest that in endothelial cells, Shroom2 is targeted to the cortical actin cytoskeleton and cell-cell junctions, likely through actin binding and a Serine/Proline-rich region shown to interact with ZO-1 [188, 203]. Shroom2 then recruits Rock to these sub-cellular locales. In these cells, transient

knockdown of Shroom2 leads to aberrant Rock distribution, a loss of cortical contractility, and increased angiogenesis [232].

While Rock1 and Rock2 are well-defined regulators of cytoskeletal dynamics and architecture, they also play important roles in centrosome biology. The centrosome consists of two orthogonally aligned centrioles which are surrounded by an amorphous protein matrix known at the pericentriolar material (PCM). The centrosome duplicates precisely once during mitosis to ensure the proper assembly of a bi-polar spindle (reviewed in [233]). Initiation of duplication is tightly controlled to ensure proper centrosome number. In mammalian cells, a procentriole forms perpendicular to the wall of each parent centriole around the G1/S transition. A primary regulator of centrosome duplication is Polo-like kinase 4 (Plk4). Inhibition of Plk4 blocks centrosome duplication, whereas overexpression induces excessive duplication, however the specific targets of this kinase remain unknown [234, 235]. Additional procentriole components have been identified which determine centriole structure such as Sas6, CPAP, and Cep135. Upon initiation of duplication, SAS6 and Cep135 form a procentriolar 'cartwheel' which forms the structural basis for the nine-fold symmetry of the microtubules [236, 237]. CPAP also localizes to the procentriole before MT assembly and is required for initial attachment of singlet MTs [238]. CPAP may also regulate centriole length as overexpression results in abnormally long centrioles [239]. By unknown mechanisms, the existence of the procentriole limits additional centriole duplication [240]. Disengagement of orthogonal centrioles during anaphase is required for initiation of duplication and relies upon the activity of separase, however the protein which engages orthogonal centrioles and the substrates of separase during disengagement are unknown [241].

Deregulation of centrosome duplication can lead to excess centrosomes which perturb proper spindle assembly, promote chromosome segregation errors, and ultimately promote chromosomal instability and tumorigenesis [242, 243]. Rock2 localizes to the centrosome and participates in centrosome duplication such that overexpression of constitutively active Rock2 promotes centrosome duplication and down-regulation of Rock2 inhibits duplication [244]. While regulators of Rock2 activity at the centrosome have been identified, such as Morgana/chp-1 and Nucleophosmin (NPM)/B23, it remains unclear how Rock2 localizes to the centrosome and which effectors mediate centrosome duplication [244, 245].

Here I present evidence through immunofluorescence, biochemistry, and domain mapping that Shroom2 is a centrosome-associated protein. In order to determine whether or not Shroom2 regulates centrosome function, I examined an endothelial cell line deficient for Shroom2 which was described in section 2.2.3. With little to no Shroom2 protein, these cells exhibit inhibited proliferation, increased multi-nucleation, and inefficient centrosome duplication. Despite the observation that centrosomes duplicate less efficiently without Shroom2, knockdown cells may assemble extra centrosomes through the accumulation of centrin aggregates. The excess centrin and centrosome phenotypes are rescued upon reintroduction of RNAi-resistant Shroom2 protein and is dependent upon the SD2 and Rock. Taken together, our results identify Shroom2 as a centrosome-associated protein which is important for regulation of centrosome duplication.

3.2 RESULTS

3.2.1 Shroom2 is a centrosome associated protein.

To confirm Shroom2 as a centrosome associated protein, I co-stained HUVECs, C166, HeLa, and NIH 3T3 cells to detect both Shroom2 and γ tubulin, a marker of the PCM (Figure 20). Results from these experiments suggest that Shroom2 is localized to the centrosome. In all cell lines tested, significantly less Shroom2 protein is observed at the centrosome during Anaphase, yet returns by G1 phase (Figure 20). To validate Shroom2 immunostaining at the centrosome, I fixed and immunostained HeLa cells with two different Shroom2 sera. Both sera mark the centrosomes (Figure 21A-B). In all cell lines tested, Shroom2 and γ tubulin immunostaining do not precisely correlate, indicating that Shroom2 does not localize to the PCM. Therefore I examined Shroom2 localization in relation to centrin, a protein incorporated into the distal ends of centrioles [246]. Shroom2 localizes precisely between the two distal centrin puncta, suggesting that Shroom2 either lies at the proximal end of centrioles or is a linker between the two centrioles that comprise the centrosome (Figure 21C). To further confirm that Shroom2 is a centrosome-associated protein, I purified centrosomes by sub-cellular fractionation of C166 cells and subjected the fractions to Western blot analysis. In this assay, Shroom2 co-sediments with γ tubulin in the fractions that correspond to the density at which centrosomes typically sediment (Figure 21D). Based on these observations, I conclude that Shroom2 is a centrosome-associated protein. Within the Shroom family, localization to the centrosome is likely specific to Shroom2 as I cannot detect either Shroom3 or Shroom4 in centrosomes (Figure 22).

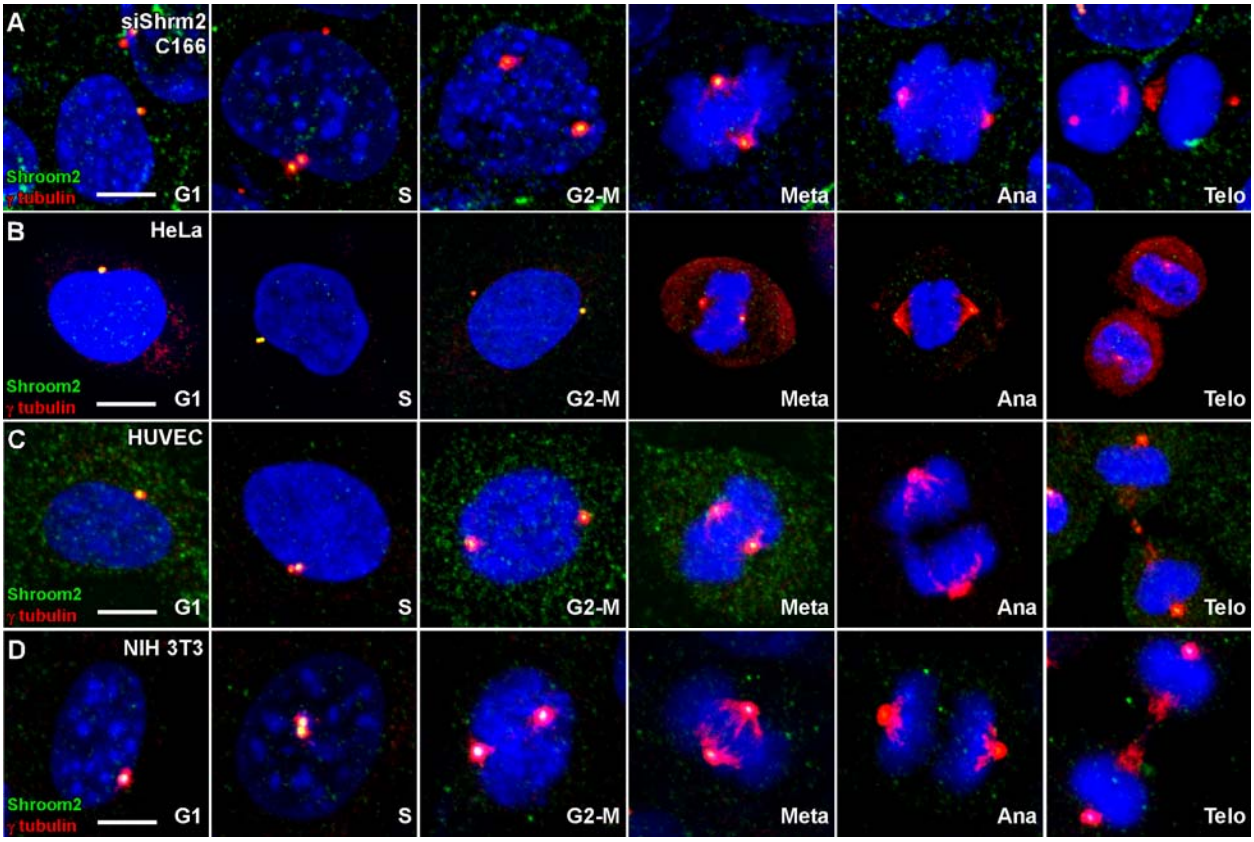

Figure 20: Shroom2 localizes to the centrosome in a variety of cell lines.

(A-D) C166 (A), HeLa (B), HUVEC (C), and NIH 3T3 (D) cells were immunostained with Shroom2 and γ tubulin antibodies and TO-PRO (blue). In all tested cell lines, centrosomal Shroom2 is diminished in anaphase and telophase. C166 cells contain a significant amount of cytoplasmic Shroom2. In A, cells were transiently transfected with siRNA specific for Shroom2 to eliminate the cytoplasmic population. Scale bars = 10μm.

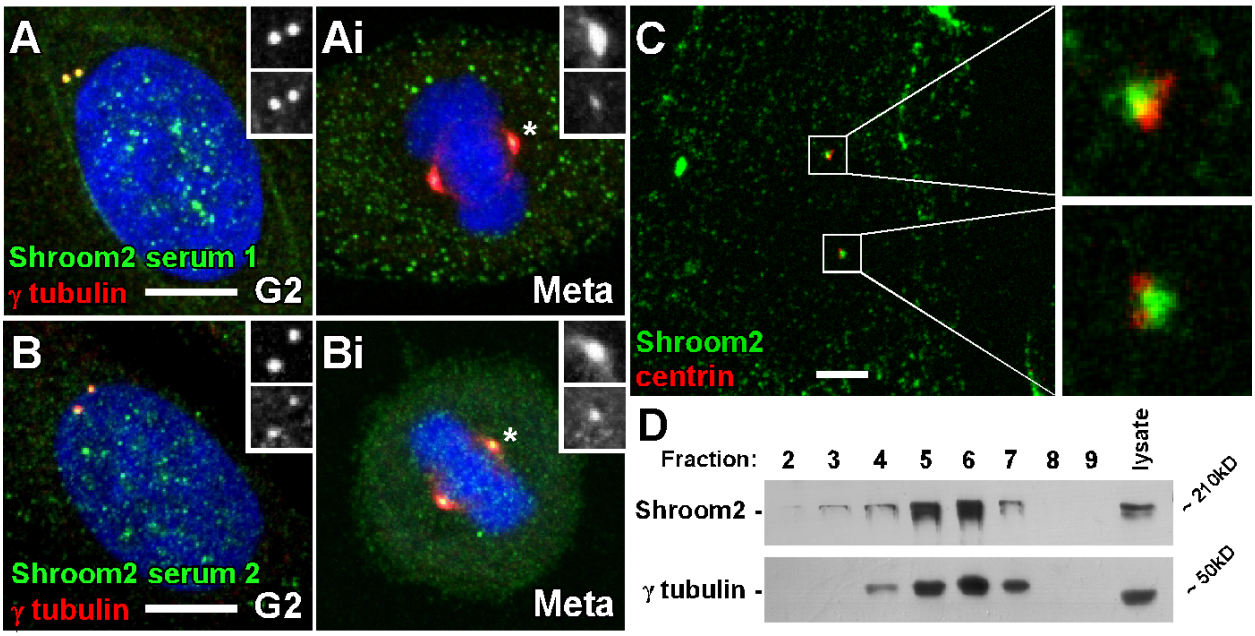

Figure 21: Shroom2 is a centrosome-associated protein.

(A-B) Hela cells stained with two different Shroom2 sera also show localization of Shroom2 at the centrosome. Displayed images are representatives of G2 (A, B) and metaphase (Ai, Bi). Cells were also stained with TO-PRO (blue). All Insets are a magnification of the centrosome denoted by an asterisk. γ tubulin (top), Shroom2 (bottom). **(C)** C166 cells were permeabilized, fixed, and immunostained with affinity-purified Shroom2 and centrin antibodies. Regions of magnification are boxed. **(D)** Centrosome isolation through sucrose density gradients indicates that Shroom2 is present in the centrosome fraction. Scale bars = 10μm.

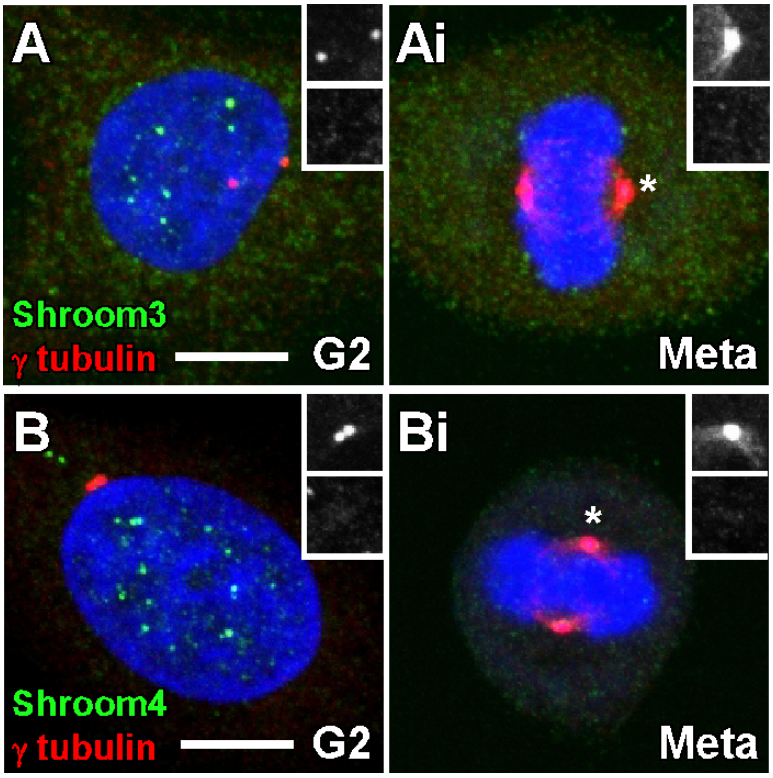

Figure 22: Localization to the centrosome is Shroom2-specific.

(A-B) Hela cells stained with Shroom3 (A) or Shroom4 (B) sera show no localization at the centrosome. Displayed images are representatives of G2 (A, B) and Metaphase (Ai, Bi). All insets are a magnification of the centrosome denoted by an asterisk. γ tubulin (top), Shroom3 or Shroom4 (bottom). TO-PRO staining is in blue. Scale bars = 10μm.

To determine the region important for Shroom2 localization at the centrosome, I generated a series of hShroom2 deletion mutations and expressed these in Cos7 cells. Beginning from the C-terminus, these constructs delete a series of motifs known to influence Shroom2 localization and function. They include the C-terminal SD2, a central, actin binding motif, a MyoVIIA binding region, a Serine / Proline-rich region (SPR) thought to interact with ZO-1, and an N-terminal PDZ domain of unknown function (Figure 23A) [188, 203]. All deletion constructs were tagged at the C-terminus with GFP protein. When expressed in Cos7 cells and subject to Western blot, all truncated proteins exhibit the appropriate molecular mass (Figure 23C). In order to determine localization to the centrosome, constructs were transiently expressed in Cos7 cells and detected by immunofluorescence. Many deletion constructs, including hShroom2 full-length (FL) GFP localize to the centrosome in a manner consistent with endogenous protein (Figure 23B, 23D-G, Figure 24). While hShroom2 1-513 GFP localizes to the centrosome (Figure 23F), hShroom2 1-128, which contains only the PDZ domain, does not (Figure 23G). These results suggest that amino acids 128-513, which contain the SPR, are important for centrosome localization of Shroom2.

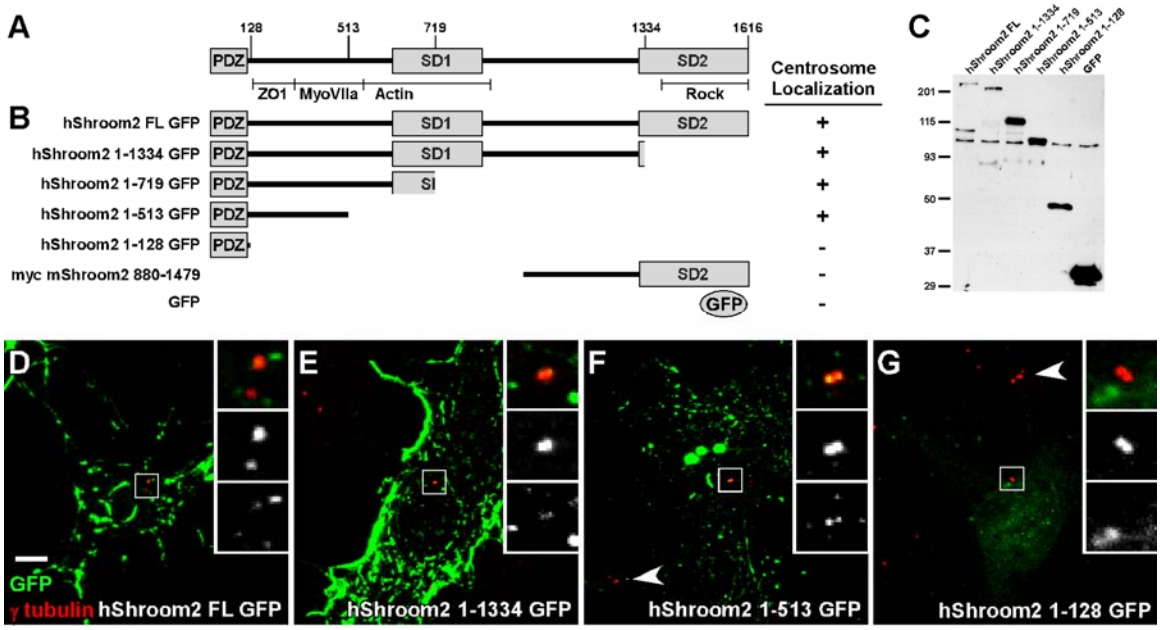

Figure 23: Characterization of Shroom2 localization to the centrosome.

(A) Schematic of known Shroom2 domains (boxes) and interactions (bars). **(B)** Schematic of deletion constructs with a C-terminal, GFP fusion or N-terminal, myc fusion and indication whether they localize to the centrosome (+) or not (-). **(C)** Western Blot (anti-GFP) of Cos7 lysate after transient transfection of the indicated constructs. **(D-G)** Representative images of Cos7 cells transiently transfected with hShroom2 FL GFP (D), hShroom2 1-1334 GFP (E), hShroom2 1-513 GFP (F), and hShroom2 1-128 GFP (G). Localization to the centrosome was visualized by immunostaining with GFP and γ tubulin antibodies. Insets are a magnification of the boxed region. Merge (top), γ tubulin (middle), GFP (bottom). Arrows indicate the centrosomes of untransfected cells with no GFP staining. Scale bar = 10μm.

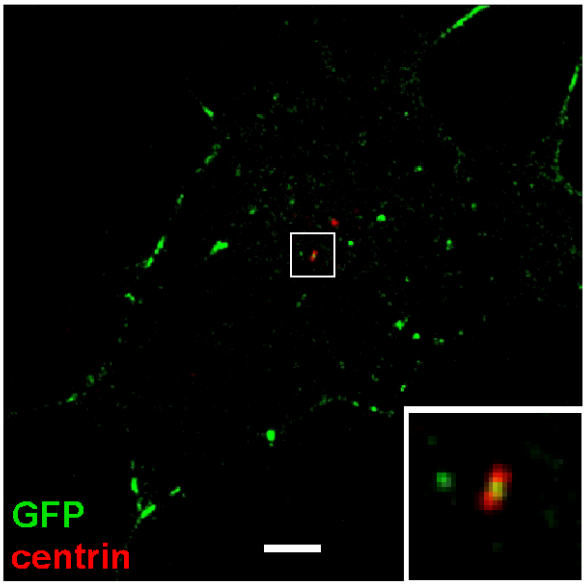

Figure 24: hShroom2 GFP constructs localize to a similar location as endogenous protein.

Cos7 cells transiently transfected with hShroom2 1-719 GFP were permeabilized, fixed, and immunostained with GFP and centrin antibodies. The region of magnification is boxed. Scale bar = 10μm.

3.2.2 Shroom2 is required for centrosome function.

To determine if Shroom2 is important for centrosome function in C166 endothelial cells, I stably knocked down Shroom2 with two different shRNAs (shShroom2-7, shShroom2-8) expressed from the pSuper vector. As described above, little to no Shroom2 protein is detected in these cells via Western blot (Figure 17A). Upon selection of stable knockdown cells, I observed decreased proliferation in both shShroom2 C166 cell lines compared to control (Figure 25A). Stable knockdown of Shroom2 also leads to a significantly higher percentage of multinucleated cells compared to control (Figure 25B). Because inhibited proliferation, multi-nucleation, and chromosomal instability can result from defects in centrosome duplication, I examined centrosome number in these cells [247]. Compared to control, in which 4% of cells have ectopic centrosomes, 28% of shShroom2-7 and 26% of shShroom2-8 cells have three or more

centrosomes, as indicated by γ tubulin immunostaining (Figure 25C-H). While many shShroom2 C166 cells lack Shroom2 staining at the centrosome and possess ectopic centrosomes (Figure 25G), some cells with normal centrosome numbers exhibit detectable Shroom2 staining at the centrosome (Figure 25E, H). In these cells, some transcripts may have escaped the RNAi machinery so that enough Shroom2 protein is made to function at the centrosome. Overexpression of Shroom2 yields no observable defects in centrosome function (data not shown). These results suggest that loss of Shroom2 at the centrosome disrupts centrosome duplication.

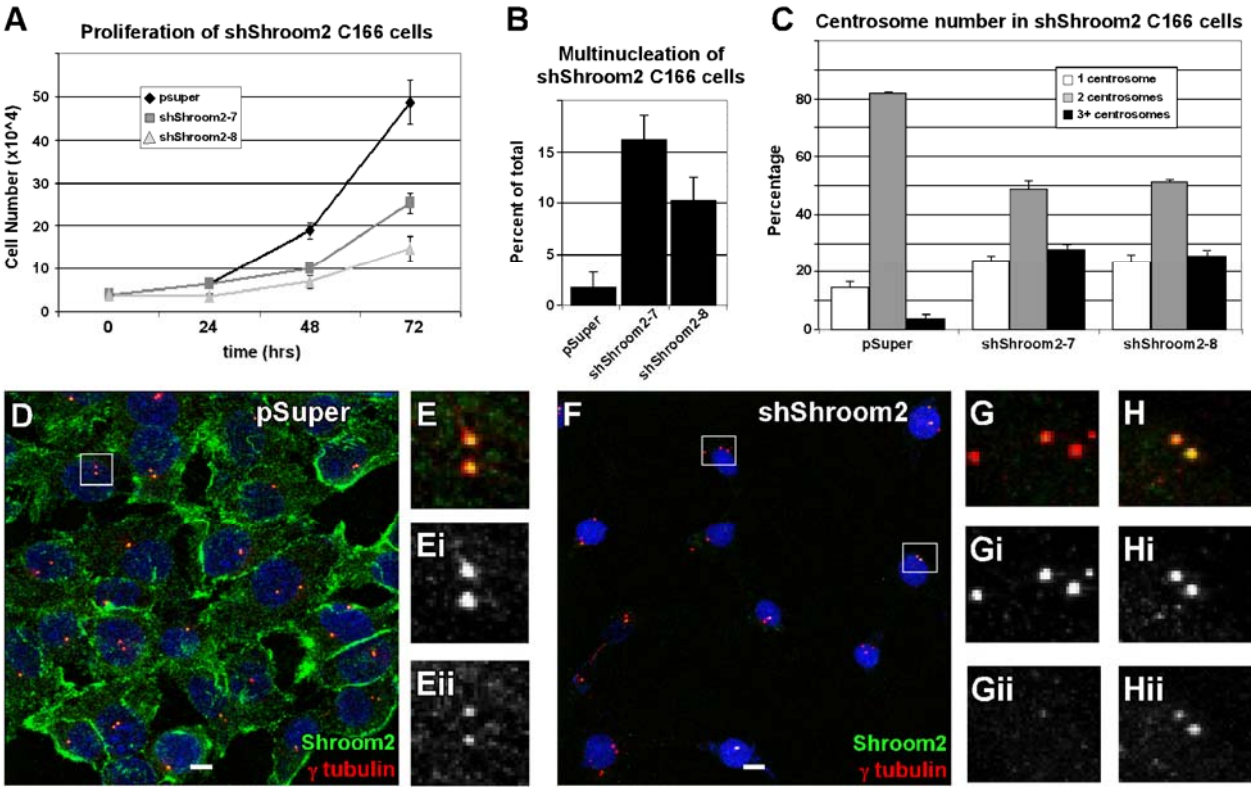

Figure 25: Shroom2 deficiency induces mitotic defects.

(A) C166 cells were stably transfected with parental vector (pSuper) or one of two shRNAs against Shroom2 (shShroom2-7, shShroom2-8). shShroom2 C166 cells proliferate less than control. Average cell number is represented ± SD of triplicate experiments. **(B)** shShroom2 C166 cells display a significant increase in multi-

nucleation compared to control cells. Population percentage is represented ± SD of triplicate experiments. **(C)** shShroom2 C166 cells were stained with γ tubulin antibodies to determine centrosome number. A higher percentage of Shroom2 deficient cells possess ectopic centrosomes compared to control. Population percentage is represented ± SD of triplicate experiments. For (A-C) at least 100 cells were counted per cell line per experiment. **(D-H)** Control (D-Eii) or shShroom2 (F-Hii) C166 cells were immunostained with Shroom2 (Eii, Gii, Hii) and γ tubulin (Ei, Gi, Hi) antibodies and TO-PRO (blue). A magnification of the boxed region represents ectopic (G) or normal (H) centrosomes. Robust Shroom2 staining can be observed at the centrosome in control cells (D,Eii). In shShroom2 C166 cells, Shroom2 is absent from the centrosomes of many cells (F,Gii). In shShroom2 cells with normal centrosome numbers, Shroom2 immunostaining resembles control (Eii,Hii). Scale bars = 10μm.

In order to determine the nature of shShroom2-induced ectopic centrosomes, I stained shShroom2 C166 cells to detect centrin. Interestingly, Shroom2 deficiency causes a striking accumulation of centrin aggregates in more than 80% of shShroom2 C166 cells, compared to 6% in control cells (Figure 26). Ectopic centrosomes, as indicated by γ tubulin, always contain centrin protein, suggesting they are not PCM fragments. However, each mature centrosome should contain two centrin puncta corresponding to the two centrioles. This is not the case, as I frequently observe small γ tubulin puncta co-localized with large centrin aggregates (Figure 26B). This suggests that ectopic centrosomes may form through *de novo* centriole assembly. In this process, centrin aggregates accumulate without a mother centriole, and over the course of the next cell cycle, mature into functional centrosomes [248]. The centrin aggregates in shShroom2 C166 cells are not pre-centriole intermediates, as they contain neither SAS-6, a proximal centriole protein, nor acetylated tubulin (Figure 27, data not shown).

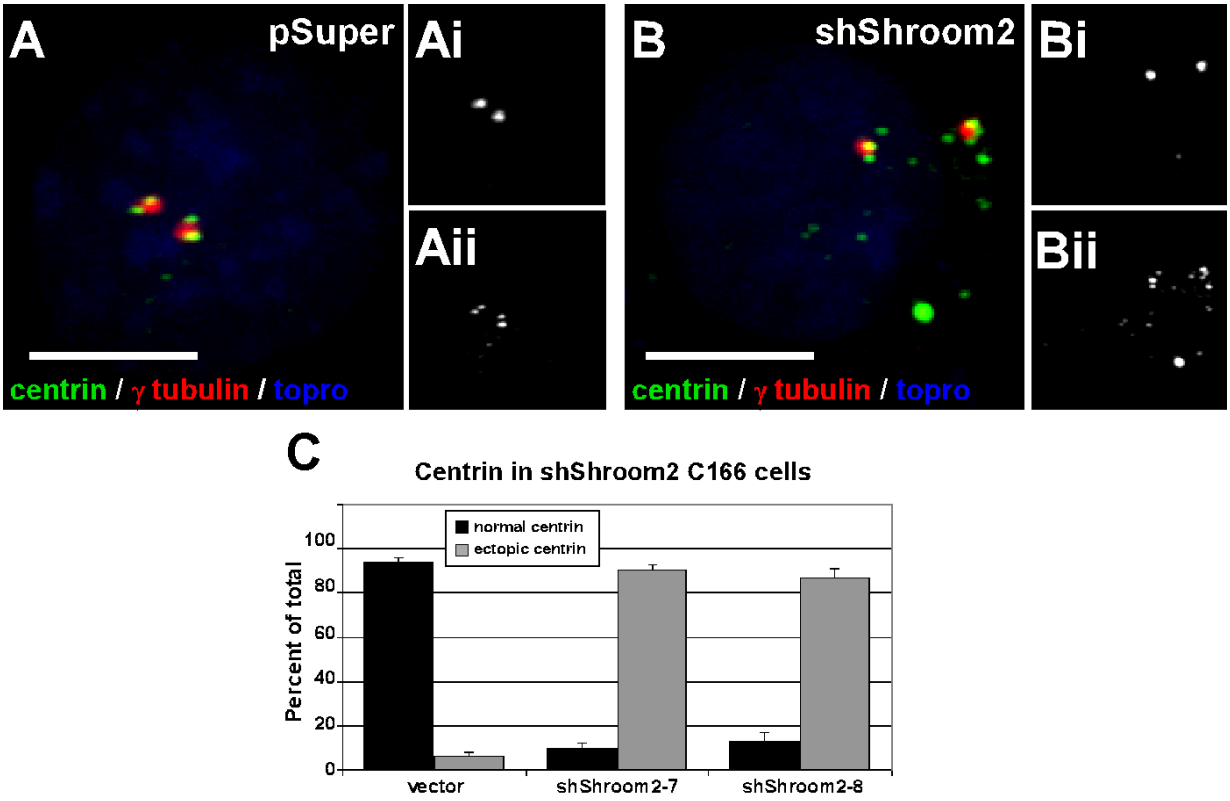

Figure 26: Loss of Shroom2 leads to ectopic centrin aggregates.

(A-B) pSuper control (A) or shShroom2 (B) C166 cells were immunostained with γ tubulin (Ai, Bi) and centrin (Aii, Bii) antibodies and TO-PRO (blue). Shroom2 deficient cells display ectopic centrin aggregates compared to control cells. **(C)** Quantification of centrin puncta in pSuper and shShroom2 C166 cells. Population percentage with normal or abnormal centrin is represented as the mean ± SD. At least 100 cells were counted in triplicate per cell line. Scale bars = 10μm.

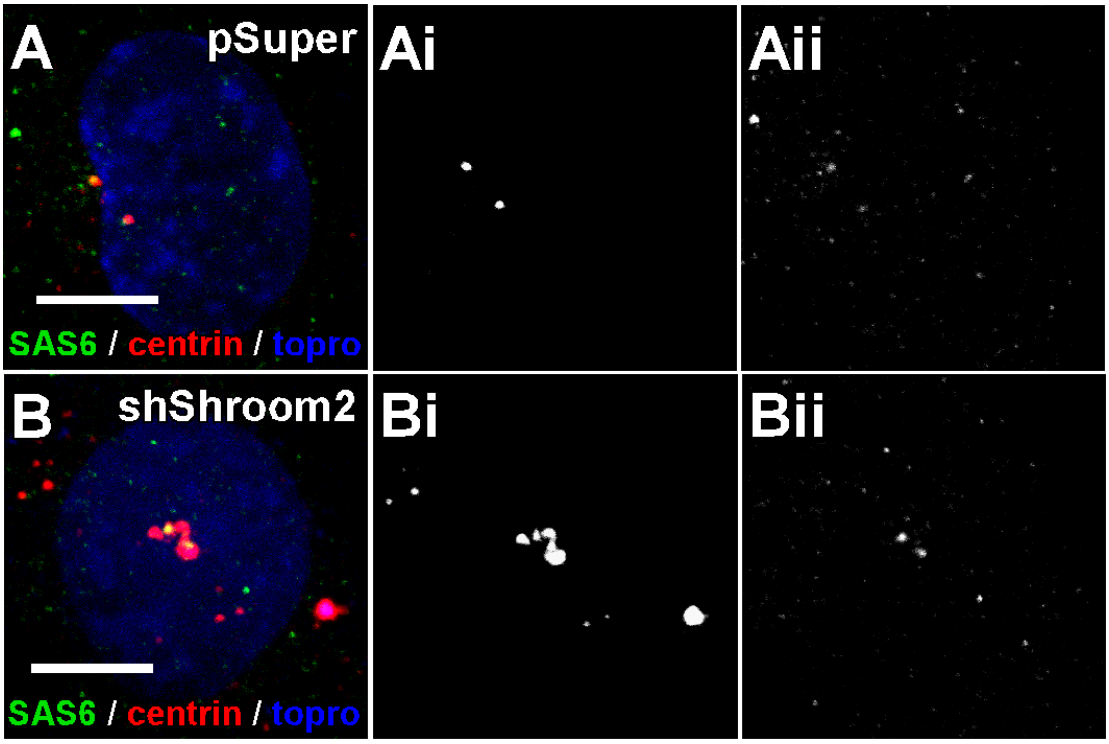

Figure 27: Ectopic centrin aggregates are not pre-centrioles.

(A-B) pSuper (A) and shShroom2 (B) C166 cells were immunostained with centrin (Ai, Bi) and SAS-6 (Aii, Bii) antibodies and TO-PRO (blue). Scale bars = 10μm.

To determine if centrin aggregates are the result of a loss of Shroom2, I attempted to rescue centrin accumulation by transiently transfecting GFP control or full-length hShroom2-GFP, which is shShroom2 resistant. Transfected cells were grown for 48 hours, stained to detect centrin and hShroom2 GFP, and binned based on either normal (one or two centrin puncta) or abnormal (excess centrin puncta) centrin staining. Full-length hShroom2 significantly rescues the excess centrin phenotype compared to control (Figure 28A, C). This activity at the centrosome is specific to Shroom2 as Shroom3 fails to rescue centrin aggregates (data not shown).

3.2.3 Shroom2 activity is required for proper centrosome duplication.

While microtubules are vital to centrosome function, cortical contractility through F-actin and MyosinII are also important for centrosome function [249, 250]. Because long term Shroom2 knockdown in C166 cells leads to changes in cortical contractility and actin organization, I sought to rule out the influence of actin dynamics by measuring duplication directly through a centrosome duplication assay [232]. Such an assay is possible in cells with defective p53 signaling where incubation with DNA synthesis inhibitors such as aphidicolin, blocks DNA synthesis but permits centrosome duplication [251]. C166 cells were created through expression of the fps/fes proto-oncogene [220] and likely are defective in p53 signaling, as centrosome duplication persists after exposure to aphidicolin (see below). For this experiment, pSuper or shShroom2 C166 cells were exposed to aphidicolin or DMSO for 48 hours at which point centrosome number was determined. The ratio of average centrosome numbers between DMSO and aphidicolin treated cells was used to determine the fold change in centrosome number. In these experiments, control C166 cells exhibit an approximate 2-fold increase in centrosome number. In contrast, cells with a Shroom2 deficiency do not duplicate centrosomes as efficiently, exhibiting only a 1.25 fold change in centrosome number (Figure 28D). These results suggest that centrosome duplication is negatively impacted by the absence of Shroom2. Because the loss of Shroom2 also yields an increase in centrin accumulation, I propose that ectopic centrosomes arise through the formation of centrin aggregates and that Shroom2 is required to maintain the efficiency of centrosome duplication.

3.2.4 Rock is required for Shroom2 function.

It has been well documented that Shroom family proteins bind and act via Rock through interaction with the C-terminal SD2 [188, 192, 232]. Because it has been shown that Rock1 and Rock2 are important for centrosome positioning and duplication respectively, I sought to determine whether Shroom2 activity is dependent upon Rock. To investigate this possibility, Shroom2 deficient C166 cells were transiently transfected with expression vectors for either GFP, full-length GFP tagged hShroom2, or a GFP-tagged hShroom2 1-1334, which lacks the SD2, and assayed for centrosome number 48 hours post transfection. Expression of GFP-hShroom2 restores normal centrin in approximately 50% of the cells. In contrast, expression of the SD2 deletion mutant fails to rescue the excess centrin phenotype compared to GFP alone (Figure 28B, C). To further show that this activity of Shroom2 is working through Rock, I performed this rescue experiment in the presence of the Rock inhibitor Y27632. Y27632 abrogates the ability of Shroom2 to rescue ectopic centrin (Figure 28C). Rock inhibition itself does not lead to ectopic centrin accumulation (Figure 29). These results suggest that Rock is essential for Shroom2 function at the centrosome. A Shroom2 construct with the N-terminal half deleted, mShroom2 880-1479, also fails to rescue the centrin phenotype (Figure 28C). As seen with other Shroom proteins, the need for full-length protein suggests that proper targeting of Shroom2 is essential for mediating Rock activity [187, 189].

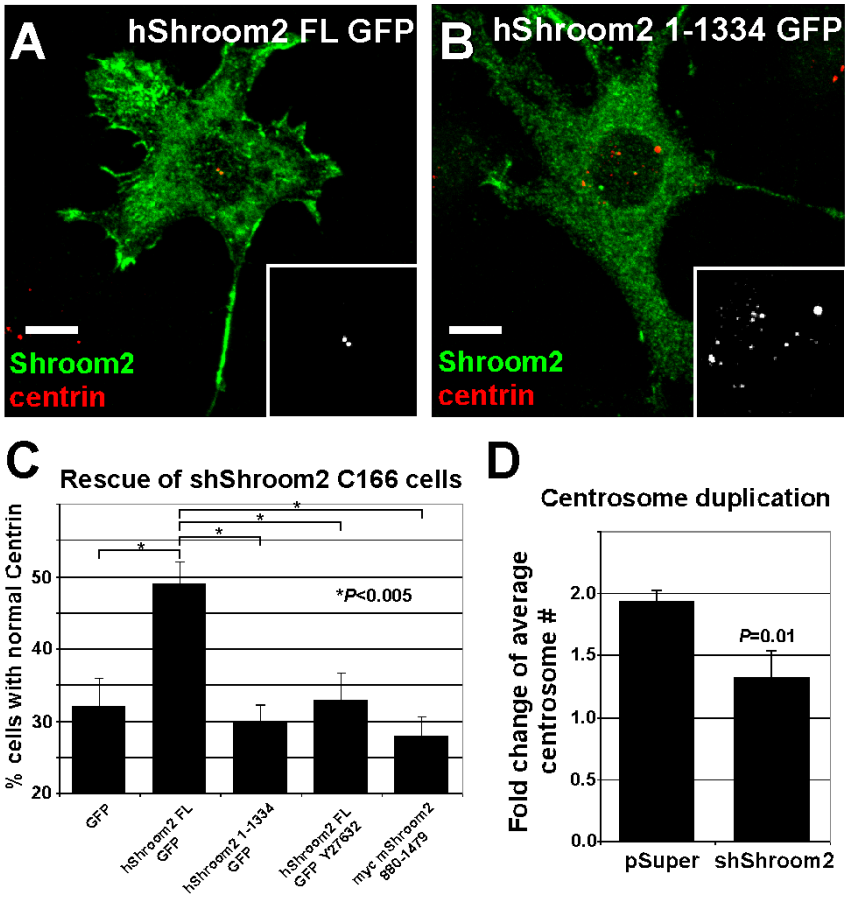

Figure 28: Ectopic centrin can be rescued by exogenous Shroom2.

(A-C) shShroom2 C166 cells were transiently transfected with GFP, hShroom2 FL GFP, hShroom2 1-1334 GFP, hShroom2 FL GFP with 10 μM Y27632, or myc-mShroom2 880-1479 and immunstained to detect centrin. (A-B) Representative images of hShroom2 FL GFP (A) and hShroom2 1-1334 GFP (B). Inset is centrin. (C) Population percentage of cells with normal centrin puncta is represented ± SD of triplicate experiments. **(D)** pSuper and shShroom2 cells were subjected to a centrosome duplication assay. The ratio of the change in average centrosome number is indicated ± SD of three experiments. For (C,D) at least 100 cells were counted per cell line per experiment. Scale bars = 10μm.

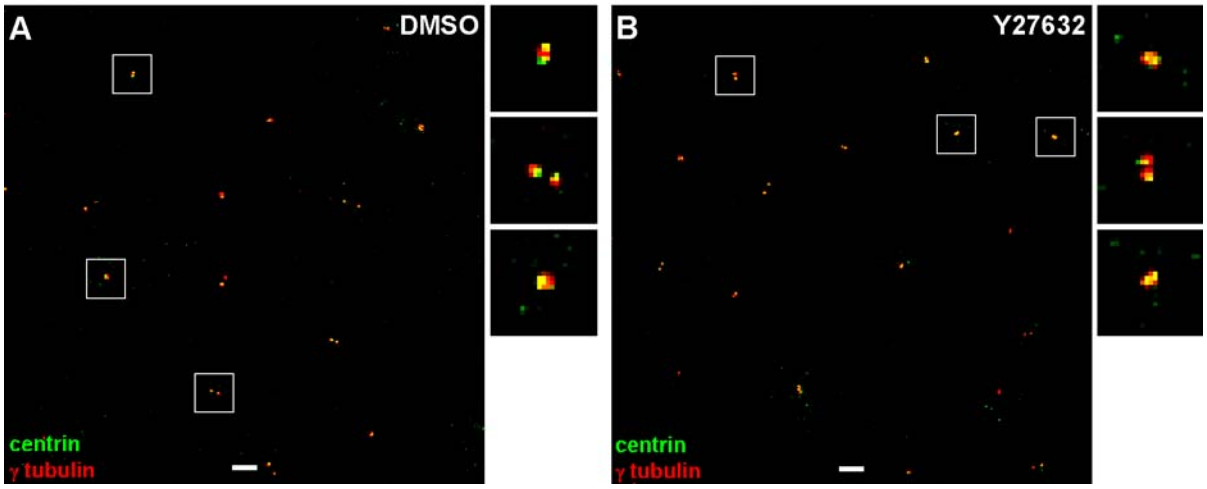

Figure 29: Rock inhibition does not lead to ectopic centrin aggregates.

(A-B) C166 cells were treated with DMSO (A) or 10μM Y27632 (B) for 48 hours and immunostained to detect centrin and γ tubulin. Scale bars = 10μm.

The presence of supernumerary centrosomes is a common trait of many human tumors [252]. In addition, centrosome amplification promotes tumorigenesis in *Drosophila*, implicating conservation of centrosome-mediated carcinogenesis [253]. While it had been believed that extra centrosomes may promote tumorigenesis through formation of multi-polar spindles and aneupoloidy, it has recently been shown that mechanisms exist to cluster multiple centrosomes into two poles [254, 255]. As a result, multipolar spindles occur infrequently, and even then, such progeny mitotically arrest or apoptose [243]. It is more likely that extra centrosomes form multipolar spindle intermediates prior to anaphase where merotelic kinetochore attachments cause lagging chromosomes, segregation errors, and chromosomal instability [243]. Ectopic centrosomes in shShroom2 C166 cells still cluster during anaphase, however chromosomal bridges caused by lagging chromosomes are frequently observed (Figure 30). In addition to spindle formation and tumorigenesis, the centrosome plays a critical role in the formation of cilia. During interphase, many mammalian cells extend a primary cilia which is nucleated by a

centrosome termed the basal body. Shroom2 localizes to the basal body in retinal pigmented epithelial (RPE) cells (Figure 31). As C166 cells do not form cilia in culture, a role for Shroom2 in ciliogenesis has yet to be investigated.

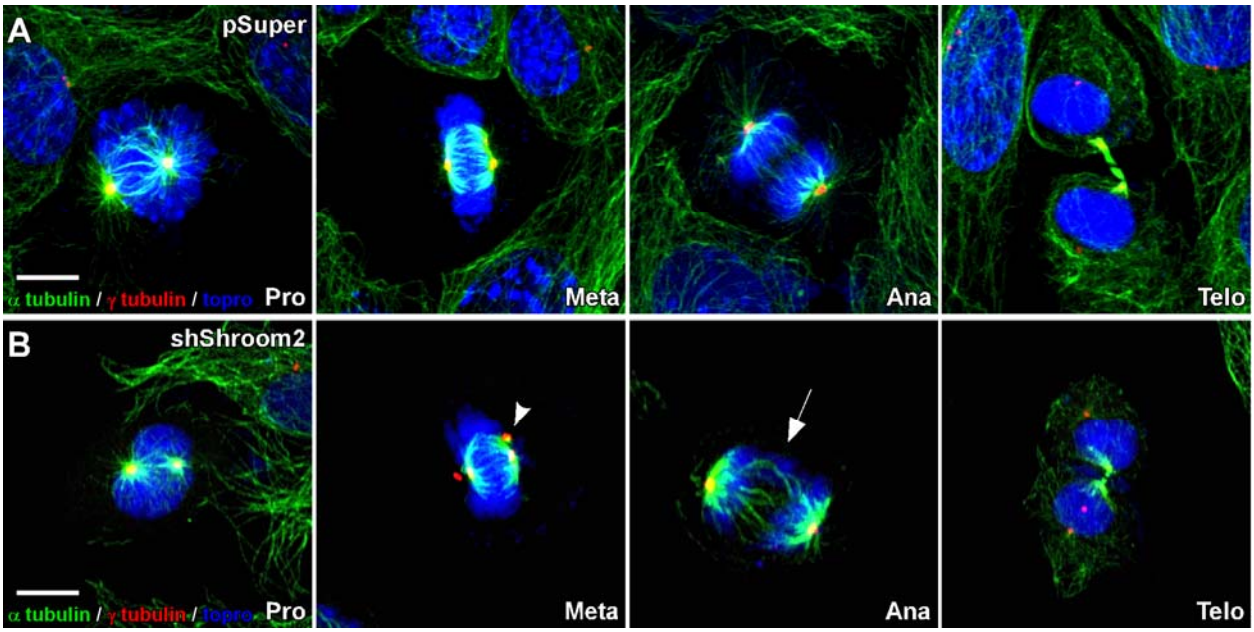

Figure 30: Shroom2 deficient cells contain lagging chromosomes.

(A-B) Asynchronous pSuper (A) or shShroom2 (B) C166 cells were fixed and immunostained for α tubulin and γ tubulin and TO-PRO (blue). Stages of mitosis are indicated. Arrowhead indicates clustering of ectopic centrosomes. Arrow indicates lagging chromosomes. Scale bars = 10μm.

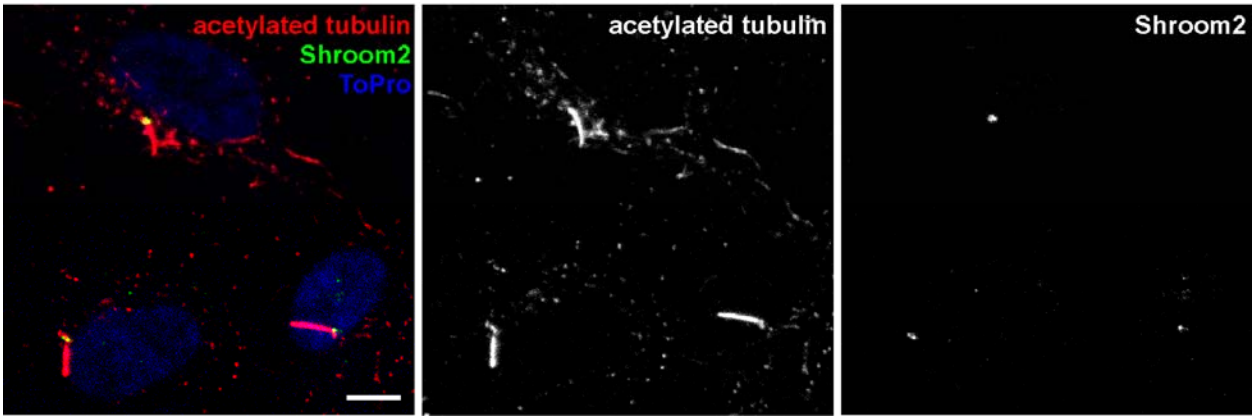

Figure 31: Shroom2 localizes to the basal body of cilia.

RPE1-hTert cells were grown to confluency, fixed, and immunostained with Shroom2 and acetylated tubulin antibodies and TO-PRO (blue). Scale bar = 10μm.

3.3 DISCUSSION

From these results, I provide the first evidence for Shroom2 as a centrosome-associated protein. Localization to the centrosome was first observed in C166 endothelial cells after transient transfection with siRNA against Shroom2 [232]. This was the first study to employ knockdown of Shroom2 in order to elucidate function in cell culture, and because cytoplasmic Shroom2 protein can obscure visualization of the centrosome, centrosomal localization may have passed unnoticed in other studies. Nonetheless, it is intriguing that centrosomal Shroom2 protein remains at the centrosome after transient knockdown. This suggests that centrosomal Shroom2 is either highly resistant to degradation or localizes with high affinity or by post translational modification, such that any protein still expressed after RNAi preferentially localizes to the centrosome. Results from several different experiments, including immunohistochemistry, biochemistry, and localization of exogenous protein, indicate that Shroom2 is a *bona fide*

centrosome protein. Although the centrosome localization domain of Shroom2 lies within amino acids 128-513, further experimentation is required to identify the resident protein(s) that recruits Shroom2 to the centrosome.

While this is the first study to implicate Shroom2 in centrosome function, a connection between Shroom2 and γ tubulin has been established in *Xenopus*. In these studies, expression of Shroom2 in blastomeres leads to apical recruitment of γ tubulin which is proposed to be essential for epithelial lengthening and pigment accumulation in Retinal Pigmented Epithalial (RPE) cells [193, 197]. Shroom3 functions similarly in this system, yet the mechanism of γ tubulin recruitment by Shroom proteins remains to be determined [216]. With regards to mammals, the only relationship reported between Shroom2 and γ tubulin comes from these experiments which demonstrates that centrosomal localization is specific to Shroom2 and not Shroom3 or Shroom4.

Stable, long term knockdown of Shroom2 in C166 cells leads to decreased proliferation, increased multi-nucleation, excess centrin aggregates, and ectopic centrosomes. However when subjected to a centrosome duplication assay, shShroom2 C166 cells duplicate centrosomes less efficiently than control. Because of these observations, it is likely that extra centrosomes derive from centrin aggregates in a process known as *de novo* centrosome assembly, whereby centrosomes are assembled in the absence of a centriole template. Under normal conditions, the mother centriole serves as a base for the formation of the daughter centriole. Mechanisms exist to ensure the proper spatial, temporal, and numerical duplication of centrioles (reviewed in [256]) as well as to inhibit *de novo* assembly [257]. In mammalian cell lines, as displayed in CHO and HeLa cells, *de novo* centriole assembly can occur in the absence of a centrosome [248, 258]. After centrosome ablation in HeLa cells, centrin aggregates converge upon each other and mature into a γ tubulin-containing centrosome over the course of the next cell cycle [248]. While

Shroom2-deficient cells still retain centrosomes, they display a striking accumulation of centrin aggregates. These observations suggest that knockdown of Shroom2 has uncoupled the mechanisms which regulate centrosome number or the *de novo* pathway. The precise nature of excess centrin accumulation and the mechanistic connection between Shroom2 and centrosome regulatory mechanisms remains to be determined.

Possible insight for Shroom2 function at the centrosome comes from previous studies of Rock2. Rock2 is a known centrosome-associated protein which specifically localizes between centrioles. Rock2 is important for centrosome function, as knock down inhibits centrosome duplication [244]. Another centrosome protein NPM/B23 is a positive regulator of Rock2 activity. Upon phosphorylation of NPM/B23 on Thr199 by CyclinE/cdk2, NPM/B23 acquires high binding affinity for Rock2, increasing its kinase activity 5-10 fold [244]. This process is opposed by Morgana/chp-1 which blocks the interaction between NPM/B23 and Rock2, inhibiting centrosome duplication [245]. The small GTPases RhoA and RhoC have also been shown to be important for Rock activity at the centrosome [259]. Though these proteins influence Rock2 activity at the centrosome, they are not sufficient for Rock2 localization. It has been proposed that an unknown interaction with the coiled-coil region of Rock2 is essential for its localization to the centrosome [244]. Interestingly, Shroom2 binds to Rock2 within the coiled-coil region [191, 192, 232]. Because the ability to rescue ectopic centrin accumulation relies upon an interaction between Shroom2 and Rock2, Shroom2 is an excellent candidate as the mediator of Rock2 localization at the centrosome (Figure 32). Due to the subcellular localization of Shroom2 during mitosis, it remains a possibility that Shroom2 and Rock mediate linkage of centrioles with subsequent regulation of Rock activity initiating centrosome duplication.

Additional studies will be necessary to characterize such an interaction and to identify kinase targets at the centrosome.

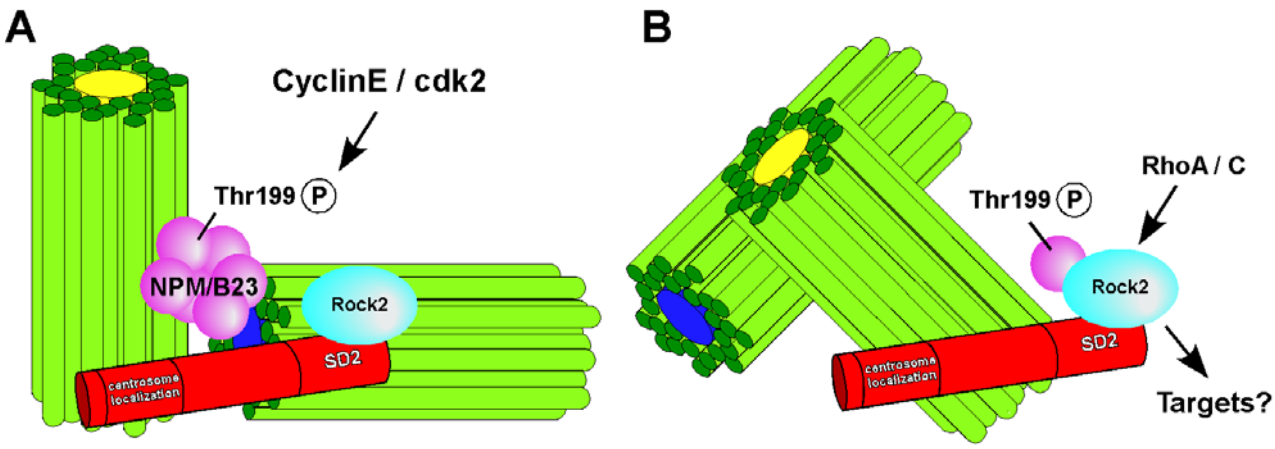

Figure 32: Model of Shroom2 function at the centrosome.

(A) Shroom2 localizes to centrosomes through amino acids 128-513 and likely recruits Rock to the centrosome through its SD2. Once CyclinE/cdk2 phosphorylates Thr199 on NPM/B23, NPM acquires high binding affinity for Rock2 and increases Rock2 kinase activity 5-10 fold. **(B)** The localization of Shroom2, Rock, and NPM/B23 are all essential for efficient centrosome duplication though their effectors remain unknown.

In conclusion, I have demonstrated that Shroom2 is a centrosome-associated protein. Long term loss of Shroom2 leads to centrosomal defects such as delayed centrosome duplication, excess centrin accumulation, hindered proliferation, and lagging chromosomes. Because Shroom2 interacts with Rock2 and this interaction is necessary to rescue ectopic centrin aggregates, I propose that recruitment of Rock2 to the centrosome is mediated by Shroom2 and is essential for efficient centrosome duplication. Because centrosome defects promote genetic instability and are a feature of many cancer cells, it will be interesting to investigate a role for Shroom2 in tumorigenesis in future studies.

4.0 CONCLUSIONS AND FUTURE HYPOTHESES

4.1 SHROOM2 MEDIATES THE SUB-CELLULAR LOCALIZATION OF ROCK

Chapter 2.0 describes the characterization of Shroom2 in endothelial cells. Because the Shroom-family proteins are capable of inducing MyosinII-dependent actomyosin networks, I hypothesized that Shroom2 was important for mediating contractility in endothelial cells. Following Shroom2 knockdown, endothelial sprouting was enhanced, suggesting that Shroom2 negatively regulates angiogenesis. As Shroom2 deficient cells cannot constrict a gel as sufficiently as control, Shroom2 likely mediates angiogenesis through the control of contractility. These results suggest that Shroom proteins may influence the contractility of an entire tissue. During the course of this work, it was shown that Shroom3 physically interacts with Rock through the SD2 [192]. Because all Shroom family members share high similarity within the SD2 [188], I tested whether or not Shroom2 also interacts with Rock. I was able to demonstrate a physical association between Shroom2 and Rock and also demonstrate Shroom2-dependent sub-cellular localization of Rock at the cell cortex, supporting a role for Shroom2 in establishing cortical contractility. These findings are significant, because Rock has historically been thought of as a cytoplasmic kinase with little to no specific localization patterns *in vivo* [229]. My results demonstrate that Shroom2 is responsible for the localization of Rock to the cortex, and this precise localization is necessary for Rock activity.

How then does Shroom2 mediate Rock activity through sub-cellular localization? There are several possibilities. First, Shroom2-dependent localization of Rock may simply place Rock in close proximity to activation signals. While RhoA is a key activator of Rock, previous studies suggest that the ability of Shroom proteins to control contraction is independent of RhoA [187, 189]. As lipids have also been shown to activate Rock [260], it could be that localization to the cortex is sufficient to allow lipid-activation of Rock. As a second possibility, the physical interaction between Shroom2 and Rock may be sufficient to induce activation. The Shroom binding domain within Rock is located in close proximity to the Rho binding domain. Because the relief of intramolecular inhibition activates Rock activity, the simple binding of Shroom2 and Rock may relieve intramolecular inhibition of Rock, leading to its activation. And last, Shroom2 may simply be a scaffolding protein which recruits all of the necessary components to establish actomyosin networks. Shroom2 binds to actin and Rock, so additional, unidentified interactions with Rock activating proteins could exist. One might speculate that the PDZ domain is important for function however it is not required for Shroom3-induced apical constriction and only mildly affects Shroom2 localization. The highest region of identity between Shroom2 and Shroom3 lies within the PDZ domain, so its function remains an intriguing question.

Regardless of how Rock is activated, there are likely additional mechanisms which mediate the interaction between Rock and Shroom2. For example, Shroom2 and Rock do not always co-localize (compare Figure 4C with Figure 10A). Post-translational modifications such as phosphorylation of Shroom2 may mediate an interaction with Rock. Another level of regulation could be through additional protein interactions like Gem/Rad which are hypothesized to block protein interactions with Rock [67].

Shroom3 interacts with Rock to induce apical constriction in MDCK cells [192]. Placed within this same system, Shroom2 does not elicit apical constriction [188]. Because Shroom2 can mediate the activity of MyosinII through Rock localization, it is likely that Shroom2 and Shroom3 enable different contractile events. Shroom3 may promote dynamic, rapid contraction, and Shroom2 may promote stable, cell tension. It will be interesting to examine the effects of Shroom2 expression in MDCK cells by measuring changes in cell rigidity through techniques such as traction force microscopy or atomic force microscopy. Although Shroom2 shows no Rock isoform specificity, we have yet to examine MyosinII isoforms. Based on the kinetic studies of MyosinIIB which implicate it in the maintenance of stable, tension generating fibers, it might be that Shroom2 promotes only MyosinIIB activity. As Shroom2 and Shroom3 are co-expressed and co-localize to the TJ of many epithelial tissues [188, 189, 192], it will be interesting to examine the relationship between the two. A combinatorial approach to studying multiple Shroom proteins in morphology has yet to be addressed.

An additional question concerning not only the Shroom proteins but the cell biology field as a whole is how actin binding proteins target specific populations of F-actin. Shroom2 and Shroom3 both contain the SD1 and can bind to actin, however they do so differently. Shroom3 can bundle actin, while Shroom2 cannot. In Rat1 fibroblasts, the SD1 of Shroom2 localizes to the cortex, while the SD1 of Shroom3 localizes to and bundles basal stress fibers. Outside of the SD1 and SD2, unique protein interactions have been identified for Shroom3 and Shroom2, so it is also likely that different protein interactions influence differential localization. Examination of the crystal structure of the different SD1 regions will provide valuable insight into the differential localization of Shroom proteins and hence the differential localization of Rock.

4.2 IMPLICATIONS FOR ANGIOGENESIS

Shroom2 influences endothelial morphology through the regulation of cortical contractility. The loss of Shroom2 abolishes cortical Rock localization and leads to changes in the actin cytoskeleton such as decreased stress fibers and increased filopodial extensions. Because contractility influences adhesion and migration, Shroom2 deficiency alters the angiogenic potential of these cells.

It is interesting that transient knockdown of Shroom2 exhibits different effects than stable knockdown in C166 cells. With normal levels of Shroom2 and high levels of contractility, endothelial cells exhibit moderate angiogenic potential. Following transient knockdown of Shroom2, contractility is reduced but not abolished, and these cells show the highest ability to undergo sprouting angiogenesis. Finally, after near depletion of Shroom2, cortical contractility is largely ablated and cells can no longer undergo angiogenesis. These results support the notion that an ideal level of contractility is essential to cell behavior, particularly angiogenesis, and by tipping the balance in one direction or the other, we can alter the outcome. These results are supported by a recent study which shows endothelial cell adhesion is regulated by varying adhesive ligand density as an independent variable in synthetic hydrogels [179]. In this model, too much or too little adhesion is detrimental to angiogenesis. As the loss of Shroom2 also leads to changes in stress fiber and FA organization, it is likely that contractility influences angiogenesis through changes in cell adhesion.

Shroom2 knockdown may also influence the stability of adherens junctions. While transient Shroom2 knockdown did not affect β-catenin localization, the impact on cadherins remains unknown. As I could not detect PECAM or VE-cadherin in C166 cells, it will be necessary to examine a more "endothelial-like" cell line which expresses these endothelial-

specific adhesion proteins. Based on the failure of shShroom2 C166 cells to form stable adherens junctions, it is likely that Shroom2 influences endothelial adhesion through the control of Rock activity and cortical contractility.

Although Shroom2 interacts with Rock1 and Rock2, it is unknown if Shroom2 utilizes both isoforms to mediate endothelial morphogenesis. As discussed in section 1.1.4.1, Rock may function redundantly *in vivo*, however in fibroblasts, knockdown of Rock1 and Rock2 have different effects. Loss of Rock1 impedes stress fiber and FA organization, while loss of Rock2 enhances the actin cytoskeleton. A role for Rock isoforms in angiogenesis has not been addressed. In the future, obtaining Rock2 antibodies for immunostaining and utilizing isoform specific siRNAs will address any differential roles for Rock1 and Rock2 during angiogenesis.

Abnormal regulation of Rock signaling is associated with various cardiovascular diseases such as hypertension, coronary and cerebral vasospasm, restenosis, atherosclerosis, stroke, and heart failure, and thus Rock inhibitors are currently under development for clinical use [261]. In addition, solid tumors stimulate angiogenesis in order to supply cells with nutrients from the body, so developing the means to inhibit angiogenesis could effectively reduce tumor size. In terms of cardiovascular disease, many drugs currently in development, such as Fasudil, are general Rock inhibitors. Due to the plethora of roles for Rock in cell biology such as contraction, actin organization, adhesion, motility, and proliferation, general Rock inhibition may lead to many detrimental secondary effects. More specific inhibitors have been developed, such as SLx2119, a Rock2 inhibitor, yet this only prevents promiscuity of interactions with other kinases and could still block all Rock2-dependent functions. Here, I report a mechanism in which Shroom2 mediates only a subset of Rock activity. These results create the possibility that by blocking the interaction between Shroom2 and Rock, we may effectively inhibit only one

facet of Rock activity in the cell, thus developing a means to specifically inhibit *in vivo* angiogenesis.

An important question remains: what role does Shroom2 play during the formation of a three dimensional vascular network. No matter the assay, angiogenesis and vasculogenesis assays rely upon cellular adhesion, migration, and sprouting to influence the resulting network. Based on evidence that Shroom2 mediates Rock activity to influence these three traits in two dimensions, it is likely that during early stages of cord-formation, decreased adhesion and enhanced migration and sprouting increases branching of the vascular network. The primary benefit of *in vitro* angiogenesis experiments lies with ease of use. *In vitro* models allow for relatively easy manipulation of cells or growth factors to visualize the impact on angiogenesis, however they restrict analysis to a preslected pool of cells which do not interact in a heterogenous cell population [262]. Ultimately the effects of Shroom2 on angiogenesis must be carried out *in vivo* to confirm the findings described above. The chick chorioallentoic membrane assay provies an *in vivo* tool in which endothelial cells can still be targeted for siRNA. But ultimately, conditional knockout of Shroom2 in endothelial cells during development will allow the best physiological evaluation of the role of Shroom2 in angiogenesis. Based on the experiments described above, a likely role exists for Shroom2 / Rock in the promotion of stable endothelial vessels. Upon reduction of Shroom2 or Rock activity, cellular adhesion structures may break down, promoting endothelial migration and subsequent sprouting angiogenesis.

4.3 SHROOM2 AND THE CENTROSOME

Chapter 3.0 describes the characterization of Shroom2 and the centrosome. I have employed several techniques to confirm that Shroom2 is a centrosome-associated protein. First, centrosome localization of Shroom2 is observed in multiple cells lines with two different Shroom2 sera. Second, biochemical purification of the centrosome through sucrose gradient centrifugation leads to the co-fractionation of Shroom2 and the centrosome. And finally, exogenous Shroom2 protein localizes to the centrosome in a manner consistent with endogenous protein. These results confirm the novel localization of Shroom2 at the centrosome. This is a unique observation as this is the first example of localization of Shroom2 which is not associated with F-actin. While we cannot exclude the possibility that actin may be present at the centrosome in very small amounts, there is no significant evidence demonstrating centrosomal actin, and treatment of cells with the actin depolymerizing drug Cytochalasin D does not affect Shroom2 localization at the centrosome (data not shown). Additionally, the region of Shroom2 responsible for centrosome localization lies outside of the actin binding domain within a.a. 128-513 which corresponds to the SPR. To identify protein interactions responsible for Shroom2 localization to the centrosome, it will be necessary to perform pull-down experiments using hShroom2 128-513 GFP with isolated centrosomes followed by mass spectrometry.

I first observed centrosomal localization of Shroom2 following transient knockdown in C166 cells. The observation that a population of Shroom2 protein persists at the centrosome after transient knockdown suggests one of two things. First, the turnover of Shroom2 protein at the centrosome may be slow, thus localization persists throughout the time course of the knockdown experiment. Second, Shroom2 may localize with high affinity such that any protein which escapes the RNAi machinery preferentially localizes to the centrosome. Stable

knockdown of Shroom2 leads to a depletion of Shroom2 at the centrosome in some cells, but residual protein in other cells still results in Shroom2 localization to the centrosome. This observation supports the hypothesis that Shroom2 has a high affinity for the centrosome. This could be tested in the future through fluorescence recovery after photobleaching (FRAP).

Long term knockdown of Shroom2 leads to centrosome defects. Shroom2-deficient cells cannot duplicate centrosomes as efficiently as control. Interestingly, a similar role for Rock2 has been proposed as knockdown or inhibition of Rock during centrosome duplication assays also hinders centrosome duplication [244]. As the ability of Shroom2 to rescue centrosome defects relies upon the presence of the SD2 and hence an interaction with Rock, Shroom2 likely mediates localization of Rock at the centrosome. Additional experiments such as examining the localization of Rock at the centrosome before and after Shroom2 knockdown will be useful to determine the relationship between Shroom2 and Rock at the centrosome. In addition, we can test if the Shroom binding domain of Rock localizes to the centrosome in a Shroom2-dependent manner. Ideally, it may act as a dominant-negative, and recapitulate the Shroom2 knockdown phenotype. The effectors of Rock which mediate centrosome duplication are unknown.

While I have shown that the loss of Shroom2 directly impacts centrosome duplication through aphidicolin treatment, the possibility remains that shShroom2-induced changes in cellular architecture still affect centrosome duplication. To exclude this possibility it will be necessary to show that other cellular processes or organelles such as the Golgi and nucleus are unaffected in the shShroom2 C166 cell line. Alternatively, targeting Shroom2 activity to the apical surface with an endolyn tag would address the need for cortical contractility in centrosome duplication. Additionally, the development of a functional dominant-negative protein which

blocks localization or activity of Shroom2 at the centrosome will address any secondary defects brought about by long-term Shroom2 knockdown.

In addition to defects in centrosome duplication, stable Shroom2 knockdown leads to the accumulation of ectopic centrin aggregates. The nature of these aggregates is unknown, and they do not appear to be pre-centriole intermediates. Centrin aggregates have also been observed in HeLa cells in which the centrosome has been ablated. Over time, the centrin aggregates mature into a functional centrosome in a process known as the *de novo* centrosome assembly pathway, the molecular regulation of which is currently unknown [248]. To determine whether or not centrin aggregates following Shroom2 knockdown participate in a similar mechanism, it will be necessary to visualize centrin and γ tubulin through live cell imaging.

Knockout mice are great models to understand gene functions *in vivo*. Unfortunately, no *shroom2* mutant mice are available. During the course of this work, I attempted to make transgenic mice through the injection of pSuper shShroom2 ES cells into blastocysts. While I did recover chimeric animals, the contribution from shShroom2 cells was low, and I never obtained mutant embryos in subsequent crosses. Given the role of Shroom2 in centrosome duplication, it is highly likely that loss of Shroom2 is deleterious to cell division and development. In moving forward with the generation of *shroom2* mutant mice, these results suggest the importance of utilizing conditional knockouts.

4.4 SUMMARY

In summary, Shroom2 is an actin and Rock binding protein which mediates the sub-cellular localization of Rock to establish specific actomyosin networks that generate cellular contractility. Through this mechanism, Shroom2 is able to mediate a subset of Rock activities which are important for endothelial morphology during angiogeneiss and centrosome duplication. As many other epithelial tissues express Shroom2, it will be interesting to identify additional roles for Shroom2 and Rock during development.

5.0 MATERIALS AND METHODS

5.1 CELL CULTURE AND TRANSIENT TRANSFECTIONS

Most cell lines were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, pen/strep, and L-glutamine. Pooled HUVECs were purchased from ATCC and cultured in Vascular Cell Basal media (ATCC) supplemented with Endothelial Cell Growth Kit (ATCC). Depending on the timing of the experiment or transfection efficiency, cells were transfected in suspension or while adherent with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Inducible Shroom2-expressing cells were made by transfecting T23 MDCK cells with pTRE2-hygro containing a full length Shroom2 cDNA. Cells were selected in EMEM/10% FBS containing 200μg/mL hygromycin and 40ng/mL doxycyclin for 10 days (d). Individual clones were isolated, expanded, and tested by Western blotting for inducible expression of Shroom2 protein.

5.2 IMMUNOHCYTOCHEMISTRY

Cells were grown on fibronectin-coated coverslips, fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton-X for 5 min or fixed in -20° MeOH for 5 min. Fixed cells were then blocked with 4% Normal Goat Serum for 20 min. Primary and secondary antibodies

were applied for 1 hour (h) each with three subsequent washes in PBT for 5 min each. For localization mapping experiments, cells were permeabilized prior to MeOH fixation with 0.1% Triton-X for 20 sec followed by two rinses with PBS. Polyclonal antibodies for Shroom2, Shroom3, and Shroom4 were previously generated in the lab [188-190]. Additional antibodies were purchased: PECAM, E-cadherin (BD Biosciences), ZO1, SAS6 – clone H300 (Santa Cruz Biotechnology), α Tubulin, γ tubulin, acetylated tubulin (Sigma-Aldrich), monoclonal γ tubulin (Abcam), Rock1, pMLC2 – Ser19, ppMLC2 – Thr18/Ser19, p-cofilin, and pFAK-Tyr397 (Cell Signaling), Rock2 (Bethyl Labs), p-MYPT, centrin - clone 20H5 (Millipore), mono- and polyclonal GFP, TO-PRO3, goat anti-mouse, goat anti-rat, or goat anti-rabbit secondary antibodies conjugated to Alexa-488 or Alexa-568 (Invitrogen).

5.3 WESTERN BLOTTING

In most cases, cells were lysed in radio immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor (Sigma), separated on 10% polyacrylamide gels, transferred to nitrocellulose, blocked in TBST + 4% milk, and subjected to immunoblotting with HRP secondary antibodies. SuperSignal West Pico Chemiluminescent Substrate was used to detect HRP (Thermo-Fisher Scientific). For Western blots of phospho-proteins, cells were directly lysed and resuspended in sample buffer (62.5mM Tris-HCL pH 6.8, 2% SDS, 10% Glycerol, 50mM DTT).

5.4 RNA INTERFERENCE

ON-TARGETplus siRNAs were ordered from Dharmacon and were tested for knockdown efficiency by immunofluorescence analysis and Western blotting. The following two siRNA duplexes were most efficient: mShroom2-7 sense sequence: AGUCAAGAUUGGCGAGA. mShroom2-8 sense sequence: GGAUAAUGUUGAACCCAAA. The ON-TARGETplus SMARTpool for hShroom2 was used in HUVEC cells. ON-TARGETplus non-targeting siRNA #1 was used as a control. C166 cells were transfected in suspension with 100nM siRNA using Lipofectamine2000. Adherent HUVEC cells were transfected with 100nM siRNA using Dharmafect #1 (Dharmacon). Cells were allowed to grow for 48-72 h before use.

5.5 STABLE TRANSFECTION OF C166 CELLS

shRNA oligos corresponding to mShroom2-7 and mShroom2-8 siRNAs (Oligoengine) were cloned into the pSuper-gfpneo^{r} vector (Oligoengine). Uncut vector and both shShroom2 constructs were individually transfected into C166 cells and after 24 h, stably incorporated cells were selected for G418 resistance. Drug-resistant cells were pooled and tested via immunofluorescence and Western blot for GFP and Shroom2 expression. Cell proliferation was measured by plating cells at the same density on day 0 and then performing hemocytometer cell counts every 24 h.

5.6 MATRIGEL ANGIOGENESIS ASSAY

A thin layer of Matrigel (BD BioSciences) was spread onto 6-well plates and allowed to harden. $2x10^6$ C166 cells or $5x10^5$ HUVECs were placed in each well with complete media. For C166 cells, multi-cellular chords form after 24 h and after ~5 d resolve into a thinner network with no change in the branching architecture [232]. Cells were photographed on an Olympus MVX10.

5.7 SPROUTING ANGIOGENESIS ASSAY

48 h after transfection with siRNA, spheroids of C166 cells were formed by re-suspending 400 cells/ well in Methocel media (20% Methocel, 80% culture media) in a non-tissue culture treated 96-well plate which was placed at 37° overnight. Spheroids were harvested and re-suspended in DMEM with 20% FBS and 30ng/mL rmVEGF (R&D Systems). 250µl of spheroids were mixed with 250µl of collagen (Upstate), neutralized with NaOH, and plated in a 24-well plate. Collagen gels were given 30 min to polymerize at 37° and were then overlaid with media and 300ng/mL rmVEGF. Sprouts were photographed after 48 h.

5.8 VASCULOGENESIS ASSAY

pSuper, shShroom2-7, or shShroom2-8 was linearized and electroporated into mouse ES cells (cell line AK7 – gift from Philippe Soriano). ES cells were cultured in DMEM with L-glutamine, Pen/Strep, 20% ES certified FBS (Thermo Scientific Hyclone), 0.1mM NEAA (Life

Technologies), and 0.1mM BME were grown on a layer of SNL fibroblasts (gift from Philippe Soriano) which had been mitotically inactivated with Mitomycin C (Sigma). SNLs were grown on gelatin-coated tissue culture plates. After 24 h, positive transformants were selected in 300ug/mL G418 for 9-11 d, changing media every day. GFP positive colonies were selected for expansion and were verified for Shroom2 knockdown via immunostaining and Western blot. Differentiation of ES cells into endothelial vessels was performed as described by Kappas and Bautch (2007) [223]. Briefly, older, more flattened ES colonies were chosen for differentiation. ES colonies were detached with Dispase and cultured on bacteriological Petri dishes for 3 d. Resultant embryoid bodies were allowed to reattach on fibronectin-coated coverslips in differentiation media: DMEM, 20% ES certified FBS, pen/strep, L-Glutamine, 300ug/mL G418, and 75μm Monothioglycerol. After 8-10 d, endothelial vessels were examined by immunostaining with a PECAM antibody.

5.9 COLLAGEN GEL CONTRACTION

1mg/mL collagen gels were prepared by diluting collagen with media, neutralizing with NaOH, and plating 500μL / well in a 24-well plate. C166 cells were used 48 h after siRNA transfection. $2.5x10^5$ cells / well in 500 μL media were placed in each well. After 24 h, the collagen gel was detached with a yellow tip pipette and photographed after 6 h.

5.10 IN VITRO BINDING

mShroom2 cDNA corresponding to amino acids (aa) 1286-1479 (SD2) was cloned into the pET151 vector which contains a His tag. hRock1 cDNA corresponding to aa 681-942 (SBD) was cloned into the pRSF vector. Both vectors were transformed into BL21 cells. Expression was induced with 0.5mM IPTG for 1 h before lysing via sonication. His-tagged SD2 was bound onto Ni-NTA resin, eluted with sample buffer, separated on a polyacrylamide gel, and coomassie stained.

5.11 GST PULL DOWN

mShroom2 cDNA corresponding to aa 1068-1480 was cloned into pGEX-2T and hRock1 cDNA corresponding to aa 593-1062 were cloned into pGEX-3X and transformed individually into RIPL cells. After IPTG induction and lysing, GST-mShroom2-SD2, GST-hRock1-SBD, and GST alone were bound to glutathione Sepharose beads (GE Healthcare) and then incubated with cell lysate at 4°C for 2 h. C166 or T23:TRE Apxl (Shroom2) cells were lysed via sonication in NETN buffer (20mM Tris pH8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40, 1:100 protease and phostphatase inhibitor cocktails(Sigma)). After incubation, glutathione Sepharose was washed 4 times in NETN buffer, resuspended in SDS sample buffer, and analyzed via Western blot.

5.12 SCRATCH WOUND ASSAY

$6x10^5$ C166 cells were transfected in suspension and plated on fibronectin-coated coverslips in a six-well plate. After 72 h, cells were scratched with a yellow tip pipette to generate consistently sized wounds. Representative wounds were fixed in PFA and stained with phalloidin. To quantify migration, live cells at the same wound site were photographed at 1, 12, and 24 h postscratch and quantified by measuring the remaining wound length.

5.13 BOYDEN CHAMBER ASSAY

At 72 h after transfection with siRNA, $1x10^5$ C166 cells or $7.5x10^4$ HUVECs were plated in the upper chamber of a fibronectin-coated, 8.0μm polycarbonate, 24-well transwell insert (Costar, Corning). Cells were allowed to migrate for 4 h. The top chamber was scraped with a Q-Tip, and the bottom cells were fixed in PFA, stained with phalloidin and TO-PRO, and photographed. Nuclei were counted from three random fields of view of two independent experiments.

5.14 GENERATION OF SHROOM2 GFP CONSTRUCTS

For rescue and localization mapping experiments, I obtained hShroom2 cDNA (Image ID: 9021734) from ATCC and through restriction digest and PCR amplification, cloned various fragments into pAcGFP1-Hyg-N1 (Clontech).

5.15 CENTROSOME ISOLATION

Isolation of centrosomes was based on protocols developed by Moudjou and Bornes [263] and described by Hsu and White [264]. 5x150mm confluent plates with exponentially growing cells were incubated with 1μg/mL cytochalasin D and 2μg/mL nocodazole in culture media for 1 h at 37°C. Plates were washed once with PBS, 0.1% PBS with 8% sucrose, and 8% sucrose in H_2O. The remaining steps were carried out at 4° C. 2mL of lysis buffer (1mM Hepes pH7.2, 0.5% NP-40, 0.5mM $MgCl_2$, 0.1%BME, 1:100 Protease inhibitor, 1:100 Phosphatase inhibitor cocktail 1) was added to each plate and placed on a shaker for 10 min. Cells were scraped and collected into a 15mL tube and centrifuged at 2,500g for 10 min to remove chromatin aggregates. The supernatant was filtered through a 50μm nylon mesh into a glass tube and was adjusted to 10mM Hepes. DNaseI (Roche, San Francisco, CA) was added to 2 units/mL and incubated for 30 min. The lysate was underlaid with 1mL 60% sucrose and centrifuged at 10,000g for 30 min. 2mL was removed from the bottom and this crude centrosome fraction was further concentrated on a sucrose gradient consisting of 500μL 70% sucruse, 300μL 50% sucrose, and 300μL 40% sucrose in 3.5mL thickwall polycarbonate tubes (Beckman, Palo Alto, CA). The discontinuous sucrose gradient was centrifuged in a Beckman Ultrafuge L8-70M with a SW55TI rotor at 120,000g for 1.5hr. Fractions were collected from the top; 1mL for fraction 1, 0.5mL for fraction 2, and 0.2mL for the remaining fractions. 30μL from each fraction was denatured in SDS sample buffer and subjected to Western blotting.

5.16 CENTROSOME DUPLICATION ASSAY

Cells were plated on fibronectin-coated coverslips and allowed to spread overnight. The following day, either DMSO or 2μg/mL aphidicolin (Sigma-Aldrich) was added per well for 48 h. Cells were then fixed and immunostained for γ tubulin and centrin in order to count the centrosome number of at least 100 cells. The experiment was performed in triplicate, centrosome numbers were averaged, and a ratio between DMSO and aphidicolin-treated cells determined fold change in centrosome number.

5.17 SCANNING ELECTRON MICROSCOPY

Embryos or tissues were fixed in 2.5% Gluteraldehyde in PBT overnight and were washed three times for 10 min in 0.1M cocadylate. Tissues were post-fixed for 2.5 hr in 1% osmium tetroxide in 0.1M cocadylate and subsequently washed 4 times for 10 min in 0.1M cocadylate. Tissues were then dehydrated in 25%, 50%, 75%, and 100% EtOH for 30 min each and incubated with 25%, 50%, 75%, and 100% HMDS in EtOH also for 30 min each. Tissues remained in hexamethyldisilazane (HMDS) overnight. Tissues were air-dried, sputter coated, and observed using a Jeol JSM6390LV SEM.

5.18 STATISTICS

All measures of significance were determined by one-tailed, unpaired *t* test.

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