CHARACTERIZATION OF THE SHROOM PROTEIN FAMILY MEMBER, SHROOM4, AND ITS ROLE IN CYTOSKELETAL REARRANGEMENTS

by

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The ability of an organism to adapt to its surrounding environment is at the essence of survival. In metazoa, this ability starts at the level of the individual cell, which utilizes a specialized set of cytoskeletal proteins to determine their overall shape and the organization of their intracellular protein complexes and organelles. During embryonic development, the dynamic nature of the actin cytoskeleton is critical for virtually all morphogenic events requiring changes in cell shape, migration, adhesion, and division. The behavior of the actin cytoskeleton is modulated by a myriad of accessory proteins. Shroom3 (Shrm3) is an actin binding protein that regulates neural tube morphogenesis by eliciting changes in cell shape through a myosin II-dependent pathway. The Shroom-related gene *SHROOM4* (formerly called *KIAA1202*) has also been implicated in neural development, as mutations in this gene are associated with human X-linked mental retardation. To better understand the function of Shrm4 in embryonic development, the mouse *Shrm4* gene was cloned and its protein product was characterized both *in vivo* and *in vitro*. Shrm4 is expressed in a wide range of tissue types during mouse development, including the vascular endothelium of the lung and the polarized epithelium of the neural tube and kidney. In endothelial cells and embryo fibroblasts, endogenous Shrm4 co-distributes with myosin II to a distinct cytoplasmic population of F-actin and ectopic expression of Shrm4 in multiple cell types enhances or induces the formation of this actin-based structure. This localization is mediated, at least in part, by the direct interaction of Shrm4 and F-actin. The actin-binding motif of mShrm4

defines a novel actin-binding element that has not yet been described in other proteins. The results described here suggest that mShrm4 is a regulator of the actin cytoskeleton and may play an important role during vertebrate development, particularly in the developing vasculature.

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PREFACE

My graduate career would not have possible without the support and patience of my advisor Dr. Jeffrey 'Jeffe' Hildebrand. We have been through a lot together over the years, as scientists and, more importantly, as human beings. Your passion for science has not gone unnoticed, as you are as enthusiastic about your research now as you were when you were fresh off of your post-doc. Being witness to this has shown me the dedication necessary to succeed and the genuine love for science required to keep plugging away, even in the face of repeated dead ends. Jeff, you have been instrumental in my growth as a scientist and as a person, over the last few years and I want to thank you for that!

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1.0 INTRODUCTION

The cytoskeleton is essential to a variety of biological processes in every living organism ranging from bacteria to vertebrates. In addition to providing a structural framework to support the cell, the various cytoskeletal networks of microtubules (MT), intermediate filaments, and actin filaments enable cells to carry out countless complex functions, including cell division, cell adhesion, and cell migration. As a testament to the importance of these dynamic macromolecules, the proteins are highly conserved from yeast to humans, 75% for tubulin and 87% for actin (intermediate filaments have not yet been described in yeast). Even though these cytoskeletal proteins have been well documented since their initial discoveries [1-3], it was not until decades later that prokaryotic homologs were finally described. FtsZ (tubulin), MreB and ParM (actin), and crescentin (intermediate filament) have all been recently discovered and these proteins share little to no sequence similarity to their eukaryotic homologs [4-7]. They do however fold into similar shaped proteins and often display similar biochemical dynamics, such as polymerizing into filamentous polymers and modulating a number of cellular processes.

Each of the of different cytoskeletal networks is necessary for normal cell function and in many cases work in conjunction with each other to maintain the cell's physiology. Microtubules are perhaps best known for their role in chromosome segregation during cell division, but they can also provide a structural role and serve as an intracellular transport system for various cargos. Intermediate filaments constitute one of the largest gene families in humans, with over

67 genes encoding intermediate filament proteins [8, 9]. The expression of these genes and the localization of the proteins are quite variable. Many intermediate filaments play general structural roles, but there are also some tissue and cell specific functions for intermediate filaments. For example, neurofilaments provide structure and protection to neuronal cells, whereas keratins play a supportive and protective role in both skin and hair. Actin filaments are well known for providing structural support for cells (stress fibers for example), muscle contraction, and cytokinesis. Actin is also at the heart of many other processes and will be discussed in greater detail in the following sections.

1.1 REGULATION OF ACTIN DYNAMICS

Actin filaments themselves are very dynamic and the underlying biochemistry is extremely interesting in its own right. Individual actin monomers, called globular or G-actin, are small 43 kDa proteins that exist in both ATP and ADP bound states and have an intrinsic ATPase activity. G-actin can polymerize into larger actin filaments (F-actin) *in vivo*, and at the right salt (and buffer) conditions, G-actin can spontaneously form filaments *in vitro*. Actin filaments have an inherent polarity due to the directed addition of subunits (which can only be added in one orientation). The fast growing or barbed (+) end is the site of net polymerization (K_d = 0.12 μM) while the slow growing or pointed (-) end represents the site of net depolymerization (K_d = 0.6 μM) [8]. This dichotomy of subunit addition at the ends of an actin filament gave rise to the idea of 'treadmilling' [9-12], where a monomer added to the barbed end will work its way towards the pointed end through the rapid addition of subunits to the barbed end and the subsequent removal of subunits from the pointed end. Once incorporated into a filament, actin will

hydrolyze ATP → ADP at a rate of 0.3 s^{-1} and release P_i much more slowly (0.002 s^{-1}) [14-16]. Much like any chemical reaction involving the addition/removal of subunits, the rate of polymerization and depolymerization reaches a steady-state equilibrium when the concentration of G-actin reaches the critical concentration. Because actin is one of the most abundant proteins in the cell and it has the ability to respond quickly to changes in its environment, actin is an excellent candidate to elicit a number of changes within the cell, a few of which will be discussed later.

1.1.1 Actin Regulatory Proteins

In the complex environment of the cytoplasm, actin dynamics are regulated by a multitude of proteins. The rate of polymerization and depolymerization of actin filaments can be manipulated within the cell by capping proteins, such as the multifunctional gelsolin [13] and CapZ [13], G-actin binding proteins, including profilin and thymosin β-4 [10], depolymerizing proteins, like ADF/Cofilin [11], nucleating proteins like the ARP2/3 complex and associated nucleation promoting factors (NPF) like WASP [12, 14, 15], as well as a host of other proteins. The list of proteins that regulate actin dynamics is immense and is the source of numerous reviews [16, 17]. The concerted efforts of these proteins allow actin filaments to respond quickly to the needs of the cell whether it is to rapidly assemble or disassemble filaments or to create branched networks of actin filaments.

One process that elegantly synthesizes the function of a number of these actin regulatory proteins is the dendritic branching of filaments. The idea of dendritic branching was proposed as an explanation for the branched actin network nucleated from the Arp2/3 complex [14]. It has since become a model for the mechanism behind cell migration and is also thought to be the

driving force behind the *Listeria* model of locomotion within the cell [18-20].

The model proposed by Mullins, *et al*, will be briefly described in order to illustrate how the actin regulatory proteins function together to rapidly rearrange the cytoskeleton. In the model, the starting point assumes a pre-existing F-actin cytoskeleton within the cell. An extracellular signal is relayed that causes the Arp2/3 complex to become active by binding a nucleating promoting factor, like WASP, and actin. The site of Arp2/3 binding to actin establishes a branch point for the polymerization of subunits into a new filament. Additionally, the Arp2/3-WASP complex mimics the pointed end of a filament, so the profilin bound G-actin subunits are rapidly being added to the barbed end of the new branch. As the filament grows, it pushes on the membrane and generates enough force to displace it relative to its original position. Capping protein binds to the growing barbed ends, terminating the filament growth and starting the depolymerization cycle. ADF/Cofilin binds the filament and promotes the release of the ADP bound subunits (an indication of 'older' filaments). Profilin then catalyzes the exchange of ADP for ATP in the monomers, thereby recharging the pool of available subunits for new filament growth. The initiation of dendritic branching and its ensuing rapid dynamics provides an excellent mechanism to drive cell migration [21-23]. In any case, the six components listed above (actin, Arp2/3, WASP, capping protein, ADP/cofilin, and profilin) are sufficient to drive the entire branching process [19, 24]. Much like every other event in the cell, the actin regulatory proteins themselves are often under the regulation of other proteins via phosphorylation, for example, and frequently by the activity of phospholipids [25]. One family of actin regulatory proteins, the GTPases, will be discussed in another section.

1.1.2 Actin Binding Proteins

The ability to bind to actin is by no means a unique feature and, in fact, there are many domains or motifs that can facilitate an interaction with actin. A common motif frequently used by actin cross-linking proteins (discussed in section 1.1.3) is the calponin homology (CH) domain. This domain is composed of about 100 amino acids that are typically found in tandem pairs and usually span about 250 amino acids [26]. The family of CH domain proteins contains calponin, fimbrin, α-actinin, dystrophyn, and spectrin [27]. These proteins can either have multiple actin-binding domains (ABD) on the same polypeptide, such as fimbrin, or they can be found on separate polypeptides that form dimers, such as α-actinin or filamin [28]. Members of the CH family of actin-binding proteins bind to F-actin at a concave surface formed by the sub-domains of neighboring actin subunits [29, 30].

The gelsolin family of actin-binding proteins, including gelsolin, severin, and villin utilize a 15 kDa module (found in three or six repeats) to differentially bind to both G- and F-actin [31]. These capping/severing proteins preferentially bind to the barbed end of F-actin where they function to block the addition of new actin subunits. In addition to the conserved module, villin contains a unique 8 kDa actin-binding motif referred to as the headpiece. This domain is actually conserved among other actin binding proteins that otherwise share no homology [32]. Members of the cofilin and ADF family of proteins bind actin with a similar domain as the gelsolin family [33]. However, the site of interaction with actin is different then that of gelsolin [34]. Profilin is a G-actin binding protein that also uses a unique actin-binding site. Profilin binds to G-actin in a way that allows the actin monomer a large amount of conformational flexibility [35]. It is believed that this ‘loose’ conformation facilitates the addition of actin monomers to the growing barbed end of an actin filament.

While the above examples do not have a standard or canonical actin-binding motif (excluding the CH domain), per se, each represents a family of proteins that share similar actin-binding properties. There are, however, a number of well-defined motifs that are known to bind to actin. One such motif is the formin homology domain 2 (FH2), a 400 amino acid sequence that is found in at least 6 classes of proteins in mammals (with each class containing multiple family members) [36]. Formins use the FH2 domain to bind to F-actin and affect cytoskeletal dynamics in a number of different ways, including increasing nucleation and polymerization rates, as well as possessing capping activity [37]. In addition to the FH2 domain, formins have an FH1 domain that binds to profilin. However, the non-conserved sequence outside of the FH domains provides the differential regulation of these otherwise conserved proteins. There are numerous actin-binding proteins in a cell and perhaps just as many unique binding motifs. It is quite striking that so many different proteins have evolved independent mechanisms to regulate the same structure.

1.1.3 Mechanisms of Actin-Bundling

There are certainly other proteins that regulate the assembly or architecture of F-actin. In some cases, actin fibers need to be bundled together, often they need to be tethered to other intracellular structures, and sometimes they are required to assume a more rigid morphology. Actin bundling or cross-linking proteins are quite numerous in the cell and enable these 'higher-order' structures to form [38]. First, it is important to mention how a protein could cross-link or bundle actin filaments. There are two mechanisms that could facilitate bundling: the first proposes that a single actin-binding protein can bind to two separate actin filaments whereas the

second scenario indicates that the actin-binding protein can bind to a single actin filament and simultaneously homodimerize (Fig. 1).

The first scenario (Fig. 1A) would allow a single actin-binding protein, fimbrin for example, to bind in a bimodal fashion to two separate actin filaments, thereby cross-linking the two. One can then envision that a second copy of fimbrin binds elsewhere on one of those filaments and brings in a third actin filament and so on [39, 40].

The second scenario (Fig. 1B) involves a protein that can bind to a single actin filament and then oligomerize to another F-actin bound copy of itself (α-actinin for example). In this case, the protein can either dimerize before binding to actin or after. Stress fibers are anti-parallel arrays of contractile F-actin bundles that use the activity of myosin II for contraction and play an important role in cell adhesion, motility, and morphogenesis. Myosin II decorates the F-actin bundles in a periodic arrangement with α-actinin and, much like the actomyosin contractile unit in a muscle sarcomere (reviewed in [41]), the myosin in a stress fiber is able to contract and create a rigid filament for use in cell migration or attachment to a focal adhesion. The mechanisms of stress fiber formation are still poorly understood, however a recent study has revealed that there are multiple mechanisms that lead to stress fiber assembly [42].

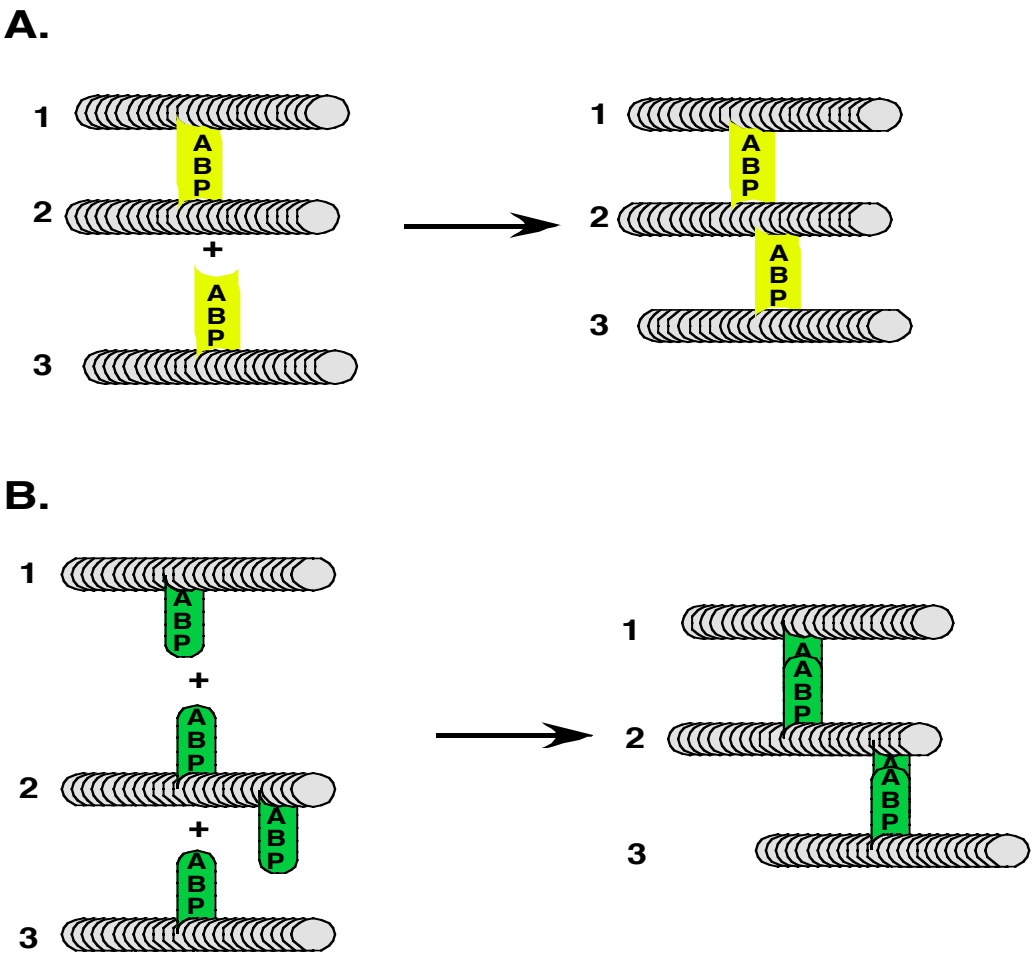

Figure 1: Mechanisms of Actin Bundling

Figure 1 Mechanisms of Actin Bundling

A. A single actin-bundling protein (yellow, ABP) can have two separate actin-binding domains, capable of binding two individual filaments (1 & 2). A second copy of ABP can bind yet a third actin filament (3) and by also binding to filament '2' will effectively begin the bundling process. **B**. In the case of actin-bundling proteins that have single binding domains (green, ABP), the bundling protein will bind a single actin filament (1) and then dimerize with another copy of ABP bound to a different filament (2). A third copy of ABP can bind elsewhere on filament 2, which will then dimerize with yet a fourth copy of ABP bound to a separate filament (3). In either A or B, the end result is the same: single actin filaments tethered together to form higher-order actin structures.

1.1.4 Regulation of Actin by G-proteins

The regulation of actin dynamics is not just a simple manipulation by direct actin-binding proteins, but rather changes in actin dynamics are often under the direct control of signaling cascades that organize these cytoskeletal rearrangements. The Rho pathway has been mentioned above and due to its important role in regulating actin dynamics, it deserves to be described in more detail [43, 44]. A classic example of the Rho pathway facilitating cytoskeletal rearrangements is the signaling cascade initiated by the extracellular ligand, *folded gastrulation* (*fog*), in *Drosophila* [45, 46]. In this pathway, the secreted protein *fog* is induced by the morphogen *twist*. *Fog* then activates *concertina* (*cta*) (a $G_{\alpha 12/13}$ protein), which in turn activates RhoGEF2. RhoGEF2 signals to ROCK, which ultimately activates myosin II on a population of F-actin that is tethered to adherens junctions [47, 48]. The activated myosin II causes the tethered actin to contract and force the apical constriction of the cells.

The Rho family consists of roughly 5 groups, Rho-like, Rac-like, Cdc42-like, Rnd, and RhoBTB subfamilies. It is the activities of the first three groups that have been well documented in their regulation of actin dynamics, including stress fiber formation and membrane ruffling/lamellipodia formation. Much like every other signaling pathway, the Rho pathway must be initiated by an external signaling mechanism to relay the requirements needed for a specific function, whether its trauma signaling for a wound healing response or morphogens (like *fog*) inducing cell movements. The Rho family of GTPases can be activated by signaling through receptor tyrosine kinases (RTKs), lysophosphatadic acid (LPA), integrins, and cadherins [49-51]. Often times, the first proteins in the Rho cascade to be activated are regulatory in nature. Nucleotide (guanine) exchange factors (GEFs), comprise a large family of proteins as

there are about 70 identified in the human genome [52]. GEFs, such as DRhoGEF2 mentioned above, are one of the first protein targets of the signaling cascade and they facilitate the exchange of GDP for GTP at the level of Rho [53, 54]. GTPase activating proteins (GAPs) function to enhance the intrinsic GTPase activity of a Rho-GTPase [55]. Additionally, there are guanine nucleotide dissociation inhibitors (GDIs), which bind to the GDP-bound RhoGTPase and inhibit the exchange of GTP for GDP. All of the above Rho-family effector proteins motioned above are in place to regulate the balance between the active and inactive forms (GTP bound vs. GDP bound respectively) [56]. It is these upstream proteins that typically provide the specificity to the extracellular signal, as Rho and ROCK are common downstream effectors for many cytoskeletal rearrangements.

In stress fiber formation, Rho functions to activate myosin II and to increase actin polymerization. The Rho kinase, ROCK, is activated by binding to GTP-bound Rho [57, 58]. Activated ROCK can then proceed to phosphorylate myosin light chain kinase (MLCK) or inhibit myosin phosphatase. By activating myosin II through this cascade, a net increase in contractile myosin occurs resulting in stress fiber and focal adhesion formation. Additionally, ROCK can activate LIM kinase, which subsequently inhibits cofilin (actin depolymerizing factor) [59], further supporting a net polymerization. Rho can further enhance stress fiber formation by activating diaphanous (mDia), an actin nucleating factor [60].

In a similar type of signaling mechanism, Rac can mobilize the cytoskeleton to generate lamellipodia or membrane ruffles. Rac signals to a complex of proteins (collectively referred to as the WAVE or SCAR complex) consisting of Arp2/3 activating protein WAVE, two Rac binding proteins (Nap125 and PIR121), and HSPC300 and Abi2 [15, 61]. Active Rac can cause this complex to dissociate, de-repressing WAVE so it can functionally enhance Arp2/3 actin

polymerization. Rac is also able to signal through other proteins, such as PAK, which signals to downstream effectors to promote actin polymerization or to at least stabilize existing filaments. It is not surprising that the Rho-family signaling pathways often converge and either directly activate each other or utilize common downstream targets to elicit a response.

1.2 THE ROLE OF ACTIN IN CELL FUNCTION

The biochemistry of actin and its ensuing dynamic nature place actin in a central role for most activities of the cell. A few cellular processes have been described above that utilize actin dynamics, such as migration, but those just scratch the surface of actin's potential. In addition to those described above, F-actin provides a rigid network which serves as an anchored support that is central for adhesion, cell polarity, signal transduction, and vesicular transport. Below is a brief description of these cellular properties and actin's role in them.

1.2.1 Adhesion Structures

Many of the adhesive properties of the cell involve either direct or indirect links to F-actin. Whether a cell needs to be anchored to the extracellular matrix (ECM) through focal adhesions or form cell-cell contacts like tight junctions and adherens junctions, F-actin is at the core of these adhesive structures [54, 62]. The following section will briefly describe the molecular composition of each of these adhesion structures, and Fig. 2 depicts the various adhesion structures discussed.

1.2.1.1 Focal Adhesions

Focal adhesions are a complex of proteins that link the ECM to the cytoplasm. Integrins are transmembrane receptor proteins that simultaneously interact both with the components of the ECM and to a complex of cytoplasmic proteins including vinculin, talin, and paxillin among others (Fig. 2B). Each of these proteins is involved in the dynamic association with actin filaments. Due to the multitude of focal adhesion associated proteins, there are different types of focal adhesions (focal complexes, focal contacts or adhesions, and fibrillar adhesions) [63]. These links to the ECM not only serve as adhesive sites, enable cell migration, or direct ECM reorganization, but they also serve as signaling centers. Many of the proteins associated with focal contacts are signaling molecules, including tyrosine kinases (focal adhesion kinase or FAK), tyrosine phosphatases, and other adaptor proteins, that can induce cytoskeletal rearrangements because of their intimate link to actin filaments [63, 64]. For example, FAK can phosphorylate α-actinin, which reduces stress fiber rigidity [65], or alternatively FAK can phosphorylate p190 RhoGEF, which increases the active myosin pool, leading to an increase in stress fiber rigidity [66, 67].

1.2.1.2 Tight Junctions

Similarly, the junctional complexes, consisting of tight junctions (TJ) (subapical complex in *Drosophila*) and adherens junctions (AJ), form adhesive structures that enable cells to link together (the 'epithelial junctional complex' or more commonly known as the apical junctional complex (AJC)). Tight and adherens junctions provide a mechanism of creating a selective cellular barrier between two spaces, such as the epithelial layer lining the gut or the endothelium that comprise the vasculature. When necessary, these barriers are able to allow the passage of small molecules, either through the cells or in between them. The arrangement of the junctions

within the cell create an inherent polarity (apical to basal), and it is this polarity that helps the cells to adhere to each other and to form the gated barrier that separates the external world from the internal one. Of course these junctional complexes must also be dynamic, and there are a number of signaling molecules associated that can respond to external signals and elicit changes within the cell [68] (Fig. 2A).

TJs, the apical most adhesive structures, are comprised of the multi-pass transmembrane proteins occludin [69] or claudin (over 24 identified) [70], or the junctional adhesion molecules (JAM) 1-4 [71], plus a number of scaffolding and signaling proteins [72, 73]. Among these are 2 well-known complexes, the Crumbs-PALS1 (Stardust)-PATJ complex and the PAR3 (Bazooka)-PAR6-aPKC complex (= *Drosophila* homologue). The proteins of these two complexes are either signaling molecules themselves (aPKC) or recruit and bind directly to other signaling molecules (PAR6 can bind to cdc42 for example). TJs also contain other scaffolding proteins such as zonula occludens -1, -2, -3 (ZO), cingulin, and many other PDZ containing proteins. PDZ motifs (PSD-95, Discs-large A, and ZO-1 being the founding members) facilitate protein-protein interactions and help to organize large protein complexes [74-77]. In general, PDZ domains consist of about 90 amino acids that contain a conserved GLGF peptide sequence [78, 79]. F-Actin binds directly to the TJ proteins occludin, cingulin, and the ZO proteins, again placing F-actin at the center of yet another adhesion structure. Regulation of actin dynamics could originate through any number of signaling molecules that have also been reported to be associated with TJs, including the Ras, Rab, and Rho-family of GTPases. These cytoskeletal rearrangements could be in response to an internal or external signal and could function to help mobilize an epithelial cell or to regulate paracellular permeability [80].

1.2.1.3 Adherens Junctions

Adherens junctions (Fig 2B) are conceptually similar to focal adhesions and TJs; they are comprised of single-pass, transmembrane cadherin proteins that are linked to the actin cytoskeleton through the catenin complex. Although there are a number of different known cadherin molecules [81] (each with a varied expression domain), they all share the ability to mediate homophilic interactions between cells via their five extracellular (EC) domains [82]. Intracellularly, the cadherins share the ability to bind to p120 catenin and β-catenin [82-84]. In fact, the cadherin molecule is required to establish the junction and recruit the necessary accessory proteins [85]. Historical thought is that α-catenin is recruited to the cadherin-β-catenin complex and this association allows a number of other actin-binding proteins to enter the complex, including vinculin, α-actinin, and formin-1 [86, 87]. It is believed that the activity of α-catenin is the source of regulation for actin dynamics at adherens junctions, either by suppressing Arp2/3 nucleation of actin or by recruiting formin-1, which nucleates unbranched actin cables [88]. Recently it has been shown that α-catenin can either bind to the adherens junction complex or to F-actin, but not both at the same time [89, 90]. It is still not exactly clear how actin is anchored to adherens junctions, but there are a few candidates and more research is necessary to determine how these links are organized.

In addition to forming cell contacts to the ECM or other cells, the distribution of adhesion structures helps to establish cell polarity. Tight junctions define the boundary between the apical domain and the lateral side of the cell, while adherens junctions mark the apico-basal boundary. Polarity is an important feature of cells that helps to drive many physiological processes, such as regulating nutrient absorption and water balance in intestinal epithelia, asymmetric cell division, and morphogenetic movements (discussed in section 1.3).

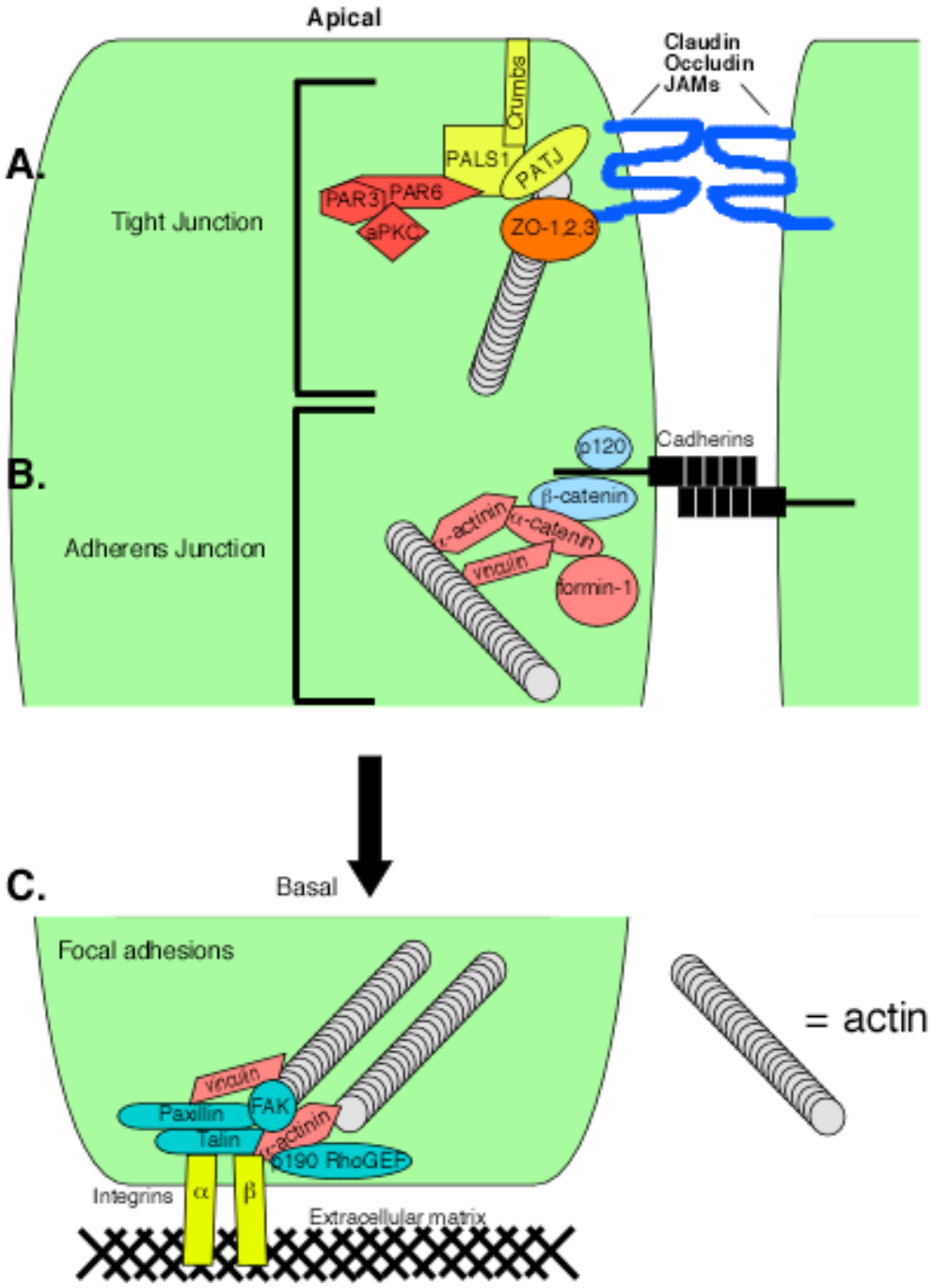

Figure 2: Model of Cell Adhesion Structures

Figure 2: Model of Cell Adhesion Structures

A. The tight junction (TJ) is the apical most adhesion structure in a polarized cell. TJs are comprised of a transmembrane adhesion protein, such as claudin, occludin, or JAM (blue), and a number of intracellular components. The conserved Crumbs-Pals1-PatJ (yellow) and Par3-Par6-aPKC (red) complexes along with other scaffolding proteins like ZO-1 (orange) help to link the entire junction to the actin cytoskeleton. **B.** Adherens junctions utilize the cadherin family of transmembrane proteins (black) to facilitate adhesion. p120 and β-catenin (light blue) bind directly to the cytoplasmic cadherin domain and together they recruit a number of actin binding proteins (pink), like α-actinin, to the complex. **C.** Focal adhesion complexes provide a means of linking the cell to the extracellular matrix. Integrins (yellow) are transmembrane proteins that bind to the ECM and a number of signaling and actin-binding proteins (blue and pink). These proteins provide a direct like to the actin cytoskeleton (vinculin) and cause rapid cytoskeletal remodeling through various signaling cascades (FAK, RhoGEF). These models are based on those in [72] [82] [91].

1.2.2 Vesicular Transport

Vesicular transport is yet another cellular process that has actin filaments as a core necessity. The random diffusion of vesicles would be extremely slow in the crowded environment of the cytoplasm and most often vesicles must be specifically delivered to a particular destination, therefore requiring the aid of molecular motors to assist in transport. Actin filaments can function as a track for vesicles to be transported along or as a scaffold to help arrange the receptor-mediated endocytic machinery [92, 93]. Myosin motors are the machinery that utilizes actin as a scaffold. There are 18 known classes of myosin molecules in the myosin superfamily, each of which is characterized by a globular motor domain (heavy chain), that binds to actin filaments in an ATP-dependent manner, and an α-helical rod domain [94]. The α-helical region facilitates oligomerization forming the coiled-coil, which tethers multiple motor domains together allowing processive movement along an actin filament. The coiled-coil domain is also able to bind to an assortment of cargo, thereby creating an intracellular transport mechanism [95]. The binding of intracellular vesicles (or organelles) to the actomyosin machinery and the ability of myosin to 'walk' along the filament could be facilitated by the host of GTPases that are present in the endocytic machinery [96]. It is still somewhat speculative as to how myosin proteins play a role in endocytosis, but regulation of actin dynamics or myosin through the Rho-ROCK pathway is one plausible mechanism [97].

It should be clear at this point that while actin plays a key role in many specific processes, it often coordinates many of the mechanisms described above into an intricate cellular response. For example, cell migration is a combination of dendritic branching of actin filaments coupled with changes in focal adhesion contacts with the ECM, changes that are orchestrated through 'outside-in' signaling. Cytokinesis is the elegant combination of actin filaments being

anchored to membrane components around the cytokinetic furrow while being constricted by myosin motors (myosin II), much like cinching a belt [15]. Signal transduction through actin-based adhesion structures can, in turn, reorganize the cytoskeleton to enable the cell to detach from its neighbors for migration or more impressively allow the cell to function as part of a unified whole. The combination of actin-based processes among cells in a given field, create some of the most phenomenal events in biology.

1.3 ACTIN AND DEVELOPMENT

One of most fundamental questions in biology is how complex multicellular organisms are created from a single diploid cell. The highly regulated and conserved signaling mechanisms that create 'microenvironments' for different populations of cells must be interpreted by those cells to elicit polarized cell divisions, to change the intracellular architecture, or to orchestrate gross morphological changes within a field of cells. These phenomena are observed in early embryonic development of many organisms ranging from *Drosophila* to mice.

The cell adhesion structures described earlier can function to direct a group of individual cells into a unified multi-cellular structure that can act as a single unit. Tight and adherens junctions physically link cells together, and in doing so they provide a means of coordinating the cytoskeletons of all the cells involved. The adhesion structures are modular complexes that require specific signals to elicit a cellular response. It is the regulated linkage of these structures to the actomyosin network that enables the large morphogenetic movements required for embryonic development.

1.3.1 Convergent-Extension

Convergent-extension (CE) is one such morphological movement during gastrulation, which is under the control of 'tissue polarity', that is now better known as planar cell polarity (PCP) (see section 1.3.2). CE refers to the process of a field of cells narrowing or converging towards its midline and then extending perpendicular to the direction of narrowing [98]. Imagine a square containing 16 cells and has a left (L)-right (R) axis and an anterior (A)-posterior (P) axis (Fig 3A). In order to make the square into a rectangle, the 4 cells lining the L border and those lining the R boarder need to converge towards the A-P axis and intercalate between the middle two rows of cells (Fig 3B). The intercalation of the L and R cells forces the A-P axis to expand, because now instead of four rows of four cells there are two rows of eight cells! This is the idea behind many of the morphological movements in metazoan development and is a well-documented process [99]. Of course, a developing embryo is a three-dimensional structure and much larger changes can be envisioned if the previous example of a square is changed to a cube that now contains 64 cells instead of 16 (Fig 3B). The underlying mesenchymal cells (red outline) generate a much larger force effect when they intercalate into a pre-existing ectodermal sheet (due to the necessary rearrangements that must occur in the ectoderm), a process that must be driven by the force generating system of the contractile actomyosin machinery.

Two models have been proposed to help explain the movements of CE: The cell-cell traction model and the cell-matrix model. In either case, the model is based on a stiff cytoskeletal network that pulls the cells across each other (traction) or across the ECM (matrix). There is evidence for both models and perhaps each has a role in facilitating morphogenesis. While CE is an incredible display of very synchronized, deliberate cell movements, there must have been a pre-existing mechanism that initially patterned the field of cells. It has recently been

established that the PCP pathway is the necessary signaling pathway that conducts groups of cells into functioning as a single unit.

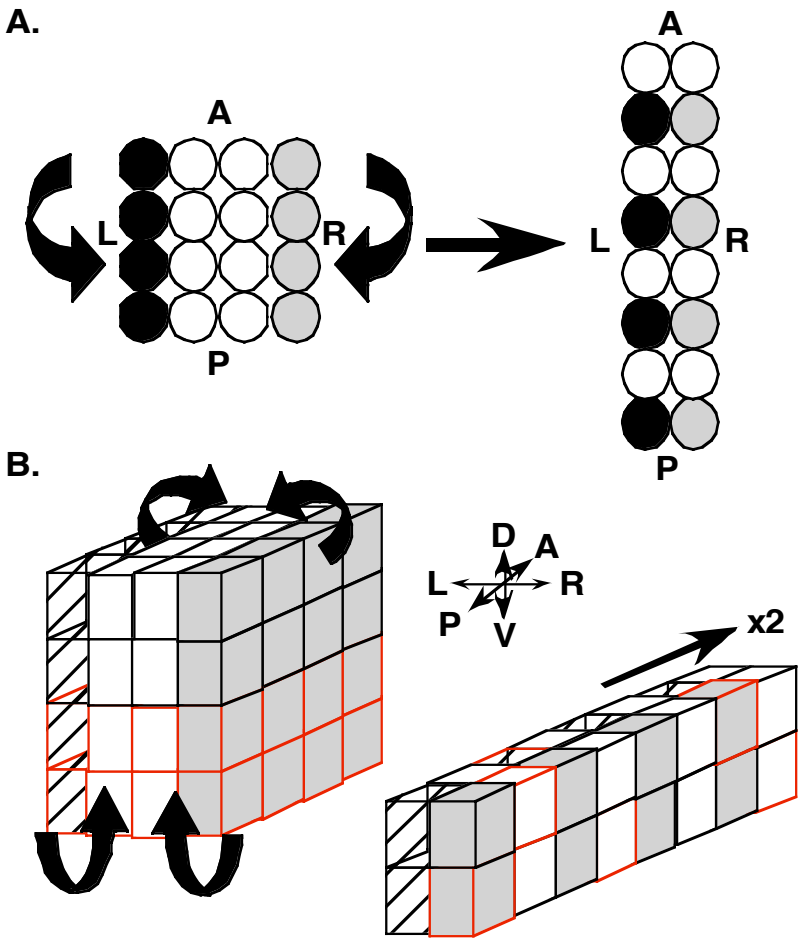

Figure 3: Mechanism of Convergent-Extension

Figure 3: **Model of Convergent-Extension**

A. A sheet of cells (represented by ovals) will extend along the U-D axis when the outermost cells intercalate towards the interior of the sheet, along the L-R axis. Colored ovals represent the exterior most cells. **B.** In a three-dimensional system, the cells will converge and intercalate along the L-R axis AND the U-D axis. This will cause the structure to extend along the A-P axis. Only half of the extended sheet is depicted, with a few of the mesenchymal cells intercalated. Black outlined cubes represent ectodermal cells, while red outlined cubes represent the underlying mesenchyme. A-P = anterior-posterior. D-V= dorsal-ventral. L-R= left-right. Model based on [99].

1.3.2 Planar Cell Polarity

Although the idea of tissue polarity was first described in 1982 [100], it has only been in the last 10 years that the PCP pathway has begun to be understood [101, 102]. Planar polarity is probably best defined as any process that affects cell polarity within an epithelial plane and involves one or more of the core PCP genes [103]. Most of the early description of PCP resulted from research examining the hairs and bristles on *Drosophila*. As is true for most signaling pathways, much of the core machinery is conserved from flies to humans. In fact, it is the non-canonical Wnt signaling pathway that is responsible for PCP. The canonical Wnt (wingless in *Drosophila*) signaling pathway is used to create a transcriptionally active pool of β-catenin through the repression of GSK-3β. When active, GSK-3β targets β-catenin for degradation, however when Wnt binds to its receptor Frizzled (Fz), the intracellular signaling molecule disheveled/Disheveled (dsh/Dvl) is phosphorylated and can then inhibit GSK-3β. The increased levels of β-catenin are then able to translocate to the nucleus, and form complexes with the TCF/LEF family to regulate transcription [104].

The non-canonical Wnt pathway uses the same basic proteins (a Wnt signal that binds to the Fz receptor and activates DVL) to elicit a much different response. However, in non-canonical Wnt signaling the pathway splits at DVL and a different cascade is activated [105]. In this pathway there are a number of 'core PCP' components involved that are not part of the canonical pathway and are conserved from *Drosophila* to humans. In addition to the canonical Wnt, Fz, and Dvl, the other non-canonical proteins include transmembrane receptors, like Strabismus and Flamingo, intracellular proteins, such as prickle and the formin homology

domain protein Daam1, and other downstream signaling molecules, including the Rho GTPase family and the C-jun N-terminal kinase (JNK) [105]. Outside of these core proteins, there are many non-conserved molecules that either function solely in *Drosophila* or specifically in mammals.

As previously described, the Rho family of GTPases is commonly used to elicit changes in cytoskeletal architecture. The PCP pathway uses the non-canonical Wnt signaling cascade to activate the Rho pathway to induce convergent-extension [98]. In this cascade of signals, Dsh interacts with Daam1 (Dishevelled-associated activator of morphogenesis), which is also able to simultaneously bind to and activate Rho [106]. As described earlier, Rho can activate ROCK, which can then affect downstream actin rearrangements. These actin rearrangements could then possibly affect the convergence of cells by contraction of F-actin elements along the medio-lateral axis and conversely, promote elongation by extending lamellipodia in the anterior-posterior directions.

This above list of PCP genes is by no means all-inclusive (for reviews see [103, 107]), but it serves to illustrate that the PCP pathway can also regulate cytoskeletal dynamics through the intracellular effectors of the pathway. The regulation of the PCP pathway is critical for normal development and defects in any component can lead to severe developmental defects.

1.3.3 Neural Tube closure

One morphogenetic process that depends on the PCP pathway and CE movements is neural tube closure. Neural tube closure (NTC) is a complex morphogenetic event that requires the function of over 190 genes, all of which exhibit a neural tube defect (NTD) phenotype when mutated in mice [108]. Impressively, this number only includes the genes that function after initial

patterning of the neural ectoderm is completed [109]. In fact, this complicated morphogenesis is so sensitive to even minor perturbations that NTDs affect nearly 1/1000 births. The neural ectoderm begins as a flat sheet of epithelial cells, also called the neural plate, which is formed through CE movements governed by the PCP genes *Van Gogh like 2* (*vghl2*), *Celsr*, *Scribble*, and *dishevelled*. Mutations in those genes (*loop-tail*, *crash*, *circletail*, and *Dvl-1;Dvl*-2 respectively [110-113]) are associated with a specific birth defect called craniorachischisis which is caused by the failure of NTC to originate at the hindbrain/cervical boundary. It is thought that the NTDs caused by these mutations are actually the result of a slightly deformed neural plate. If the neural plate is properly patterned, NTC results from the bending of the neural ectoderm, the subsequent elevation and further bending of the neural folds, and finally fusion at the dorsal midline [114]. Fusion of the neural folds is initiated at a few specific locations along the neural axis and proceeds in a zipper-like fashion in both the anterior and posterior directions. NTDs in the anterior most region of a mouse embryo are known as anencephaly or exencephaly, whereas NTDs in the posterior of the embryo are more commonly known as spina bifida.

It can be imagined that this large-scale morphogenesis must be well coordinated and depends on the function of cytoskeletal components to provide the necessary tension and force to facilitate such a drastic rearrangement. Indeed, mutations in various proteins known to regulate cytoskeletal dynamics have been shown to result in NTDs. Among these are vinculin (focal adhesion associated protein) [115], MARCKS (PKC target and F-actin cross-linking protein) [116], p190 (RhoGAP) [117], Mena/profilin double mutants [118], and the non-receptor tyrosine kinases Abl and Arg [119] (for a review of all the known genes see [108]). Shroom3 is another actin-regulatory protein that is required for NTC [120] and will be discussed in greater detail in the next section. In addition, treatment of embryos with cytochalaisin D (a toxin that causes F-

actin to depolymerize [121]) also leads to similar NTDs [122, 123]. Interestingly, all of these mutations affect cranial NTC, but spinal NTC remains relatively unaffected, indicating a differential requirement for the contractile actomyosin belt that is positioned at the apical surface of the neuralepithelial cells.

1.3.4 Actin and Myosin II in Other Developmental Processes

A number of other mechanisms depend on the activities of a fully functional and tightly regulated actomyosin network. Eyelid closure, a process akin to NTC, in mice requires the contractile ability of the actomyosin network [124, 125]. In this example, the activity of ROCK-I and ROCK II are required to stimulate myosin light chain (MLC) phosphorylation, which in turn facilitates the cross-linking of actin filaments in the eye epithelium. The activated myosin then triggers the assembly of actomyosin bundles. These bundles then provide the tension necessary to drive eyelid closure prior to birth (the mutant phenotype is referred to as 'eyes open at birth' or EOB). The signaling pathway leading up to eyelid closure is initiated extracellularly by epidermal growth factor (EGF) binding to the EGF receptor (EGFR) [124, 126-128]. The EGF stimulus actually provides a positive feedback loop for the formation of actomyosin cables. Interestingly, but not too surprisingly, in the ROCK-I deficient mice, a similar defect is observed during the umbilical ring closure.

As indicated above, the development of the embryonic nervous system is incredibly complex and NTC is by no means the only process governing neural development. Neuronal cells have to migrate to the correct locations within the developing organism, create the appropriate cell junctions, and ultimately be able to release neurotransmitters into the synaptic junctions. Of course, all of these processes require actin and myosin. Cell migration is a process

that involves many of the components discussed elsewhere (see section 1.1), however a recent study in mice has placed this mechanism at the heart of a neural development. In this study, the n-cofilin knockout mouse displays neural tube closure defects, but it also exhibits a specific defect in neural crest cell (NCC) migration [129]. NCCs from these mice not only fail to migrate from cultured explants, but they lack any F-actin based structures. Axon guidance also depends intimately on an actomyosin network. Migrating axons are constantly encountering guidance cues that promote migration or retraction (repulsive cues). These cues are often mediated through the Rho GTPases to affect cytoskeletal changes. As an example, the guidance cue semaphorin-3A triggers the RhoA-ROCK pathway to activate myosin II and, in this case, create a unique F-actin bundle. This bundle provides the scaffolding for the myosin-facilitated retraction [130].

As described above, the ability of actin to elicit changes within a cell or a field of cells in response to external signals is essential for many biological processes to occur normally. The regulation of actin is at the heart of its function and thus it makes sense that there are hundreds of proteins that affect actin dynamics or function in some fashion. Often times, the proteins that can affect actin dynamics are classified into families that represent a conserved mechanism or some conserved homology specifically among those in the group. The next section will describe in detail a family of proteins that are involved in regulating the actin cytoskeleton with mutant phenotypes that affect various aspects of neural development.

1.4 THE SHROOM FAMILY OF PROTEINS

The Shroom (Shrm) family of proteins is a small family of actin binding proteins that are able to induce cytoskeletal changes and thereby influence cellular function. There are four Shrm family members in vertebrates, and these were originally identified as Apx [131], Apxl [132], Shroom [120], and KIAA1202 [133] and have recently been renamed Shrm1, Shrm2, Shrm3, and Shrm4 respectively [134]. The family is defined by the conservation of at least 2 of 3 domains: an N-terminal PDZ domain, a centrally located Apxl/Shrm Domain (ASD) 1 domain, and a C-terminal ASD2 domain (Fig. 4A). PDZ domains are well known for their ability to serve as protein-protein interaction motifs, however the binding partners for the PDZ domains of the Shroom family have not yet been identified (Fig 4B). The ASD1 domain of Shrm2 and Shrm3 has been experimentally shown to bind directly to actin and is able to target the proteins to the proper sub-cellular localization [135]. However, this motif displays a differential ability to bundle actin. The ASD1 element from Shrm3 is able to facilitate the bundling of actin filaments, whereas the ASD1 element from Shrm2 is not competent to do so [135]. This difference could perhaps reside in the sequence surrounding the ASD1 domain. The ASD2 domain is the most conserved domain, as all Shrm family members have one, and has quite elegantly been shown to facilitate an actomyosin-based constriction event [135, 136] (Fig. 4C).

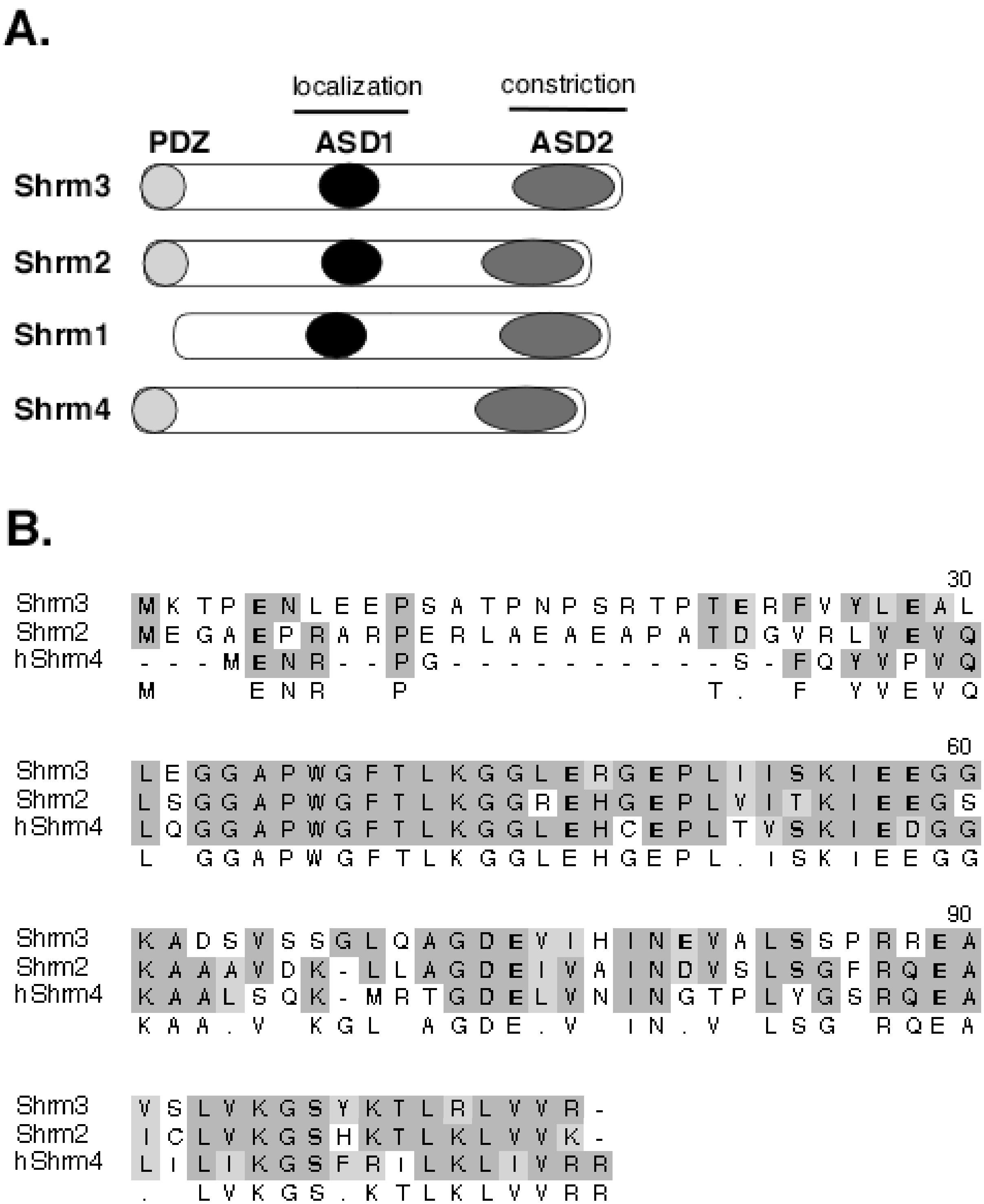
A.
localization
constriction
PDZ
ASD1
ASD2
Shrm3
Shrm2
Shrm1
Shrm4
B.
30
Shrm3 M K T P E N L E E P S A T P N P S R T P T E R F V Y L E A L
Shrm2 M E G A E P R A R P E R L A E A E A P A T D G V R L V E V Q
hShrm4 - - - M E N R - - P G - - - - - - - - - - S - F Q Y V P V Q
M E N R P T . F Y V E V Q
60
Shrm3 L E G G A P W G F T L K G G L E R G E P L I I S K I E E G G
Shrm2 L S G G A P W G F T L K G G R E H G E P L V I T K I E E G S
hShrm4 L Q G G A P W G F T L K G G L E H C E P L T V S K I E D G G
L G G A P W G F T L K G G L E H G E P L . I S K I E E G G
90
Shrm3 K A D S V S S G L Q A G D E V I H I N E V A L S S P R R E A
Shrm2 K A A A V D K - L L A G D E I V A I N D V S L S G F R Q E A
hShrm4 K A A L S Q K - M R T G D E L V N I N G T P L Y G S R Q E A
K A A . V K G L A G D E . V I N . V L S G R Q E A
Shrm3 V S L V K G S Y K T L R L V V R -
Shrm2 I C L V K G S H K T L K L V V K -
hShrm4 L I L I K G S F R I L K L I V R R
. L V K G S . K T L K L V V R R

Figure 4: The Shroom Family of Proteins

Figure 4: The Shroom family of Proteins

A. Domain map of the vertebrate Shroom proteins. The PDZ domains (light gray oval) and **A**pxL/**S**hroom/**D**omain 1 motifs (ASD1, black oval) are conserved in most of the Shrm family members. The ASD2 domain (dark gray oval) is conserved in all known family members. **B**. Sequence alignment of the conserved PDZ domains from Shrm3, Shrm2, and hShrm4. Note the number of conserved G-L-G-F residues (hallmark of PDZ domains). **C.** Sequence alignment of the conserved ASD2 domains. Both vertebrate and invertebrate sequences are compared.

1.4.1 Sub-cellular Function of Shroom

Shrm3 is able to target to the apical surface of epithelial cells and cause the apical surface to constrict relative to the basal surface (Fig 5A) [136]. Expression of a Shrm3 protein construct lacking the ASD1 domain does not cause constrictions when transiently transfected into MDCK cells. This is most likely due to improper targeting. Likewise, the expression of a Shrm3 construct that lacks the ASD2 domain fails to cause constrictions in tissue culture cells even though it is apically localized. Additionally, in these experiments it is shown that Shrm3 directs the apical positioning of F-actin and non-muscle myosin II (NMMII) creating a contractile apical actin 'belt' (Fig 5B) [136]. The ability of Shrm3 to induce constrictions depends on the activity of ROCK (but not RhoA). It is the ability of Shrm3 to localize to the apical surface and facilitate actomyosin-based constrictions that allows it to govern the morphogenesis of the neural tube from the neural plate (discussed below).

When ectopically expressed in MDCK cells, neither Shrm2 nor Shrm4 are able to induce a constriction event. In fact, neither of these proteins is targeted to the apical surface. However, chimeric proteins containing the localization motif (ASD1) of Shrm3 and the ASD2 domain of either Shrm2 or Shrm4 are able to trigger an apical constriction event in MDCK cells. These data show that the function of the ASD2 domain is conserved among the Shrm protein family members, and it is the differential expression and sub-cellular localization that causes them to assume different roles [135]. Interestingly, some Shrm proteins have been shown to affect other cytoskeletal components in addition to actin. It has recently been shown that Shrm1, Shrm2, and Shrm3 can regulate the distribution of γ-tubulin in developing eye epithelial cells [137, 138], however the mechanism (and purpose) of this event is still ill-defined. In fact, neither Shrm2 nor Shrm3 is able to recapitulate this event in MDCK cells (Hildebrand, unpublished data).

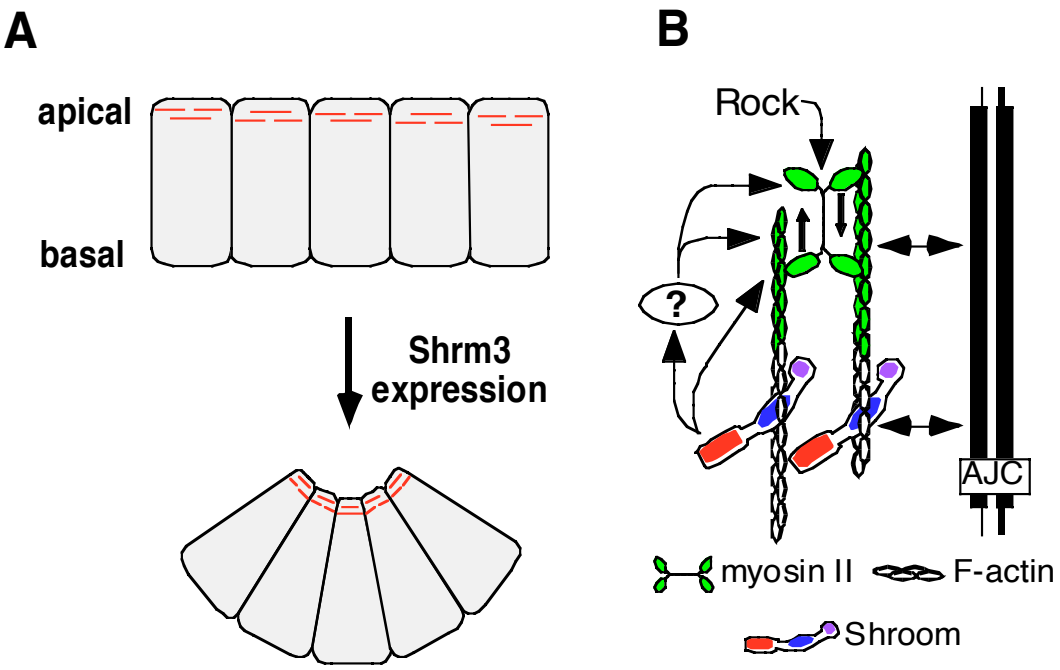

Figure 5: Sub-cellular Function of Shrm3

Figure 5: Sub-Cellular Function of Shrm3

A. Shrm3 is able to effect cell shape change in epithelial cells. The expression of Shrm3 causes the apically located actin (red lines) to constrict, forcing the entire apical surface to constrict. **B.** Shrm3 elicits acpical constriction through myosin II, by way of the ROCK pathway. Shrm3 binds to apical actin via its ASD1 motif (blue). Additionally, Shrm3 recruits myosin II through the function of the ASD2 domain (red). However, the details of this recruitment are unclear. The constriction event is dependent upon ROCK signaling. AJC = apical junctional complex. Figure based on that from [136].

1.4.2 Shroom is Evolutionarily Conserved

The Shrm family is conserved across many species with orthologs found in organisms ranging from *Drosophila* to *Homo sapiens*. As described above, many vertebrates have most, if not all, of the family members, while the invertebrates seem to have one Shrm related ortholog (Fig. 4C). The Shrm related proteins found in invertebrates all seem to contain a conserved ASD2 domain, and a few have a conserved PDZ domain (*Ciona* and sea urchin) with little homology to any Shrm protein outside of these domains. The *Drosophila* Shroom (dmShrm) protein cannot induce apical constriction in transiently transfected MDCK cells. However, when the dmShrm ASD2 domain is targeted to the apical surface (in the same type of chimeric experiments described above), it is able to facilitate an apical constriction event. The developmental role of the invertebrate Shrm orthologs is currently under investigation, but these proteins could provide great insight into evolutionarily conserved mechanisms of development.

When comparing all of the Shrm family proteins, it is quite apparent that they fall into two categories: Those with an ASD1 domain and those without one. This makes for quite an interesting observation, as the only vertebrate Shrm protein that lacks the ASD1 domain is Shrm4. Shrm4 could perhaps be the most 'ancestral' of the Shrm proteins since its protein structure most closely resembles that of the invertebrate Shrm ortholog. In fact, a gene duplication of the early Shrm protein most likely resulted in the dichotomy, giving rise to versions found in vertebrates, those without ASD1 (Shrm4) and those with (Shrm2 and Shrm3) [135]. Outside of the conservation of the ASD2 domain, little is known about the invertebrate Shrm orthologs. However, the vertebrate Shrm proteins have been well described in the literature, and most play key roles in specific developments processes.

1.4.3 Developmental Role of the Shroom Family of Proteins

As described above, the actin cytoskeleton plays a critical role in many developmental processes. Proper regulation of cytoskeletal dynamics (both spatially and temporally) is essential for the normal development of an organism as well as the homeostasis of adult tissues. Many of the vertebrate homologs of the Shrm proteins have been implicated in regulating specific aspects of development. Shrm3 is required for proper neural tube closure in mice, Shrm2 is required for normal retinal development, and Shrm4 has been implicated in X-linked mental retardation.

1.4.3.1 Shroom3 and NTC

As described previously, NTC is a complex morphogenetic process that intimately depends on actin dynamics to complete its formation. Shrm3 deficient mice fail to close their neural tubes and display various open neural tube phenotypes such as spina bifida, anencephaly, and exencephaly [120]. In *Xenopus*, xShrm3 is required for NTC since morpholino knockdowns and expression of a dominant-negative xShrm3 cause NTDs [139]. Shrm3 is localized to the apical junctional complex (TJs) of the neuralepithelia during neurulation and is able to direct actomyosin-based apical constrictions (see above). These two mechanistic details (expression domain and cellular function) along with the open neural tube phenotype, show that Shrm3 plays a direct role in this crucial morphogenesis and is absolutely required ensure it is properly completed.

1.4.3.2 Shroom2 and Eye Development

The retinal pigment epithelium (RPE) is a layer of epithelial cells that is required to prevent the scattering of light within the retina [140]. In order to function normally, this layer of cells

requires pigmentation by melanosomes during retinal development. Many genes have been identified that regulate both the biogenesis of melanosomes as well as their proper localization, including myosin VIIa [141, 142]. Mutations in the genes encoding these proteins have been implicated in many human disorders affecting retinal development. xShrm2 has recently been shown to be required for RPE development in *Xenopus* [137]. Morpholino knock-down of xShrm2 results in reduced retinal pigmentation due to aberrant lamination, abnormal RPE structure, and defects in the position and maturation of melanosomes within the RPE. Interestingly, *hShrm2* is located on the X chromosome in a region that is associated with Ocular Albanism (OA1), a disease state that is characterized, in part, by hypopigmentation and defective melanosomes [143]. No direct evidence has been discovered that implicates *hSHROOM2* in OA1, but the fact that the gene covers over 70% of the OA1 critical region makes it an interesting candidate. It is important to remember that Shrm2 expression in MDCK cells is not sufficient on its own to drive apical constrictions and similarly, xShrm2 expressing cells do not exhibit apical constrictions in developing Xenopus embryos. So unlike Shrm3, where the ability to direct apical constrictions is at the heart of its function, Shrm2 most likely elicits its function through a different mechanism, perhaps utilizing γ-tubulin to regulate melanosome localization.

1.4.4 Shroom4 : XLMR and Initial Characterization

While gross morphological aberrations can cause severe and often lethal birth defects, more subtle types of mutations can lead to non-lethal, yet debilitating maladies. One consequence of irregular neural development is mental retardation, which is reported to affect 1-3% of the world's population [144]. Of these cases, more than 10% have been attributed to mutations in genes on the X chromosome, also referred to as X-linked mental retardation (XLMR). The

mutations leading to XLMR fall into quite a few gene categories, and it is not surprising that a number of these are gene products that affect actin dynamics at some level, including the Rho effectors *ARHGEF6*, *ARHGEF9*, *FGD1*, *OPHN1*, and *PAK3*, and actin binding proteins *FLNA* (filamin) [145].

Recently it has been reported that mutations in *hShrm4* (*SHROOM4*) are also associated with XLMR [133]. In this study, two unrelated patients are carriers for mutations that result in XLMR, while 6 other families harbor innocuous polymorphisms. The two XLMR mutants are a silent E474E (A>G) mutation and a missense S1089L (C>T) mutation. hShrm4 cDNA (6,029 bp) was first isolated from a screen from a large-insert adult brain library [146]. Five different isoforms were isolated, one of which corresponded to the cDNA and encoded a 1498 AA protein. *hShrm4* consists of 10 exons, with the majority of the coding region found in exon 4. The intron-exon structure closely resembles that of Shrm3, with exons one and two separated by over 117,000 bp and exons 2 and 3 separated by about 57,000 bp. hShrm4 has the conserved N-terminal PDZ domain and C-terminal ASD2 domain and a putative PDZ binding site as the last 3 AA. In addition, hShrm4 contains a potential EVH1 binding site (FPPPP) and a large stretch of glutamine and glutamic acid residues. Expression analysis of the RNA by Northern blot reveals ubiquitous, but seemingly differential, expression of hShrm4 in adult tissues (including 8 sub-regions of the brain).

Analysis of mShrm4 (the basis of this dissertation) similarly revealed ubiquitous expression of the RNA in all adult tissues examined as well as embryonic expression beginning at E8.5 and persisting throughout adulthood. The disease causing mutations in *hShrm4* lie in a region that is roughly 80% identical to *mShrm4* and *rShrm4*, but the actual AA change is in a non-conserved serine residue (proline in both mouse and rat). Subcellular localization studies of

hShrm4 show that it is coincident with actin and collapses with actin upon latrunculin B treatment. In all, these studies not only place hShrm4 into the Shrm protein family, but also establish yet another neural phenotype associated with a mutation in a Shrm family protein.

1.5 AIMS OF DISSERTATION RESEARCH

The importance of the Shroom protein family in neural development is highlighted by the discovery and initial characterization of Shrm3. Since that time, a number of other proteins have been identified that share homology with Shrm3 and have been ascribed to the Shroom family of proteins. Shrm1 and Shrm2 had already been somewhat described in the literature, but the less similar Shrm4 protein had not. In fact, at the onset of this work, Shrm4 was little more than an uncharacterized ORF discovered in a screen of large-scale cDNAs from an adult human brain library.

The aim of this dissertation research is to identify and clone the murine *Shrm4* gene, and to characterize the corresponding gene product. My research on mShrm4 is in agreement with what was reported in the original work on hShrm4, but more importantly it has provided greater insight to understand how Shrm4 functions and allows for speculation as to how it may play a role in XLMR.

2.0 MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY

cDNA representing bases 3433-4429 of *mShrm4* was isolated from a mouse brain cDNA λ library (Stratagene), using the human *SHRM4* cDNA as a probe (a gift from the Riken Corporation). PCR was used to amplify bases 3480-4429 with primers (engineered restriction enzyme sites underlined): Fwd -λ 5'GCCCCgAATtCAGAGGAACAGCC3' and Rv-λ 5'GGTGCAGTGGCATACTCGAGGTGC3'. cDNA containing bases 268-3885 were obtained from the RIKEN corporation (D430043L16, GenBank accession BB487710) and bases 861-3480 were PCR amplified for cloning using the following primer set: Fwd-Riken 5'GCCTTCCGGCCTCTAGAGCCC3' and Rv-Riken 5'GGCTGTTCCTCTGaATTcGGGGC3'. The 5' cDNA corresponding to bases 1-861 were PCR amplified from random hexamer primed RNA. Primers for PCR were designed based on sequence deposited on Ensembl (ID #: ENSMUSG00000068270) with engineered restriction sites for cloning. Fwd-ATG 5'GCCCAGatCtAGCCGAGGATGGAGAGC3' and Rv-ATG 5'GGGCTCTAGAGGCCGGAAGGC3'. The three overlapping cDNAs were cloned together into pBluescript. *Shrm4* was then sub-cloned into the pCS3mt eukaryotic expression vector. The sequence for *mShrm4* has been deposited under GenBank Accession DQ435686.

cDNAs encoding fragments of mShrm4 protein were generated using PCR with engineered restriction sites, using the following primer sets: 1-522 Fwd-λ and Rv 5'GGAAgAAtTcGCAGAGGGCTGC3'; 522-1180 Fwd 5'GCAGCCCTCTGCgAaTTcTTCC3' and Rv-Riken, 436-966 Fwd 5'GGCAGCAAGGGGATGAATTCGCCAATTGGG3' and Rv 5'CCTGGATTTCCAAGAATTCCCAGGTGCC3'. CS3mt-Shrm4 1180-1475 was generated by cloning the insert isolated from the λ library into pCS3mt. For bacterial expression of Shrm4 protein, *mShrm4* DNA fragments encoding amino acids 350-878 and 1152-1311 were PCR amplified and cloned into the pGEX3X expression vector.

2.2 ANTIBODY PRODUCTION AND PROTEIN EXPRESSION

Mouse Shrm4-specific sera (UPT114 and UPT155) were generated in rabbits (Cocalico) using an antigen consisting of amino acids 1152-1311 (UPT114) and 350-878 (UPT155) fused to GST. Antigenic protein was expressed in BL21pLys *E. coli* and induced with 250 μM IPTG overnight at 30°C. Cells were harvested by centrifugation, resuspended in NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.05% NP-40) containing a protease inhibitor cocktail, and lysed by sonication. Lysate was cleared by centrifugation and incubated with glutathione-sepharose beads at 4°C for 2 hours (Amersham). Sepharose beads were collected by low speed centrifugation and washed with ice cold NETN. GST-mShrm4 fusion proteins were eluted from the beads with Elution buffer (20 mM glutathione, 100 mM Tris [pH 8.0], and 120 mM NaCl) with protease inhibitors. Eluted protein was subjected to SDS-PAGE followed by Coomassie blue staining to determine concentration and integrity. Shrm4-specific antibodies were purified

from rabbit sera (UPT114 and 155) by chromatography using their respective antigens coupled to CNBr-sepharose (Amersham).

2.3 CELL CULTURE

Brain endothelial (bEND), C166 yolk sac endothelial, and RAT1 fibroblastic cells (ATCC) were maintained in DMEM supplemented with 10% FBS, pen/strep, and L-glutamine at 37^{o}C and 5% CO_2. HUVEC cells were grown in ECM supplemented with BulletKit (Cambrex) at 37^{o}C and 5% CO_2. Cells grown on gelatin coated glass cover slips were transfected with 1-2 μg of DNA using Lipofectamine 2000 according to manufacturer's recommendations (Invitrogen). For drug treatments, cells were treated with DMSO (vehicle), 2 μM cytochalasin D (CD, Sigma), or 200 μM blebbistatin (Calbiochem) for 20 or 120 minutes at 37^{o}C, respectively. Washout experiments were completed as described above, except following the drug treatment, the cells were washed 3 times in PBS and allowed to recover in DMEM or DMEM + 200 μM blebbistatin for 3 hours. Calyculin A (Calbiochem) was used at 4 nM for 45 minutes and Y-27632 (Calbiochem) at 20 mM for 1.5 hours. Nocodazole was used at 33 μM on ice for 30 minutes.

2.4 WESTERN BLOTTING

Adult mouse tissue lysates and e10.5 day mouse embryo lysates were generated by homogenizing isolated tissues in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris [pH 7.5], 4 mM EDTA, and 1.0% NP-40) on ice with a Tissue Terror/mechanical

homogenizer. Lysate was cleared by centrifugation and supernatant was stored at -80^{o}C. Cells ectopically expressing mShrm4 proteins were lysed in RIPA buffer, scraped from the surface of the dish, and centrifuged to clear the lysate. Equal volumes of lysate were loaded and resolved on a 10% SDS-PAGE gel, then transferred to a nitrocellulose membrane. Endogenous and exogenous mShrm4 was detected using affinity purified rabbit anti-Shrm4 or mouse anti-myc (9E10) antibodies at a dilution of 1:200 in TBST + 4% milk. Primary antibodies were detected using HRP-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Amersham) diluted 1:2500 in TBST, followed by ECL (Amersham).

2.5 IMMUNOCYTOCHEMISTRY

Cells grown on glass cover slips were washed three times with phosphate buffered saline (PBS) and fixed with either -20^{o}C methanol for 5 minutes or 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. PFA fixed cells were permeablized for 5 minutes in PBS + 0.2% Triton-X 100. Cells were stained with primary antibodies diluted in PBS + 0.1% Tween 20 (PBT) supplemented with 1% goat serum (Jackson Labs) for one hour at room temperature. The following antibodies were used: rabbit anti-mShrm4 (1:200), mouse anti-non-muscle myosin II (CMII 23, Developmental Studies Hybridoma bank), rabbit anti-non-muscle myosin II-B (Covance, 1:200), rat anti-PECAM (BD Pharmingen, 1:400), mouse anti-caveolin (Transduction Laboratories, 1:200), mouse anti-Tubulin (a gift from Dr. Charles Walsh, University of Pittsburgh), mouse anti-giantin (a gift from Dr. Adam Linstedt, Carnegie Mellon University), and mouse anti-rab5 and mouse anti-rab7 (a gift from Dr. Kirill Kiselyov, University of Pittsburgh). Following primary antibody incubation, cells were washed three times in PBT and

stained for 1 hour with fluorescently labeled secondary antibodies (Molecular Probes) diluted 1:400 in PBT. Secondary antibodies were either Alexa-488 or Alexa-568 conjugated to goat anti-rabbit, goat anti-mouse, or goat anti-rat IgG. Actin was detected using either TRITC or Alexa-633 conjugated Phalloidin (Sigma and Molecular Probes, respectively). Cells were then washed as above and mounted on microscope slides with Vectashield (Vector Labs).

For staining tissue sections, embryos or adult tissues were isolated and fixed in 4% PFA for 1-3 hours, depending on the size of tissue, at 4^{o}C. Samples were then washed extensively in cold PBS, equilibrated in 30% sucrose overnight at 4^{o}C, embedded in OCT compound, and stored at -80^{o}C. Samples were cut into 10-12 μM sections on a Leica cryostat at -20^{o}C and placed onto glass microscope slides. Sections were rehydrated in PBS for 5 minutes prior to use, incubated in blocking buffer (PBT + 1% goat serum) and stained overnight at 4^{o}C with primary antibodies diluted in blocking buffer. Sections were washed in PBT for 15 minutes at room temperature, incubated with secondary antibodies and/or TRITC-phalloidin for 2 hours, washed, and mounted using Vectashield. Images were acquired on a Biorad Radiance 2000 Laser Scanning Confocal System mounted on a Nikon E800 microscope (40X oil objective), using LaserSharp 2000 software. Adobe Photoshop software was used to process images.

2.6 ACTIN CO-SEDIMENTATION ASSAY

GST-Shrm4 fusion proteins consisting of amino acids 436-966 and 436-1180 were prepared as described above, with the exception that cells were lysed using a French press. F-actin (0.2 μM, 10-20 μm filaments) was prepared according to manufacturer's recommendations (Cytoskeleton). Purified GST-Shrm4 and GST alone (control) were pre-cleared at 100,000 x g.

The control (GST-Shrm4, GST, GST + actin, and actin alone) and experimental (GST-Shrm4 + actin) samples were incubated at room temperature for two hours in F-actin buffer (Cytoskeleton). The supernatant and pellet fractions were separated at 100,000 x g, resolved by SDS-PAGE electrophoresis, and visualized by Coomassie blue staining. For bundling assays, samples were prepared as above, except the separation occurred at 10,000g. In order to fluorescently label F-actin bundles, 30 μL of the sample (before centrifugation) was incubated with 1 μL of TRITC-phalloidin for 1 hr and placed directly on a glass slide. Images were acquired as described above.

2.7 WHOLE MOUNT IN SITU HYBRIDIZATION

Zebrafish embryos were generously provided by Dr. Beth Roman, University of Pittsburgh. zShrm4 cDNA was cloned by PCR from a 36 hpf cDNA library (M. Tsang, U. of Pittsburgh), using primers designed from the zebrafish Shrm4 sequence on Ensembl.org [147]. The forward primer: 5'CCAGCGGTCGTTTAGGTCGATCGGATGAC3' and the reverse primer: 5'CTGCAGTACAGGATCCTTTTGGTCCC3' were used to amplify bases 2624 to 5469 (end) of zShrm4. This PCR product was digested with SacI and BamHI to generate a fragment of about 600 bp and cloned into pBluescript (Stratagene). The plasmid was linearized with SacI for riboprobe synthesis. Digoxygenin-labeled antisense RNA was transcribed using T7 RNA polymerase (Stratagene) and Digoxygenin RNA label mix (Roche) according to manufacturer. In situs were performed according to [148]. Briefly, embryos were fixed in 4% PFA and hybridized overnight with 1:50 dilution of riboprobe in HB4 buffer (50% formamide, 5X SSC, 5mg/ml yeast RNA, 50 μg/ml herparin, and 0.1% tween). Following hybridization, embryos

were washed with 50% formamide/2X SSC/0.1% Tween, and incubated with the anti-digoxygenin antibody overnight. Staining was observed by incubation with NTMT followed by NBT/X-phosphate.

3.0 CLONING AND INITIAL CHARACTERIZATION OF MURINE SHROOM4

3.1 INTRODUCTION

The Shroom family encompasses a relatively small number of proteins that share sequence identity in a few conserved domains. Shrm4 was initially identified as an uncharacterized ORF in the human genome that shared homology to the other known Shrm family members. Since nothing was known about Shrm4 functionally, the gene had to be cloned so that a number of tools could be created to assist in the elucidation of Shrm4's function. The murine *Shrm4* gene was cloned, because most of the ensuing *in vivo* types of experiments were to be performed in the mouse. After cloning the mShrm4 cDNA, antibodies were raised against specific non-conserved regions and were used to investigate the tissue distribution and sub-cellular localization of the Shrm4 protein product. The following sections will describe the cloning of mShrm4 and the initial characterization of the protein product.

3.2 CLONING OF SHRM4 AND ANTIBODY PRODUCTION

3.2.1 Cloning of mShrm4

The mouse *Shrm4* gene was cloned using the cDNA corresponding to the ASD2 sequence of hShrm4 as a probe for a λ-library screen. The library we had in our collection did not reveal a full-length cDNA clone, so the central portion of the cDNA was obtained from the RIKEN Corporation. The PDZ domain was not present in this clone either (see below), so RT-PCR was used to clone the corresponding sequence using the data provided from the Ensembl database (www.ensembl.org v.45) [147]. Cloning of mouse *shrm4* reveals an open reading frame of 4425 bp encoding a predicted protein of 1475 amino acids with a predicted molecular mass of 163 kDa (Accession number: DQ435686) [149]. Mouse Shrm4 mirrors the organization of the other Shrm family proteins [134], such that it contains an N-terminal PDZ domain and a C-terminal ASD2 motif, both of which are approximately 60% identical to those of mShrm3 (Fig 6). Like mShrm3 and hShrm4, mShrm4 contains putative binding sites for EVH1 domains (FPPPP) and PDZ domains (SNF) [150, 151] (Fig 6A). In addition, mShrm4 has a leucine zipper heptad repeat (bZIP) [152], which is conserved in most of the known ASD2 domains across species (Figs 4C and 6A asterisks). A stretch of glutamines (Q) and glutamic acids (E) (QLQQQQQQQQQQQQQRCEEEEEKEQEEEGEKEE) is located between the EVH1 binding site and the ASD2 domain. This sequence motif is not present in other known Shrm-related proteins but is conserved in hShrm4. While most of the proteins containing polyglutamine stretches are regulators of transcription, none of the Shrm proteins have displayed any transcriptional activity. mShrm4 shares 98% and 94% identity to the hShrm4 PDZ and ASD2 motifs, respectively. Notably, Shrm4 lacks the central ASD1 (actin-targeting) domain. Even

with the high degree of sequence identity between the mouse and human Shrm4 sequences, the region in between the PDZ domain and the ASD2 motif only retains about 70% identity. Shrm4 is conserved in a number of non-mammalian vertebrates including *Xenopus* and *Danio* (Fig 7A,B,C). The full protein sequences for both *Danio* and *Xenopus* can be found in Appendix A.

It is interesting to note that polyglutamine repeats are associated with numerous neurodegenerative diseases (NDD) [153], and the number of CAG repeats in Shrm4 falls within the "normal" distribution of repeats found in the proteins implicated in NDD. As previously mentioned, this repeat is not conserved across the Shrm family and in fact, only the mammalian *Shrm4* genes seem to retain this sequence repeat.

mShrm4 is located on the X-chromosome (like *hShrm4* and *Shrm2*), and its genomic organization closely resembles that of *hShrm4* and the other *Shrm* family members (Fig 8A). Like the other *Shrm* genes, there are two exons that encode the PDZ domain, which are roughly 120 kb apart, and exons 2 and 3 are another 60 kb apart. Initially, many of the reported Shrm4 sequences in the database did not include the sequence from the first two exons, indicating that the presence/absence of these two exons may represent alternatively spliced isoforms. This makes sense based on what is reported for hShrm4 and for Shrm3. Exon 4 contains over 2400 bp of the 4425 bp coding sequence. It is this exon that will be targeted when creating the *Shrm4* knockout mouse. In fact, a probe specific for exon 4 was used to isolate a genomic clone of *mShrm4* from a genomic library. Since it is necessary to have upstream (5′) and downstream (3′) regions of homology for creating a targeting vector, the isolated genomic clone was probed either for exon 3 (Fig. 8B) or exon 5 (not shown). Southern blot analysis revealed that the clone was positive for exon 3 sequences, but that is did not include sequence pertaining to exon 5. The prospect of this experiment will be described in greater detail in section 6.4.

A.

MESRPGSFQYVPVQLQGGAPWGFTLKGGLEHCEPLTVSKIEDGGKAALSQKMRTGDELVNING
TPLYGSRQEALIIKGSFRILKLIVRRRNTPVSRPHSWHVAKLLEGCPDVATTMHFPSEAFSLSWH
SGCNTSDVSVQWCPLSRHCSTEKSSSIGSMESLEQPGQPTYEGHLLPIDQNMYPSQRDSAYSS
FSASSNASDCALSLKPEEPPSTDCVMPGPGPIKVTDDQANVSENSGSSHSTSEDHVTSTSHASS
YSDEGHHSGPAKMARGPPEPPVRSDSLPASRAQLLNGEQHRASEPVDSLPQKEKPGLETVLPP
RSSNQFCCLSGQDQVTDEDHQNCELSKPSESSQDDCEHLLIEDSSKALDSPKAHDKGSNKEFG
LLKEASADLANTLNFGAIPHLRGTMEHRHSAPEQLLASHLQQVHLDSRGSKGMELPIGQDGHQ
WTVSPLHNNPKGKKSPSLPTGGTQDQTRKERKTTPLDDKLMASVHQSQSDVLLGEVDGHPNR
AGRASSDLTSQQPSATCSSVQQTRDFLSAHKIVDHTEASEEGDNEPKECGRLGGRRSGGPRG
RSIQNRRRSERFATNLRNEIQRRKAQLQKSKGPLSQLCDTNEAVEETQEPPESPPLSASNASLLP
SYKNVPSPGDKVFNKSMILRARSSECLSQASESSKARGGVEGRMSPGQRSGQSSLALNTWWK
ASDSSTLDTEKANAHHGVCRGHWRWSPEHNAQPQVALSTEAPSNPDDSKELKTSTPQAGEEA
VLMPFADRRKFFEESSKSLSTSHLPGLTTHNNKPFIQRQKPIDQNFQSVSYRDLRCHPLDQSYH
SADQSYHAADQSYHSLSPLQSETPTYPECFATKGRDNSLCCKPVHHGDCDYHRTCSHPCSAQ
GTVRHDPCICCSGEICPALLKRNLLPKCHNCRCHHHQCIRCTGCCHGPQHSAHEDSSMAPGNA
WKSRKAAIQEFPVDKWKPITGNRKTSHSGREMAHSKAGFSLSTPFRPCIENPALDLSNYRAVSS
LDILGDFKRASNKPEESSVYEDENSVASMPRPLRSRAFSESHISLEPQNTQAWGKHQRESFSKG
SETQPDTLGARKKV**FPPPRPPPPP**NWEKYRLFRAAQLQQQQQQQQQQQQQQRCEEEEEKEQE
EEGEKEEDLPPQYFSSELTGSCAPNTEEQPQSLKMGHQEASRQGSQSLQEQEAFALHPSNFYP
PVRGCTVPQPEKAQHPCYYGTHGLWRTTEQEATVTPKQEFQHFSPPKGASGIPTSYSAYYNISV
AKAELLNKLKQQPEMAEAGLGEEGVDYELAQKKIQLIESISRKLSVLREAQRGLLDDINANAALGE
EVEANLKAVCKSNEFEKYHLFIGDLDKVVNLLLSLSGRLARVENALNSIDSESNQEKLVLIEKKQQ
LTNQLADAKELKEHYDGREKLVFGMVSRYLPQDQLQDYQHFVKMKSALIEQRELEEKIKLGEEQ
LKCLKESLHLGPSNF

B.

Figure 6: Cloning of mShrm4

Figure 6: Cloning of Murine *Shroom4*

A. The *mShrm4* encodes a protein of 1475 amino acids that has a number of features that are conserved among the Shrm family of proteins, an N-terminal PDZ domain (shaded in yellow) and a C-terminal ASD2 motif (shaded in gray). There is also a putative EVH1 protein-binding site (FPPP) (in bold text) and a potential PDZ domain ligand (boxed). The non-conserved run of glutamine (Q) and glutamic acid (E) residues are underlined. The leucines that make up the leucine zipper within the ASD2 domain are marked with an asterisk. **B**. The protein map of mShrm4, which will be used to designate the protein throughout. The PDZ domain and the ASD2 motif (yellow and gray ovals respectively), the EVH1 site (shaded arrowhead), the Q/E run (open arrowhead), and the C-terminal PDZ binding site (arrow) are all indicated. The red lines mark the regions to which the antibodies were generated.

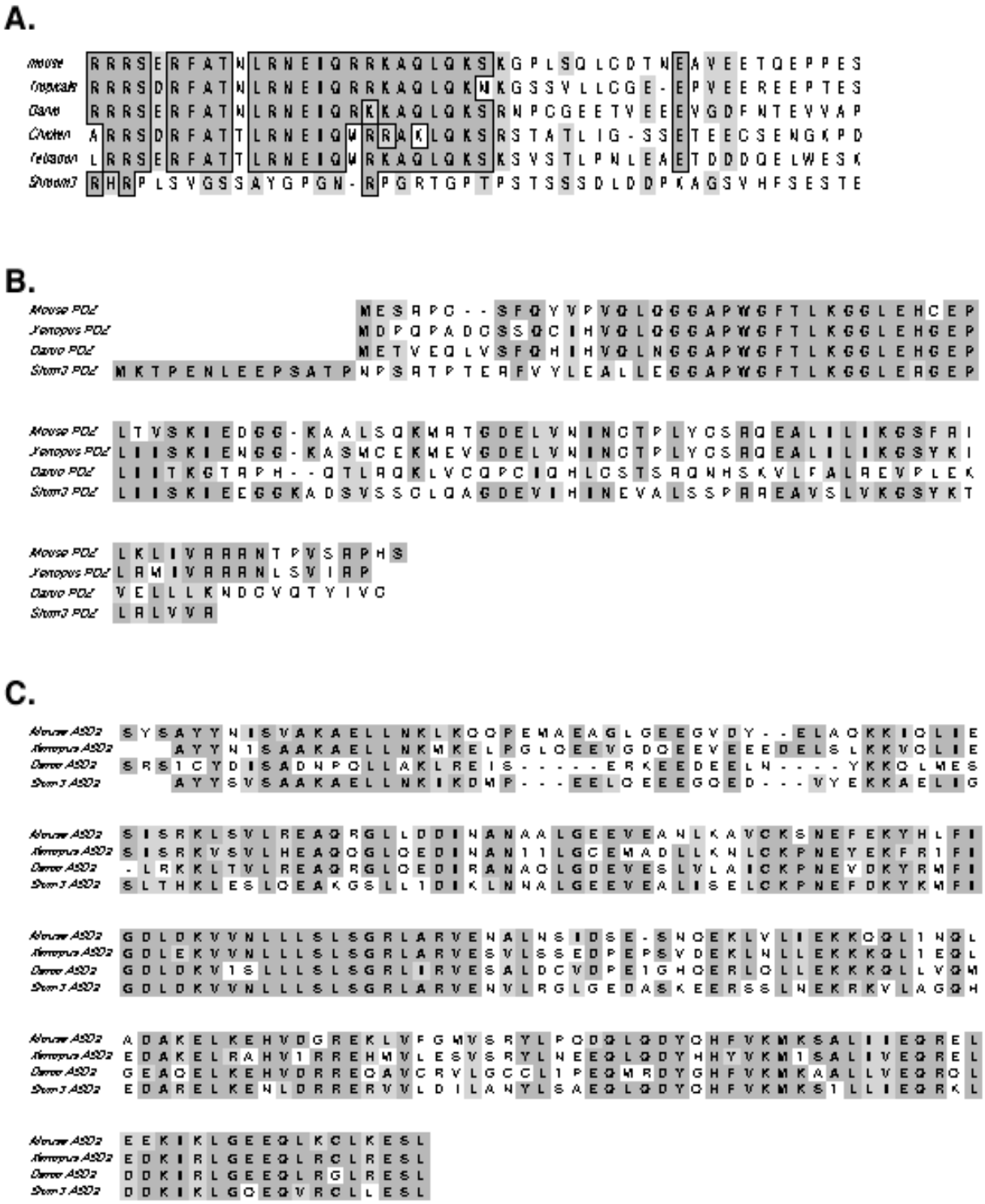

Figure 7: Alignment of Shmr4 Sequences Conserved Among Species

Figure 7: Alignment of Shrm4 Sequences Conserved Among Various Species.

A. The relatively non-conserved central domain of Shrm4 does have a short sequence conserved in Shrm4 orthologs, which is not conserved in Shrm3. Sequence corresponds to amino acids 575 to 622 of the mShrm4 protein. **B**. The PDZ domains of mShrm4 and xShrm4 are more similar than zShrm4. The mShrm3 PDZ is shown for comparison. **C**. The ASD2 domains are also conserved among Shrm4 orthologs. Again, the mShrm3 domain is shown for comparison. The leucine zipper motif is conserved among species as well.

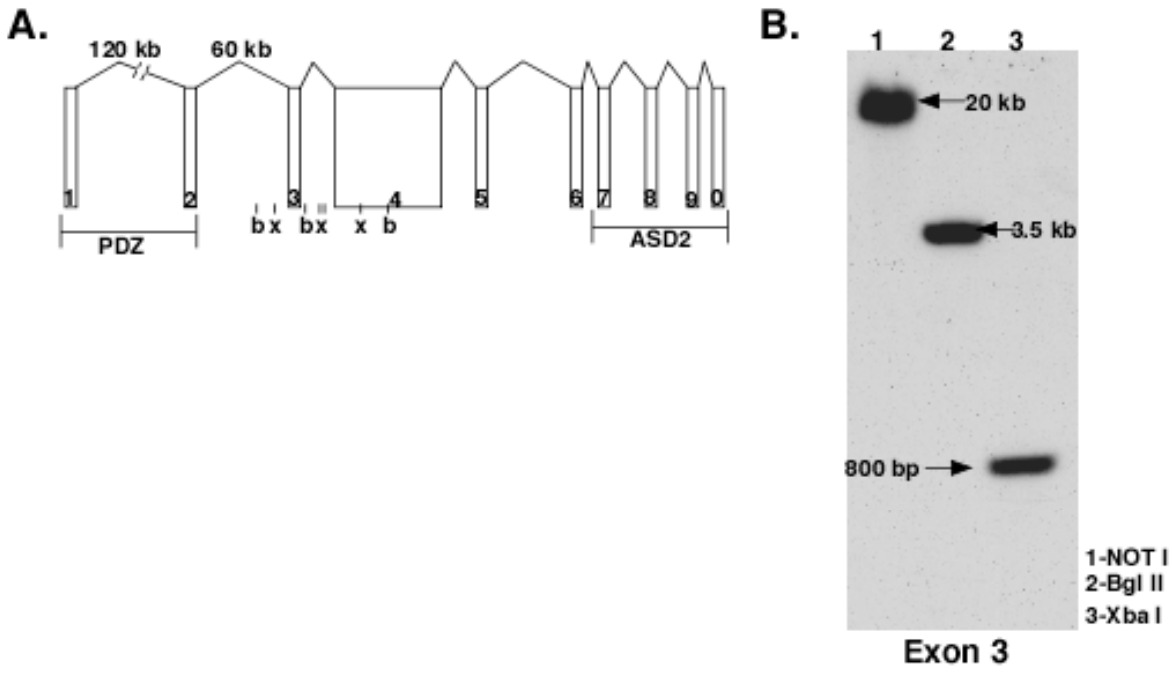

Figure 8: Genomic Organization of mShrm4

Figure 8: Genomic Organization of mShrm4

A. The intron/exon boundaries are based on the sequence from Ensembl (www. ensembl.org). The exons are numbered and the lines attaching them (above) represent the intervening intronic sequence (distances are given for the large introns in kb). The PDZ and ASD2 coding exons are noted as well as the predicted restriction sites for the BglII and Xba I restriction nucleases. **B**. The represented genomic clone recovered in a genomic library screen with an exon 4 specific probe. The 20 kb insert (lane 1) is flanked by Not I sites in the λ-library cloning vector. The clone was digested with the indicated enzymes and analyzed via Southern blot with a radiolabeled probe specific for exon 3.

3.2.2 Shrm4 Antibody Production

Polyclonal antibodies were raised in rabbits against the protein product generated from the mShrm4 cDNA. Two different constructs were used for expressing portions of the mShrm4 protein that are not conserved among the Shrm family (Fig. 6B, red lines). The N-terminal antiserum was raised against amino acids 350-878 and the C-terminal antisera was raised against amino acids 1152-1311. The specific antibodies were affinity-purified and both recognize a band of about 200 kD from HEK293 cells transiently transfected with a cDNA encoding full-length mShrm4. This is much larger than the predicted molecular mass of 163 kD, however all the Shrm family proteins migrate much slower in an SDS-PAGE gel than would be predicted. This is possibly an indication of post-translational modifications, such as phosphorylation, but it is not yet known what causes this large discrepancy.

In order to better characterize the specificity of the antibodies, all of the Shrm4 expression constructs contain an N-terminal fusion to a 6X myc-tag which is recognized by the 9E10 anti-myc monoclonal antibody (data not shown). The N-terminal mShrm4 antibody recognizes constructs expressing amino acids 1-522 and 522-1180 of Shrm4, but not amino acids 1180-1475. Conversely, the C-terminal antibody recognizes the 1180-1475 construct, but neither the 1-522 nor the 522-1180 construct (Fig. 9A, B). In cell culture, HEK293 cells were transiently transfected with an N-terminally myc-epitope tagged mShrm4 construct and stained for mShrm4 expression (Fig. 9C). The 9E10 and the N-terminal mShrm4 antibodies recognize the same protein in the cell, indicating not only that the antibody recognizes mShrm4 *in vivo*, but also that the expression construct is able to produce and express a full-length protein in cell culture. Since the antibodies are able to recognize mShrm4, they were next used to examine the endogenous tissue distribution and sub-cellular localization of Shrm4.

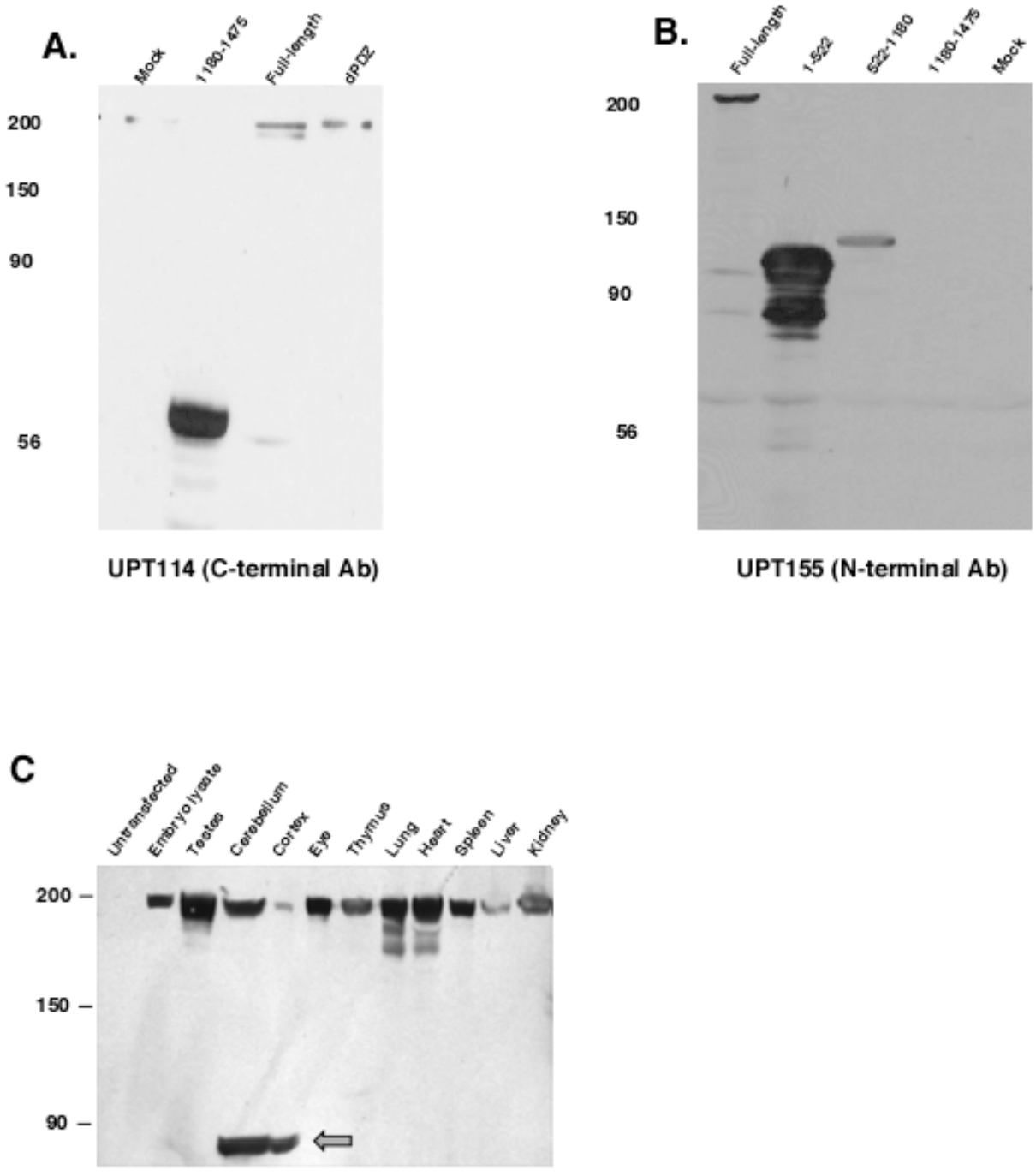

Figure 9: Analysis of mShrm4 Using Affinity Purified Anti-Shrm4 Antibodies

Figure 9: Analysis of mShrm4 Using Affinity Purified Anti-Shrm4 Antibodies

A. The C-terminal affinity purified anti-Shrm4 antibody recognizes Shrm4 protein in lysates from transiently transfected HEK293 cells. The full-length Shrm4 and a construct lacking the PDZ domain (dPDZ) migrate around 200 kDa. A construct consisting of amino acids 1180-1475 is also recognized. An untransfected cell lysate was loaded as a control. **B**. Similarly, the N-terminal affinity purified antibody also recognizes Shrm4 protein in lysates from transiently transfected HEK293 cells. The protein constructs 1-522 and 522-1180 fall within the antigenic region of the antibody, whereas the 1180-1475 construct does not. **C.** HEK293 cells were transiently transfected with the full-length mShrm4 construct (containing an N-terminal myc epitope tag) and stained to detect myc (red) and mShrm4 (green). The N-terminal anti-mShrm4 and the 9E10 anti-myc primary antibodies were used and detected with Alexa-488 or Alexa-568 secondary antibodies. **D**. Western Blot of lysates from e12.5 whole embryo and adult tissues. mShrm4 was detected using the C-terminal mShrm4 antibody and a HRP-conjugated secondary. For all blots, equal volumes of lysate were loaded onto the gels, resolved by SDS-PAGE, transferred to nitrocellulose, probed with the primary Shrm4 antibody, and were detected using an HRP conjugated secondary and enhanced chemiluminescence (ECL).

3.3 EXPRESSION AND LOCALIZATION OF ENDOGENOUS mSHRM4

3.3.1 Tissue Distribution of Shrm4 in Embryonic and Adult Tissues

The distribution of mShrm4 in a number of different tissue types was examined both by Western blot (WB) and by immunofluorescence (IF). All the tissues examined (both embryonic and adult) were positive for Shrm4 by WB (Fig. 8D). These data are in agreement with the ubiquitous expression observed for the Shrm4 mRNA [133]. In brain, the Shrm4-specific antibodies reproducibly detect a protein with an apparent molecular mass of 90 kDa (Fig. 8D, arrow). It is unclear if this protein species results from alternative RNA splicing, post-translational modification of full-length Shrm4, or represents an unrelated protein with a shared epitope.

Based on the ubiquitous expression of Shrm4 and the known expression pattern of Shrm2 and Shrm3 [120, 135, 136], the cellular distribution of Shrm4 within the tissue architecture was

examined by IF (Figs. 10 and 11). Frozen sections from embryonic day (e) 12.5 and e15.5 mouse embryos and adult mouse tissues were stained to detect Shrm4, along with a number of marker proteins. Shrm4 is expressed in the developing eye epithelium in e15.5 mouse embryos (Fig. 10 A and B). It appears that Shrm4 is expressed at the apical surface in these cells as well as along the lateral membrane of these cells (Fig. 10A, arrowhead and arrow respectively). However, in single optical sections, it appears that Shrm4 localizes in puncta just basal to the apical actin network in these cells (Fig. 10B, box). This punctate staining is also observed in the neural tissue as well (Fig. 10D, box). In e12.5 mouse embryos, Shrm4 is enriched at the apical surfaces of the neural epithelium (Fig. 10C, D arrowhead). Strikingly, Shrm4 is prominently expressed in the developing vasculature of the neural epithelium (Fig. 10D). Shrm4 is in the endothelial cells of the vasculature as it is co-expressed with PECAM. Similarly, Shrm4 is co-incident with PECAM in the highly vascularized adult lung (Fig. 11A). Similar to the observations in the embryonic eye and neural tissue, Shrm4 is also enriched at the apical surface of the adult kidney tubule epithelium as well (Fig. 11 B, C). In all tissues observed, Shrm4 co-localizes with actin (Figs 10A, B and 11B, C and data not shown). In addition, in kidney tubules, Shrm4 is detected in a punctate pattern in the cytoplasm and co-localizes with actin at the basal surface of these cells (Fig 11C). A transverse section through the adult epidermal layer reveals that Shrm4 appears only to be expressed in the polarized cell populations of the tissue (Fig 11D), as Shrm4 only co-distributes with E-cadherin (Fig 11D, arrows). It is interesting to note that Shrm4 seems to selectively be expressed in polarized endothelial and epithelial cells, as indicated by co-incidence with PECAM, β-catenin, ZO-1, and E-cadherin. The IF of various tissue sections gives an informative, yet incomplete picture of Shrm4 localization. In order to

better understand Shrm4 function, an understanding of the sub-cellular localization of Shrm4 is necessary.

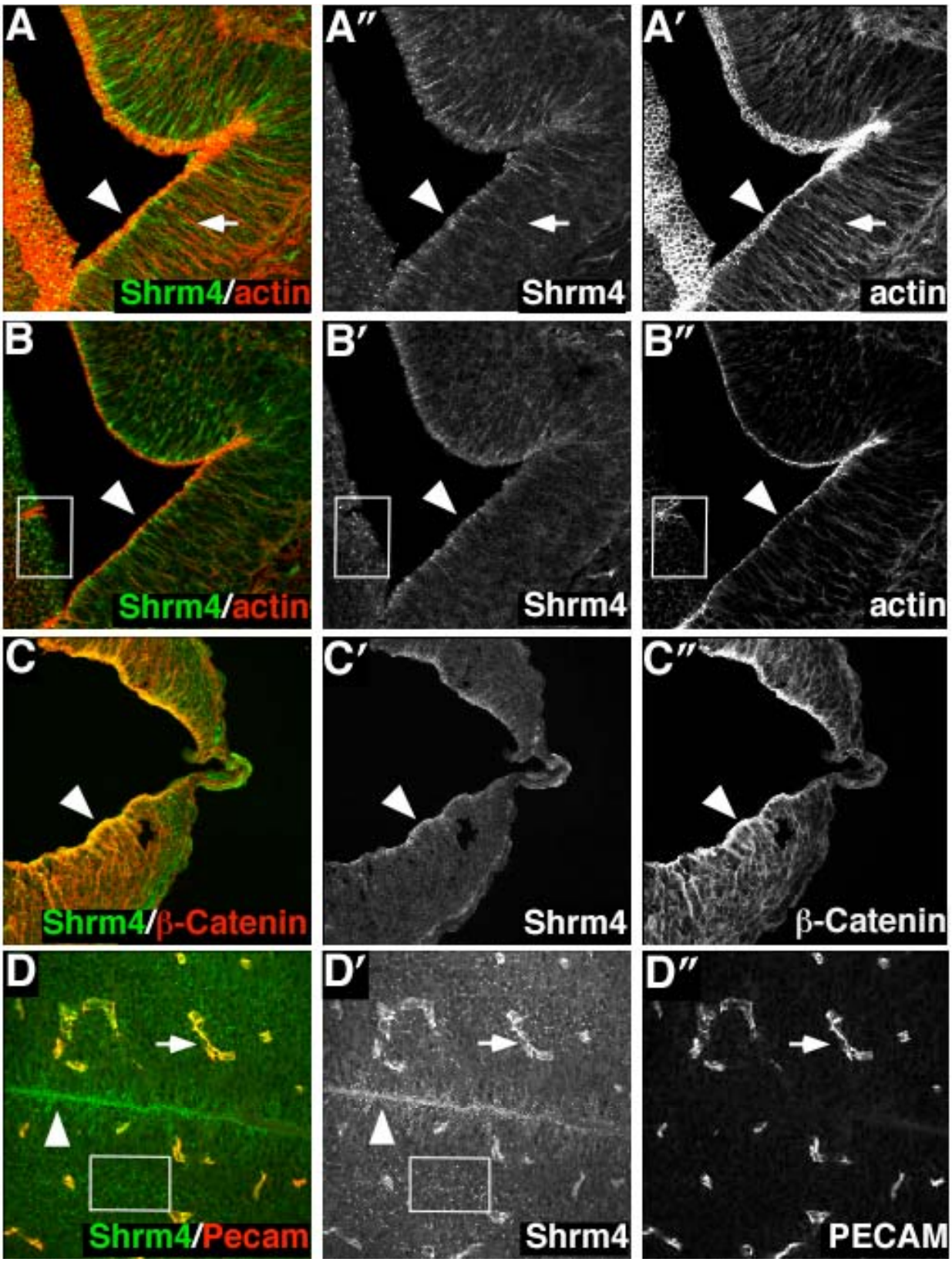

Figure 10: mShrm4 Localization in Embryonic Tissue Sections

Figure 10: Shrm4 Localization in Embryonic Tissue Sections.

Cryo-sections of e15.5 (**A** and **B**) or e12.5 (**C** and **D**) mouse embryos were stained to examine Shrm4 localization in the developing embryo. In the developing eye epithelium, (**A** and **B**), Shrm4 is enriched at the apical-basal boundary (arrowheads). The boxed region in **B** shows the punctate localization at the basal surface of these epithelial cells (compare the actin staining (red) at the right of the boxed area to the left of the boxed area). Panel **B** is a single optical section from the Z-series used to make Panel **A**. In the head region of an e12.5 mouse embryo (**C** and **D**), Shrm4 (green) localizes to the apical surface of the neural epithelium (**C** and **D**, arrowheads). β-catenin (**C**, red) marks the AJ in the neuroepithelial cell. Shrm 4 is also expressed in the endothelial cells of the vasculature as indicated by PECAM (**D**, red) expression.

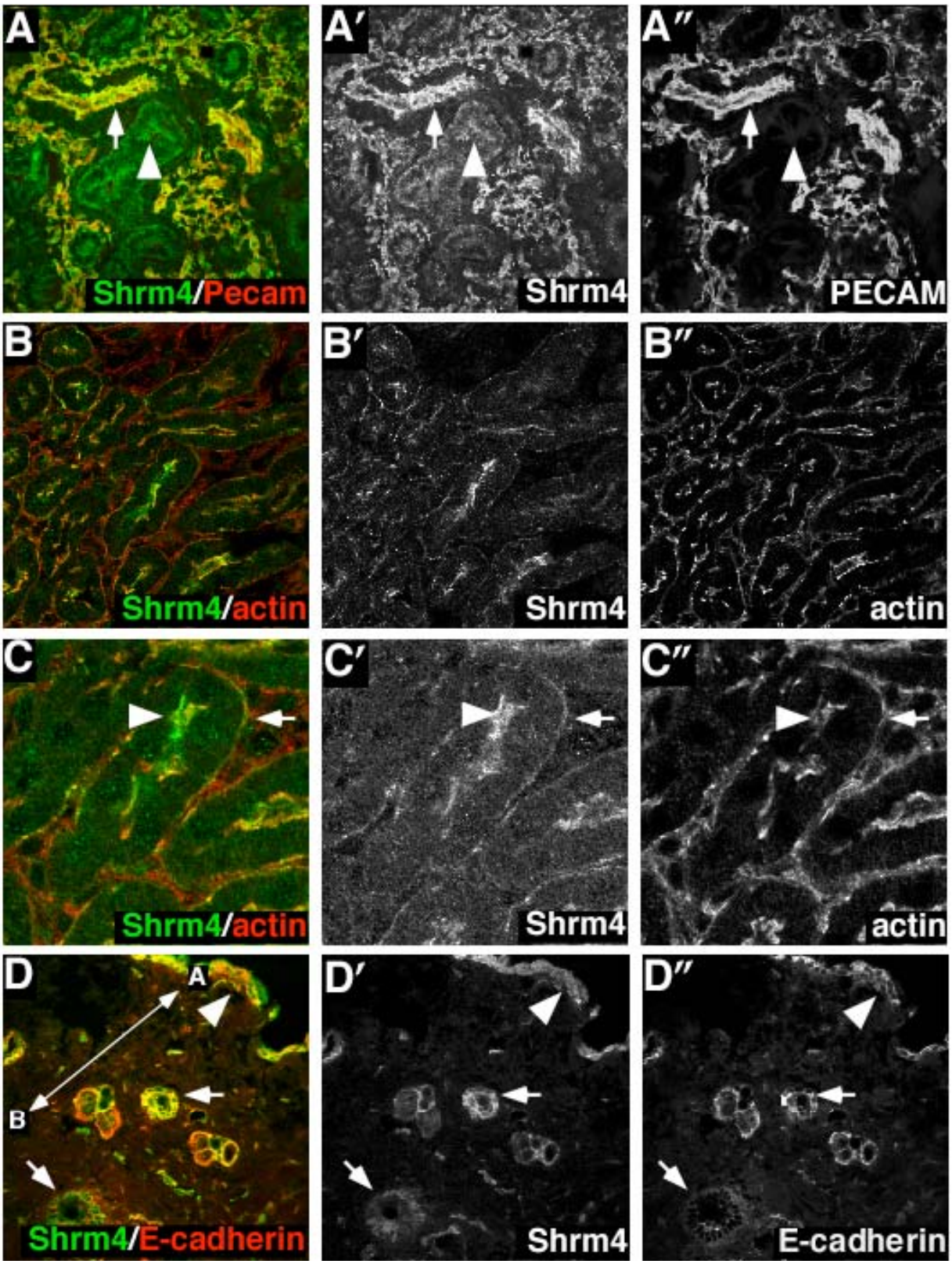

Figure 11: mShrm4 Localization in Adult Tissue Sections

Figure 11: Shrm4 Localization in Adult Tissue Sections

Cryo-sections of adult tissues were used to examine Shrm4 localization in the adult lung (**A**), kidney (**B** and **C**) and epidermal layer (**D**). Shrm4 (green) is expressed in the endothelial population of the lung vasculature (PECAM, red) (**A**, arrow). Shrm4 is also expressed at the apical surfaces lung epithelial cells (**A**, arrowhead). In the adult kidney (**B** and **C**), Shrm4 (green) is expressed in the apical and basal surfaces of the epithelium comprising the kidney tubules. Shrm4 is largely coincident with actin (red). Panel **C** shows a higher magnification of the Shrm4 distribution in kidney tubules. In the adult epidermal layer (**D**), Shrm4 (green) is expressed in a number of structures comprised of epithelial cells (arrows) including the outer most layer of the skin (arrowhead) as indicated by the co-distribution with E-cadherin (red). The apical-basal architecture (A-B) of the whole tissue is noted (double-ended arrow).

3.3.2 Sub-Cellular Localization of Endogenous Shrm4

To more closely examine the sub-cellular distribution of Shrm4, primary mouse cells were cultured and stained to detect Shrm4 and F-actin. In primary mesenchymal cells, Shrm4 is typically detected in a pattern that is reminiscent of extended vesicles or short, dense filaments (Fig 12A-C). In addition, Shrm4 is observed in patterns that are reminiscent of focal adhesions (Fig 12D) as well as membrane localization (Fig 12E). Since the filamentous pattern is the most commonly observed, that will be the focus of the remainder of this research. These structures can be found in either the cytoplasm or in close proximity to the plasma membrane (compare Fig 12A to 12C). Co-staining to detect Shrm4 and F-actin indicates that these Shrm4-containing structures are typically co-incident with a distinct compartment of the actin cytoskeleton (Fig 12F). This actin population is not particularly obvious and is only apparent in light of the localization of Shrm4. The Shrm4-associated actin is often diffuse and typically has “cloud-like” appearance (Fig 12F, inset). These actin clouds are often positioned at the ends of actin stress fibers, suggesting that actin filaments may either radiate from, or terminate in, these structures. Shrm4 is also associated with tightly bundled actin filaments that are more reminiscent of stress

fibers, although they are not on the surface of the cell contacting the underlying substrate. Stress fibers are composed of a contractile acto-myosin network [154], so IF was used to determine if non-muscle myosin II (NMMII) is a component of these Shrm4-actin structures. Indeed, myosin II is a part of the Shrm4-actin based structures (Fig. 12G). This result is not too surprising, based on the ability of Shrm3 to recruit myosin II to the apical surface of MDCK cells in an ASD2 dependant manner [136]. Since primary mouse cells are often not a homogenous population of cells (sometimes comprising dozens of cell types), it was important to find a cell line that endogenously expresses Shrm4. Based on the expression of Shrm4 in the vasculature, various endothelial cell lines were examined for Shrm4 expression.

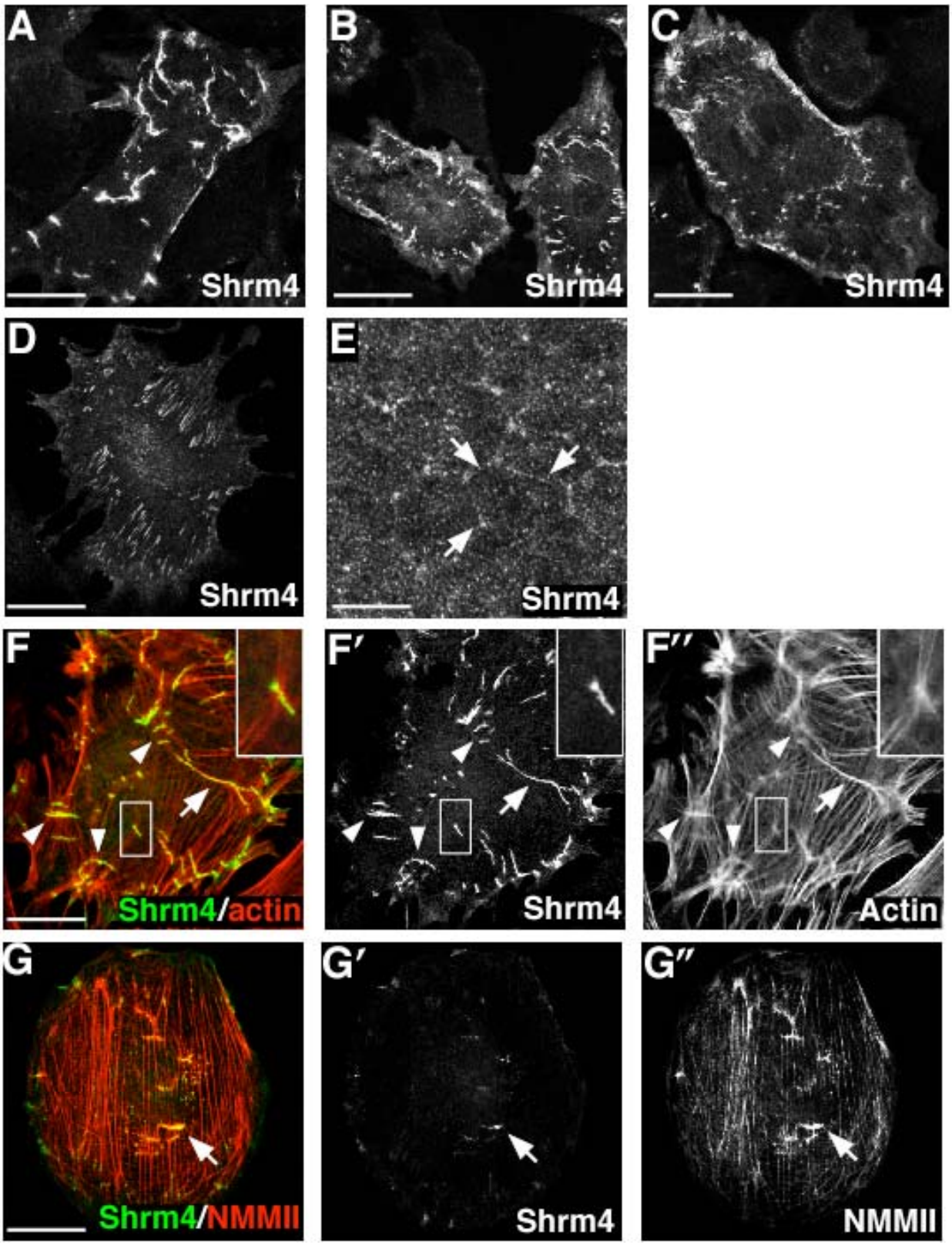

Figure 12: Sub-Cellular Localization of mShrm4 in Primary Mouse Cells

Figure 12: Sub-cellular Localization of Shrm4 in Primary Mouse Cells

Cell derived from e10.5 mouse embryos were stained to detect either Shrm4 alone (**A-E**), Shrm4 and actin (**F**, green and red respectively), or Shrm4 and non-muscle myosin II (NMMII) (**G**, green and red respectively). In primary mouse cells, Shrm4 most often displays a filamentous/vesicular distribution (**A-C**), however patterns reminiscent of focal adhesions (**D**) and membrane localization (**E**) are also observed. The Shrm4 filaments are coincident with a unique population of F-actin (**F**, arrow), which are sometimes observed as actin "clouds" (**F**, arrowheads). The boxed region is shown enlarged in the inset. Shrm4 also co-localizes with a population of NMMII that is distinct from the stress fiber population (**G**, arrow). Scale bar equal to 15 μm (**A-D**, **F**, **G**) or 7.5 μm (**E**).

3.3.3 Shrm4 is Endogenously Expressed in Some Endothelial Cell Types

In the quest to find cell types that endogenously express Shrm4, a number of different endothelial cell types were examined based on the observation that Shrm4 is co-expressed in cells expressing PECAM. Embryonic brain endothelial cells (bEND) and yolk sac endothelial cells (C166) from mice, rat lung microvessel endothelial cells (RLMVEC), and human umbilical vein endothelial cells (HUVEC) were all examined for expression of Shrm4 (Fig. 12). Shrm4 expression is detected in the bEND and RLMVEC cells by IF and Western blot (Fig 12A, B, E). However, Shrm4 is not detected in the C166 or HUVEC cells (Fig 12C and D). In bEND and RLMVEC cells, Shrm4 localizes into the short dense filaments (Fig 12A and B, arrows) similar to those observed in the primary mouse cells (Fig 11F). In addition, the Shrm4 filaments in the bEND and RLMVEC cells contain a unique population of F-actin that is not observed in the Shrm4 negative C166 or HUVEC cells.

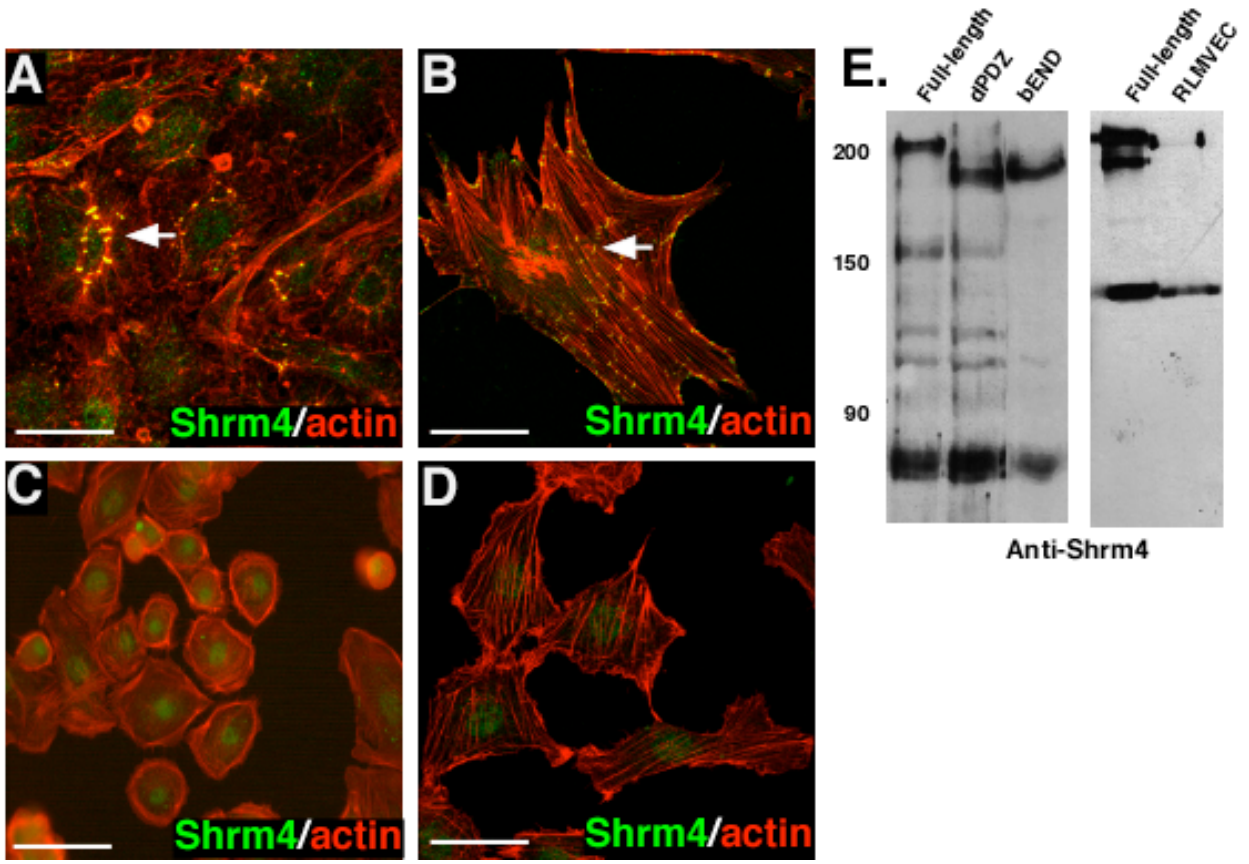

Figure 13: mShrm4 is Expressed in Endothelial Cells

Figure 13: Shrm4 Expression in Endothelial Cells.

Immortalized endothelial cells from mice (bEND and c166), rat (RLMVEC), and human (HUVEC) were stained to detect Shrm4 (green) and actin (red). Shrm4 is detected in bEND and RLMVEC cells by IF (**A, B**) and from cell lysates (**E**). No Shrm4 expression is detected in c166 or HUVEC cells (**C, D**). The bEND and RLMVEC cells express a protein of the predicted molecular size when examined by Western blot (**E**). Shrm4 negative RAT1 fibroblast cells were transiently transfected with Shrm4 (full-length or dPDZ) and used as a positive control. Equal volumes of the cleared cell lysates were loaded onto the gel and resolved by SDS-PAGE. The N-terminal anti-Shrm4 antibody was used for the WB. Scale bar equal to 15 μm.

3.4 DISCUSSION

mShrm4 encodes a 1475 amino acid protein that contains an N-terminal PDZ domain and a C-terminal ASD2 motif, each of which are about 60% similar to the other Shrm family members yet notably lacks the conserved ASD1 motif. Since it does contain the PDZ and ASD2 elements, as well as similarly located EVH and PDZ binding sites, it can be placed in the Shrm family of proteins. The presence of a leucine zipper is curious, since leucine zippers are most commonly found in regulators of transcription [155]. However this function has not been identified for any Shrm protein. It is most likely that this domain is facilitating a protein-protein interaction through the formation of a coiled-coil interaction [156], or it is a coincidental stretch of leucine residues. The mShrm4 protein is roughly 80% identical to hShrm4, with the PDZ and ASD2 motifs at 98% and 94% identical, respectively. As mentioned previously, this domain structure is more reminiscent of the invertebrate orthologs [135], indicating a gene duplication event. Even though there seems to be no identifiable 'domain' in the central region of Shrm4 and no homology to any known proteins, there does appear to be a short (~20 amino acids) sequence that is conserved. Short sequences such as these could perhaps reveal a yet unidentified functional domain, but more research is necessary to determine if these stretches of sequence represent a conserved function or are merely a coincidence.

The Shrm4 protein is ubiquitously expressed during early embryonic development and persists into adulthood. Much like the other Shrm family members, Shrm4 is expressed in tissues that contain highly polarized cell types (such as the lung and kidney) and in fact is co-incident with the epithelial cell markers β-catenin, ZO-1, and E-cadherin and the endothelial marker PECAM. Expression of Shrm4 in the vasculature could be the explanation for the

observed ubiquitous expression, since all tissues are vascularized. It will be interesting to determine if Shrm4 does play a role in vascular development (discussed in section 6.4).

The sub-cellular localization of Shrm4 is also quite interesting. In both the primary mouse cells and the endothelial cell lines, Shrm4 is co-incident with a distinct population of actin. This is striking because the ASD1 domain, which is required for targeting Shrm2 and Shrm3 to actin, is not present in the Shrm4 protein. The co-incident localization of Shrm4 with NMMII is not too surprising since it is the ASD2 domain that facilitates this interaction for Shrm3. The association of Shrm4 with actin and NMMII will be described in section 4.3, but it is important to address the unique actin clouds. The 'actin-clouds' described in the primary mouse cells are composed of a dense actin core with filaments of F-actin seeming to radiate out of this structure. These structures are quite reminiscent to those seen in the early assembly of filopodia [157] [22]. In these studies, the actin 'stars' represent the early formation of filopodial-like bundles (in the absence of capping protein), and their genesis is dependent on the Arp2/3 complex, as well as the EVH1 domain protein WASP. mShrm4 contains an EVH1 binding ligand (which have been shown to be critical for actin dynamics [158]) and can bind (and bundle) F-actin (Figs 24 and 25) [149]. Based on the sequence elements and the sub-cellular localization alone, Shrm4 seems a likely candidate for being a regulator of F-actin dynamics. It is interesting to speculate that Shrm4 may play a pivotal role in directing the architecture of actin filaments to facilitate the development and maintenance of the vasculature.

4.0 SUB-CELLULAR LOCALIZATION AND FUNCTIONAL STUDIES OF mSHROOM4

4.1 INTRODUCTION

The endogenous distribution of mShrm4 in tissue sections and in cell culture makes it an intriguing candidate for regulating the actin cytoskeleton in the vasculature. The sub-cellular localization pattern of mShrm4 does not resemble the sub-cellular localization patterns observed for any of the other Shrm proteins [120, 135]. Shrm2 and Shrm3 both localize to the AJC at the plasma membrane, where they are coincident with the TJ marker ZO-1 and with the cortical population of F-actin [120, 135]. In addition, Shrm2 is associated with a basal population of F-actin in endothelial cells [135], and Shrm3 can bind to the actin-rich stress fiber network [120]. It is hypothesized that the sub-cellular localization may be a critical regulatory step that allows these proteins to function properly within the cell and effect cell shape [135, 136]. mShrm4 does not endogenously localize to the AJC nor does it decorate actin stress fibers. However, mShrm4 does co-localize to distinct sites that are composed, in part, by a dense actomyosin network [149].

In order to better comprehend the sub-cellular localization pattern and function of mShrm4, it is first necessary to understand the nature of the observed Shrm4-actin structures. The endogenous localization of mShrm4 often appears vesicular or assumes the morphology of

elongated or tubular vesicles/organelles [159, 160]. In addition, the co-localization of mShrm4 with actin is extremely interesting as it does not contain a conserved actin-binding ASD1 domain, nor does it share similarity to any known actin binding protein. As mentioned previously, actin does play a major role in the endocytic pathway, and the associated vesicles are typically linked to a population of actin [161]. The following section will examine the nature mShrm4 sub-cellular localization.

4.2 ECTOPICALLY EXPRESSED mSHRM4 MIMICS THE ENDOGENOUS LOCALIZATION

4.2.1 Ectopic Expression of mShrm4

When expressed in HEK293 cells, the myc-mShrm4 construct produces a protein product that is the correct size (Fig 9A, B) and localizes in a similar sub-cellular pattern as the endogenous mShrm4 protein (compare Figs 9C and 12). Does mShrm4 localize to unique F-actin based structures and is this a cell type specific event? To address this question, the myc-mShrm4 construct used previously was transfected into a number of cell types, including endothelial and fibroblastic cells. Indeed when mShrm4 is transiently transfected into bEND, C166, HUVEC, and RAT1 fibroblasts, the sub-cellular localization of ectopically expressed mShrm4 is indistinguishable from that of the endogenous pattern (compare Fig 11F to Fig 14A-D) (NOTE: In the bEND cells, the endogenous mShrm4 protein is expressed at significantly lower levels compared to the ectopically expressed protein). Not only does exogenous Shrm4 exhibit the correct distribution in these cells, but it can also enhance the formation of the actin-based

structures (Fig 14A″-D″, arrows). This enhancement is most apparent in the C166, HUVEC, and RAT1 cell lines, where the untransfected cells do not obviously contain these structures (compare the starred cell in Fig 14B″ or 14C″ to the other cells in those panels). In fact, greater than 80% of the cells expressing ectopic mShrm4 exhibit these actin-based structures (which also contain mShrm4), while less than 3% of the untransfected cells display these F-actin structures (n = >> 50 transfected or control cells using RAT1 and HUVEC cells in multiple independent experiments). These results indicate that mShrm4 may be inducing cytoskeletal reorganization or stabilizing/amplifying a pre-existing structure. However, since these structures still have the appearance of elongated tubules or vesicles, it was next necessary to establish if this observation were valid.

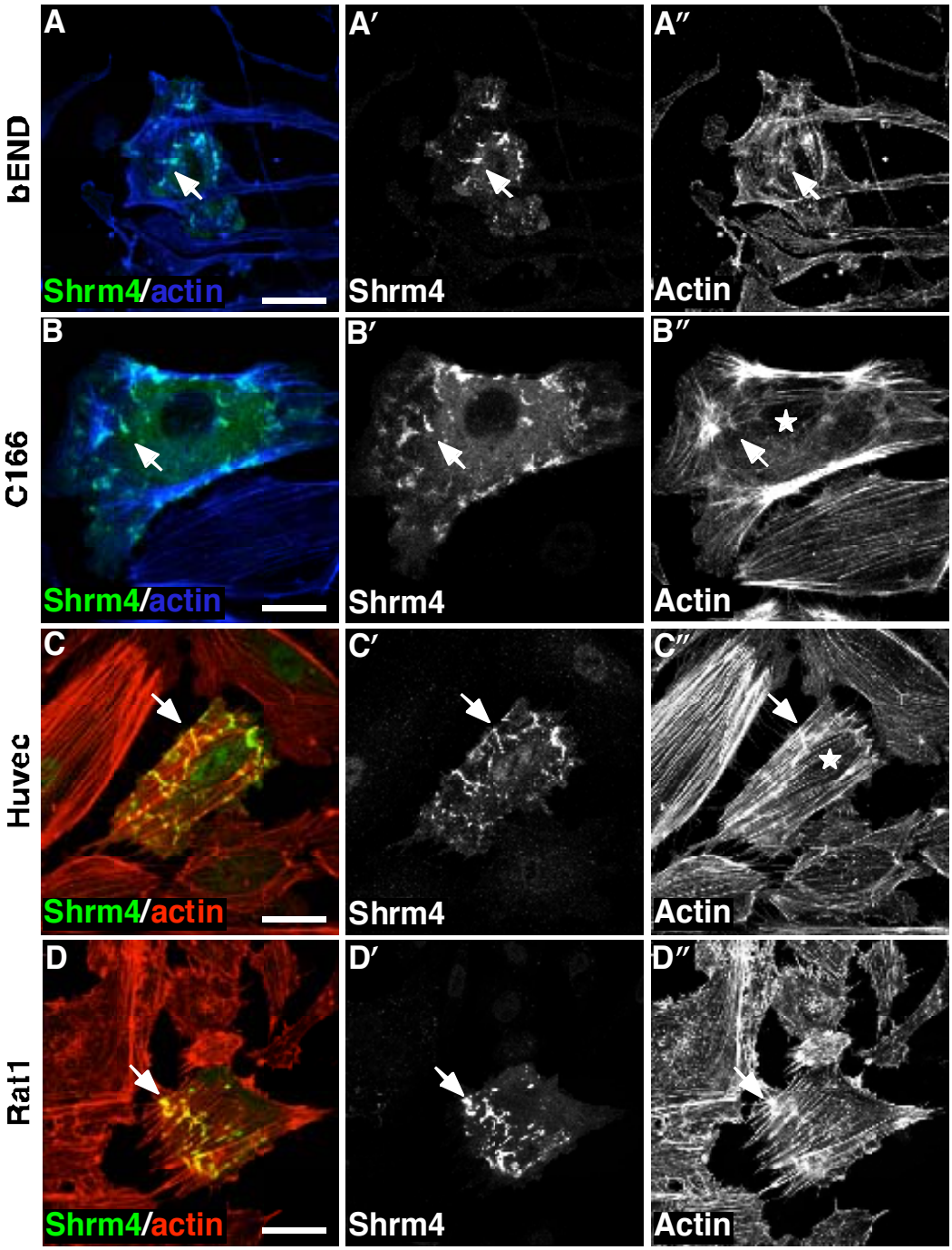

Figure 14:Ectopic Expression of mShrm4

Figure 14: Ectopic Expression of mShrm4

Ectopic mShrm4 localizes to actin-rich structures. bEND (**A**), C166 (**B**), HUVEC (**C**), and RAT1 fibroblast (**D**) cells were transiently transfected with full-length Shrm4 expression vectors and stained to detect both Shrm4 (green) and actin (blue, **A** and **B**; red **C** and **D**). Arrows indicate the co-localization of Shrm4 with actin. In C166 and HUVEC cells, these actin-based structures (arrow) are seen in transfected cells (star) but not in untransfected cells (see text). Scale bars equals 15 μm.

4.2.2 mShrm4 is Not Associated with Endosomal Components

As previously described, the endocytic pathway depends on the actin cytoskeleton and the activity of various myosin motors [159]. The short dense actin-rich mShrm4 structures are somewhat reminiscent of endocytic vesicles or elongated organelle structures. Many of the components that comprise these vesicles are well documented and serve as excellent markers for comparison. The GTP binding proteins Rab5 and Rab7 are associated with early and late endosomes, respectively, and can be used to mark these two parts of the endocytic pathway [162]. Ectopically expressed mShrm4 does not co-localize with Rab5 or Rab7 (Fig 15A, B), indicating that mShrm4 is not part of the endocytic pathway. To more fully explore the possibility that mShrm4 is associated with vesicles, cells transiently transfected with mShrm4 were incubated with TRITC-dextran for 24 hours to label all intracellular vesicles. Dextran is internalized by macropinocytosis from the surrounding media and subsequently incorporated and recycled through the endocytic pathway. mShrm4 does not co-localize with any TRITC-dextran positive structure (Fig. 15C). An alternative pathway that cells will often utilize for intracellular transport is that of the caveolae-coated vesicles. mShrm4 does not associate with caveolin coated vesicles (Fig. 15D). These results suggest that mShrm4 is not associated with components of the endocytic or caveolin-mediated intracellular transport pathways. Based on the localization pattern of mShrm4 that frequently appears globular or tubular, it still remains possible that mShrm4 is associated with membrane-bound organelles, such as the Golgi apparatus or the mitochondria. These organelles often assume a vesicular type of appearance themselves, which sometimes makes separating organelle from vesicle an arduous task.

To test this possibility, mShrm4 was transiently co-transfected into HEK293 cells with an ornithine transcarbamylase (OTC) expression construct that has an N-terminal fusion to green fluorescent protein (GFP) (Fig 16A). OTC is a mitochondrial enzyme involved in the urea cycle, and therefore this construct will label the mitochondria. There is no apparent co-localization between the GFP-OTC and mShrm4 (Fig 16A). Additionally, mShrm4 does not co-localize with the Golgi marker, giantin, nor do the structures show any morphological changes when subjected to treatment with brefeldin A (a toxin that blocks vesicular transport) (Fig 16B, C)[163, 164]. These data indicate that mShrm4 is not associated with membranous structures, such as intracellular vesicles or organelles, and that the structures with which mShrm4 is associated, are more likely 'mechanical' in nature. Since the population of actin that is co-incident with mShrm4 is unique, it is necessary to determine if mShrm4 localization depends on an intact cytoskeleton.

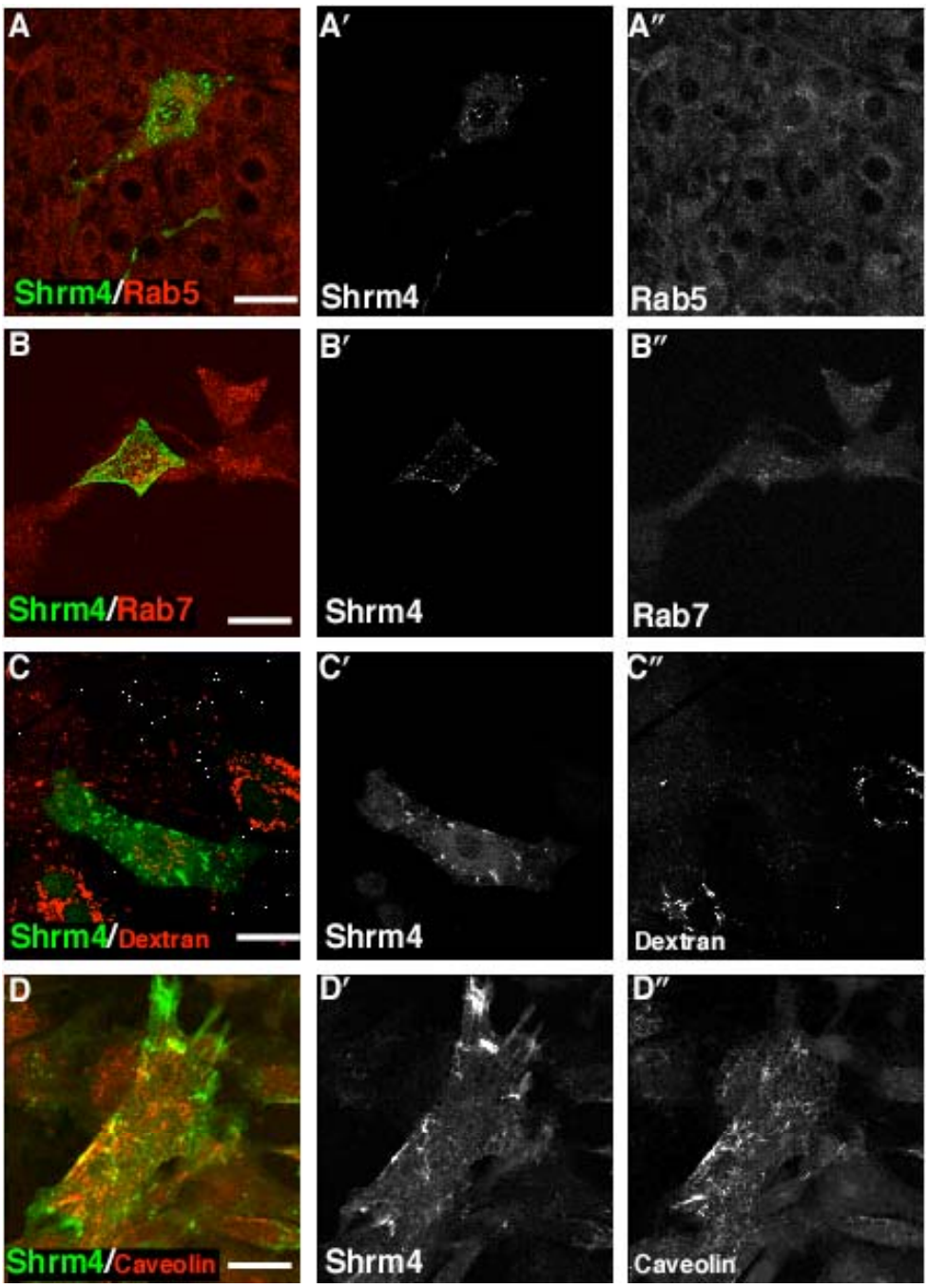

Figure 15: mShrm4 is Not Associated with Endocytic Vesicles

Figure 15: mShrm4 is Not Associated with Endocytic Vesicles

HEK293 cells were transiently transfected with mShrm4 (green) stained to detect Rab5 (**A**), Rab7 (**B**), or Caveolin-1 (**D**) (red). mShrm4 expressing HEK293 cells were also incubated with TRITC-dextran (red) for 24 hours and stained to detect mShrm4 (green) (**C**). mShrm4 was detected with the mShrm4 specific primary antibody. Secondary antibodies were either Alexa-488 (green) or Alexa-568 (red) conjugated. Scale bars are equal to 15 μm (**A-C**) or 10 μm (**D**).

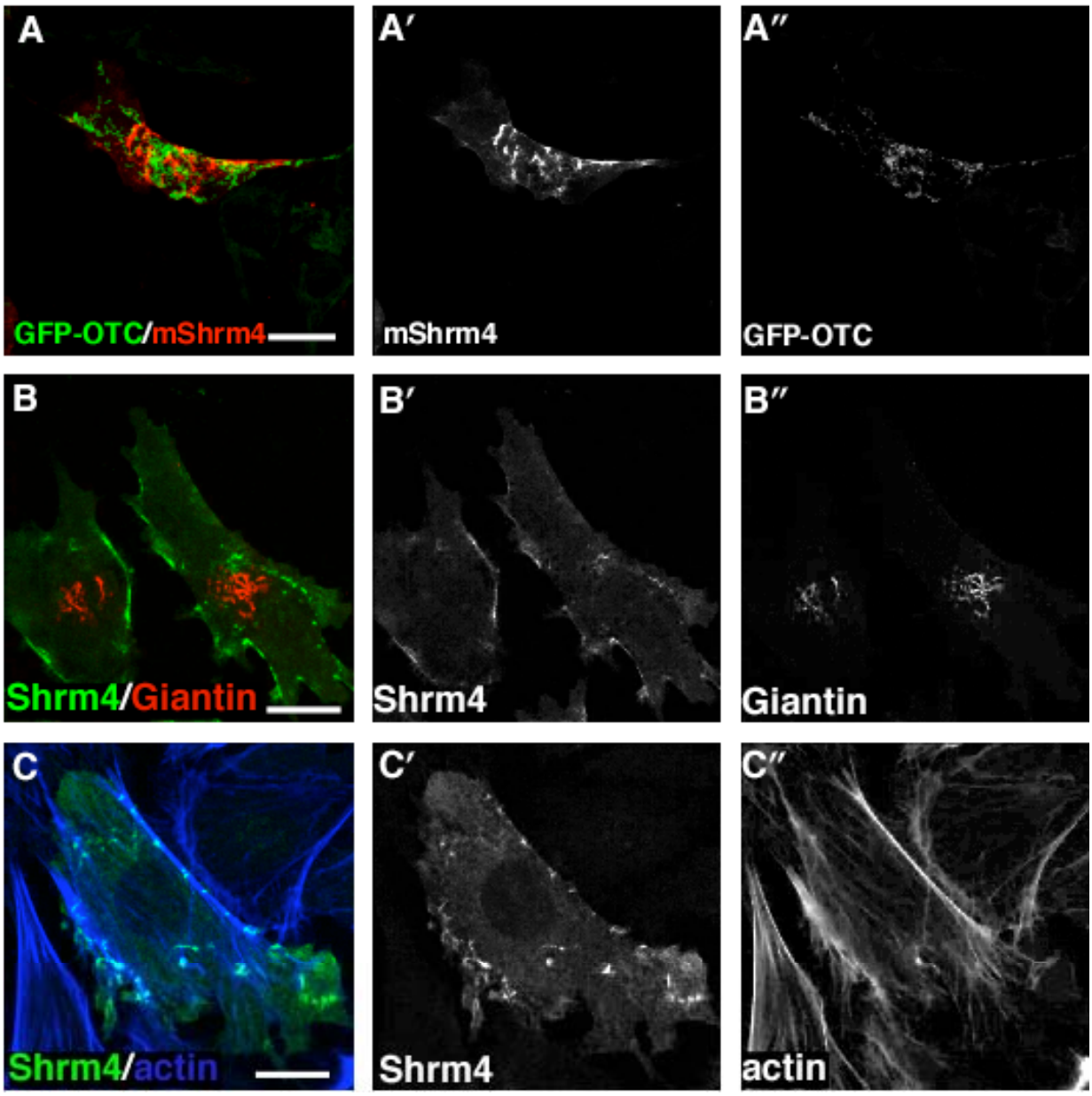

Figure 16: mShrm4 is Not Associated with Organelles

Figure 16: mShrm4 is Not Associated with Organelles

HEK293 cells were transiently transfected with mShrm4 and GFP-OTC (**A**) or mShrm4 alone (**B, C**). mShrm4 (red) does not co-localize with mitochondria (green) (**A**). mShrm4 (green) is not associated with the Golgi (red) (**B**) nor is its localization effected by treatment with brefeldinA (**C).** Scale bars equal 15 μm.

4.3 mSHRM4 LOCALIZATION DEPENDS ON THE ACTIN CYTOSKELETON

4.3.1 An Intact Actin Cytoskeleton is Necessary for mShrm4 Localization

The next step in understanding the sub-cellular function of mShrm4 is to determine the relationship between F-actin organization and mShrm4 distribution. CytochalaisinD (CD) is a well-known inhibitor of actin polymerization and is commonly used to cause the breakdown of F-actin based structures [121]. Treatment of HUVEC cells expressing exogenous mShrm4 with 2 μM CD completely disrupts the distribution of both mShrm4 and F-actin (Fig 14C versus Fig 17A). However, despite the overall lack of cytoskeletal organization following CD treatment, Shrm4 remains co-distributed with F-actin. This observation indicates that the sub-cellular distribution of mShrm4 is dependent on an intact actin cytoskeleton and that mShrm4 may be in a complex with F-actin. In contrast, mShrm4 localization is not dependent on microtubules, as treatment with nocodozole (33 μM) does not alter its localization (Fig 17B). Endogenous mShrm4 localization is also dependant on an intact cytoskeleton, as treatment of bEND cells with 2 μM CD causes mShrm4 to collapse with actin (Fig 17C). This phenotype is not permanent and can be rescued by removing the CD and allowing the cells to recover (Fig 17D). Since the mShrm4-actin structures are also co-incident with a population of non-muscle myosin II, it is necessary to examine the role of myosin II in the establishment of these structures.

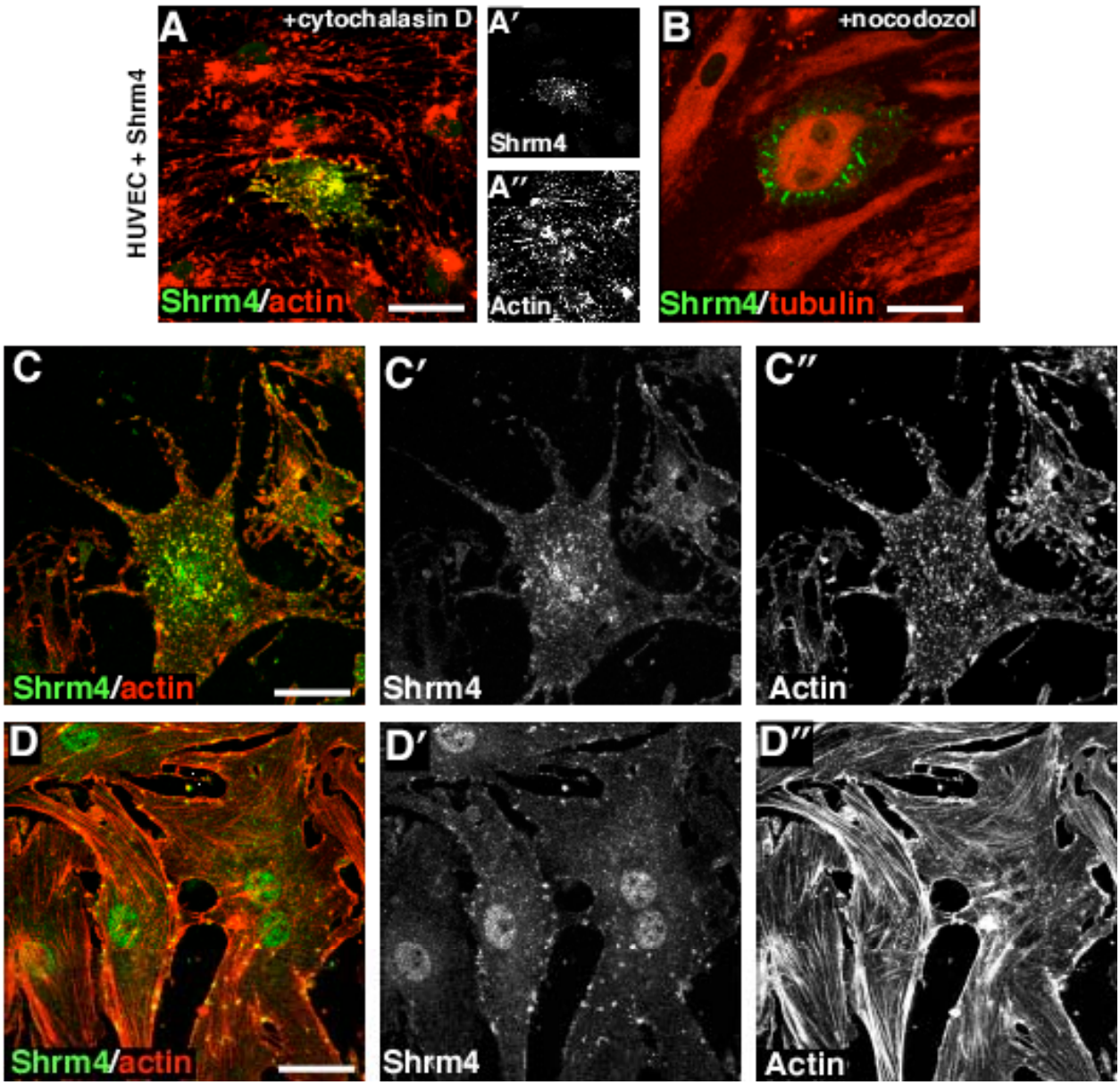

Figure 17: mShrm4 Localization is Dependant on the Actin Cytoskeleton

Figure 17: mShrm4 Localization is Dependant on the Actin Cytoskeleton

(**A** and **B**) HUVEC cells transiently transfected with Shrm4, were treated with either 2μM cytochalasin D (**A**) or 33 μM nocodozole (**B**) and stained to detect Shrm4 (**A** and **B**, green), actin (**A**, red), or tubulin (**B**, red). (**C** and **D**) bEND cells were treated with 2μM CD and either stained to detect mShrm4 (green) and actin (red) or the CD was removed from the media, the cells allowed to recover for 3 hours, then stained as in **C**. Scale bar equal to 15 μm.

4.3.2 The Morphology of mShrm4 Structures Requires Functional NMMII

It has been shown that Shrm2 and Shrm3 co-distribute with myosin II in non-muscle cells and that Shrm3 requires myosin II to cause constriction [135, 136]. Since some F-actin based structures (such as stress fibers) are dependent on the activity of myosin II, the localization of mShrm4 in vascular endothelial cells was examined following the treatment of cells with blebbistatin, an inhibitor of myosin II activity [165]. In both C166 and bEND cells, ectopically or endogenously expressed mShrm4 and myosin II co-distribute as is seen in primary fibroblasts (Fig. 18A, B versus Fig. 11G). In fact, mShrm4 appears to recruit myosin II to this locale, as very little or no myosin II exhibits this localization in untransfected cells (Fig. 18A, starred vs. unstarred cell). Treatment of bEND and transfected C166 cells with blebbistatin effectively eliminates actin stress fibers but does not eliminate the association of Shrm4 with actin or the formation of the actin structures to which mShrm4 and myosin II are localized (Fig. 18C and 18D). However these structures are less prominent and are typically more globular, particularly in cells over expressing Shrm4 (Fig. 18C). Similarly, when cells are treated with CD and blebbistatin simultaneously, followed by removal of CD and recovery in the presence of blebb, the characteristic mShrm4 structures do not reform (Fig 19A). Yet, mShrm4 still associates with actin, again in a dense globular type of pattern (19A, arrow). These data suggest that myosin II activity is required for formation, and likely, the maintenance of the Shrm4 -F-actin structures.

In addition, bEND cells treated with effectors of the myosin II pathway (both positive and negative) disrupt the normal localization pattern of mShrm4 without disrupting the association with F-actin. Rho activation of myosin II through ROCK is a well-known pathway (described above) [166], and much effort has been put forth to generate specific inhibitors and activators to each step. The ROCK inhibitor, Y-27632, effectively blocks all ROCK-dependant

activities [167]. When bEND cells are treated with this compound, the stress-fiber network breaks down, but the cortical population of actin remains intact. However, mShrm4 remains associated with the cortical population of F-actin in a globular morphology, similar to the blebbistatin treated cells (19B). Conversely, activation of myosin II with calyculin A, an inhibitor of myosin phosphatase [168], seems to stimulate the number and density of the mShrm4-actin structures (Fig. 19C). In addition, the mShrm4 seems to localize specifically to a population of F-actin that forms a circle around the center of the cell. (*These results have not been quantified and more repetitions are necessary to validate this hypothesis). While the Shrm-4-actin-myosin structures are maintained in the presence of blebbistatin, it is currently unclear if myosin II activity is required for the formation of these structures.

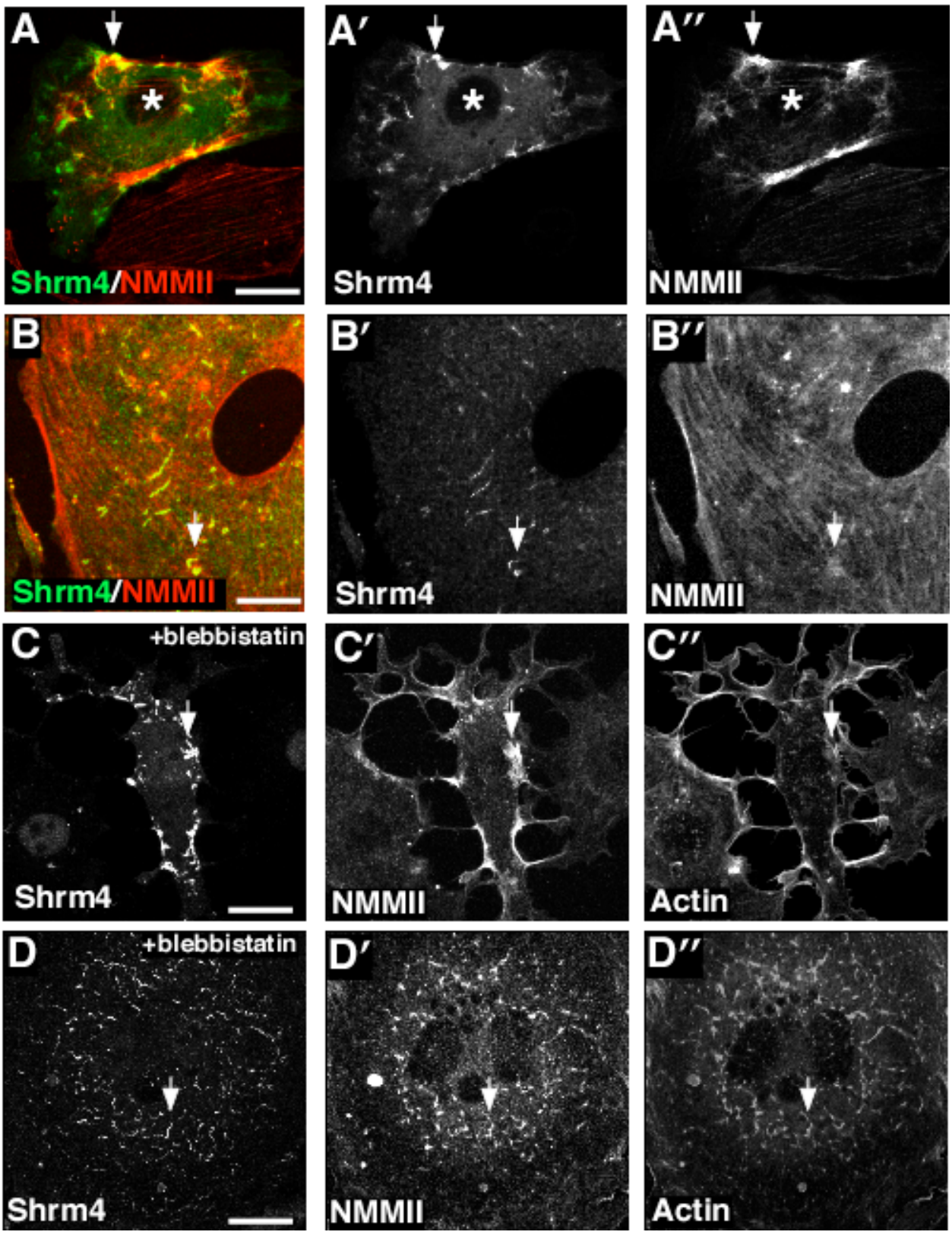

Figure 18: Morphology of mShrm4 Structures Depends on NMMII

Figure 18: Morphology of mShrm4 Structures Depend on NMMII

C166 (A and C) and bEND (B and D) were used to examine mShrm4 and NMMII interactions. C166 cells were transiently transfected and stained to detect mShrm4 (green) and NMMII (red). bEND cells were stained to detect endogenous mShrm4 (green) and NMMII (red). mShrm4 co-localizes with NMMII in dense structures (A and B, arrows) that are also positive for actin (not shown). In fact, ectopically expressed mShrm4 causes the formation of these large NMMII structures when compared to the untransfected control (A, starred cell vs. unstarred cell). Treatment with 200 nM blebbistatin for 90 (C) or 180 (D) causes the reduction/loss of stress fibers, but mShrm4 remains associated with NMMII in more globular type structures (arrow). Scale bars equal 15 μm, except in B (5 μm).

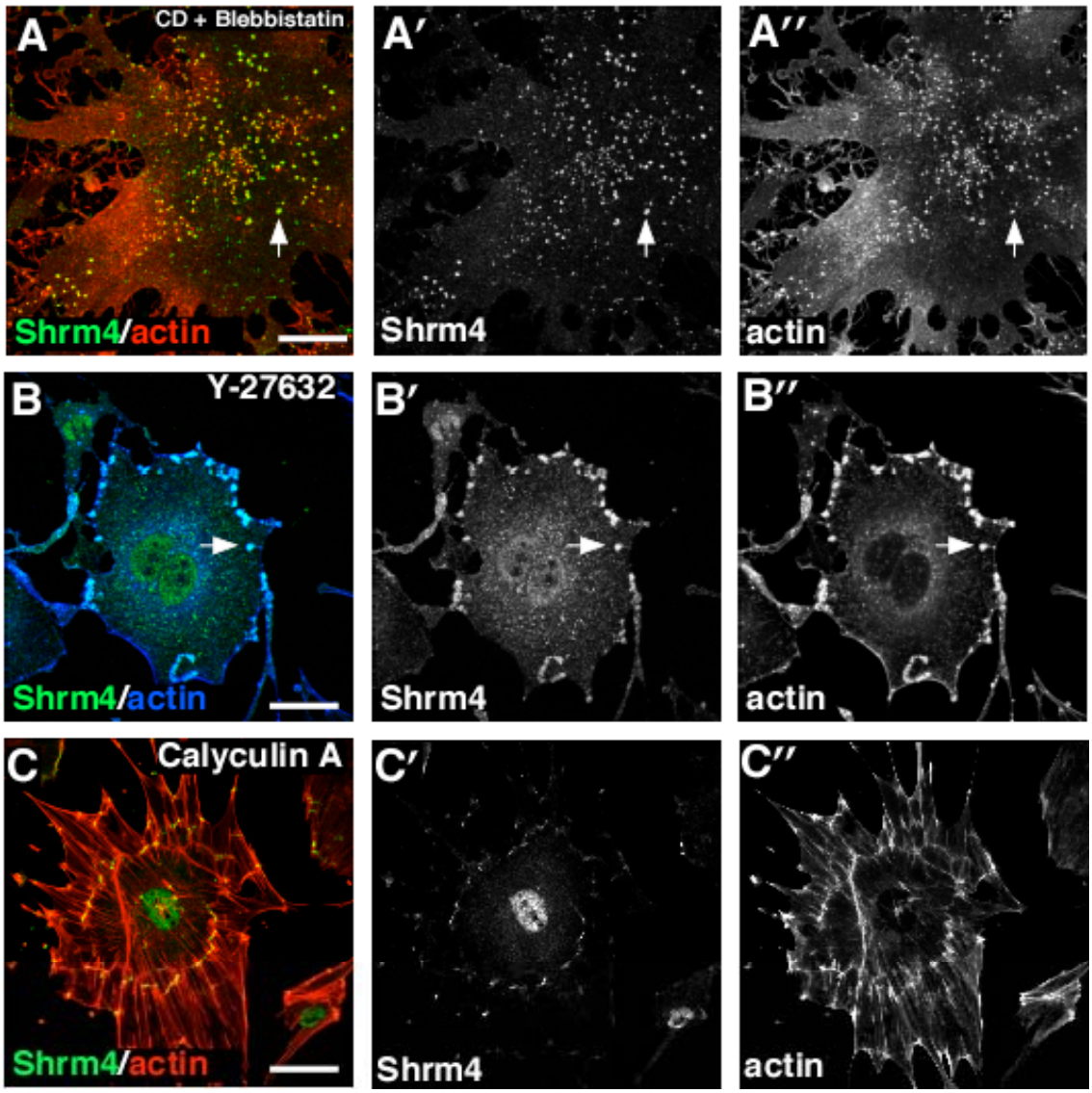

Figure 19: mShrm4 Localization and Morphology Depend on Functional NMMII

Figure 19: mShrm4 Localization and Morphology Depend on Functional NMMII

bEND cells were treated with various chemicals that effect the Rho-ROCK pathway. Cells were treated with CD and recovered in the presence of blebbistatin (CD+blebbistatin**, A**), the ROCK inhibitor Y-27632 (**B**), or the myosin phosphatase inhibitor CalyculinA (**C**) and stained to detect mShrm4 (green) and actin (red in **A, C** and blue in **B**). mShrm4 remains associated with actin after treatment with CD+blebbistatin or Y-27632, but assumes a globular morphology (**A** and **B**, arrows). Constitutively active myosin II causes the stress fibers to contract and shorten, however the mShrm4-actin structures seem more prominent and perhaps elongated (**C**). Scale bars equal 15 μm.

4.4 DISCUSSION

The ability of the Shrm family of proteins to reorganize the actin cytoskeleton has been well documented and the mechanism of function is slowly being elucidated [135, 136]. Shrm3 binds to a population of F-actin at the apical surface of cells, recruits myosin II, and facilitates the constriction of the apical actin belt. This process has been shown to be dependent on ROCK activity, but not its activator RhoA. While the localization pattern of mShrm4 is unlike any of the other Shrm proteins, the resultant structures share the components that are key to Shrm function, actin and myosin II.

mShrm4 can induce the formation of short, dense actomyosin structures (which also contain mShrm4). Exogenous expression of mShrm4 in cells that do not normally express Shrm4, causes a robust increase of these actin structures (Fig 14C, D) and endogenous mShrm4 is almost always associated with unique actin rich structures. In fact, mShrm4 is not just associated with actin, but it is dependent on an intact actin cytoskeleton for proper localization. Since myosin II is present in these structures, a similar question can be asked about myosin II's role in the mShrm4 structure. Indeed, mShrm4 requires the activity of myosin II to maintain the normal morphology of the structures. Treatment of cells with inhibitors to the myosin II pathway leads to the formation (or the "un-formation") of small, globular structures that remain rich in actin, myosin II, and mShrm4. These data indicate that mShrm4 probably facilitates the formation and/or maintenance of the actin-rich structures through the activity of myosin II, not unlike the mechanism observed for Shrm3 [136]. The mShrm4-actin structures do not appear to play a role in vesicular transport, so they are perhaps playing a role in the global cytoarchitecture. The rigid association of mShrm4 with actin is quite curious, since it lacks the ASD1 actin-binding domain and it has no homology to any other known actin-binding protein.

The collapse of a protein with actin in response to CD treatment is a hallmark phenotype of an actin-binding protein. This observation begs the question: Does mShrm4 bind directly to actin?

5.0 mSHRM4 BINDS ACTIN WITH A NOVEL ACTIN BINDING DOMAIN

5.1 INTRODUCTION

Actin regulation is best accomplished by proteins that can bind directly to G-actin or to F-actin. These proteins (discussed in more detail in section 1.0) not only regulate the kinetics of actin assembly [169] but also facilitate the 'downstream' function of actin filaments, such as muscle contraction or cytokinesis. Both Shrm2 and Shrm3 are actin-binding proteins that affect cell shape change through an actomyosin-based mechanism [136]. The Shrm proteins bind actin via their ASD1 motifs; and the ability to bind to actin is critical for Shrm function [120, 135]. It has been shown that Shrm3 binds actin in an ASD1-dependent event and recruits myosin II to the Shrm3-actin complex. In this complex, myosin II is activated by way of ROCK and causes constriction of the actin filaments [136]. In addition to binding directly to actin, Shrm3 (but not Shrm2) is able to induce actin bundles, a function also mediated by the ASD1 motif [135]. The conserved ability of the Shrm family to bind to actin via the ASD1 motif posits an interesting scenario.

Shrm4 has many of the conserved features of the Shrm family, including the PDZ domain and the ASD2 motif, but lacks the actin-binding region of ASD1. Surprisingly, mShrm4 remains closely associated with regions of dense actin structures in cell culture and remains associated even when the cells are treated with chemicals that disrupt the normal organization of F-actin.

mShrm4 has a unique sub-cellular distribution compared to that of Shrm3 and of Shrm2, and it is most likely that this distribution provides the differential function of the proteins. Since mShrm4 belongs to a family of actin-binding proteins and displays an actin dependent sub-cellular localization, the question still remains as to whether or not mShrm4 can directly bind to actin, despite lacking the ASD1 motif. The following section will investigate the actin binding ability of mShrm4.

5.2 mSHRM4 HAS AN ACTIN LOCALIZATION MOTIF

To define the localization motif, a series of vectors were generated that express different regions of mShrm4 protein, each containing an N-terminal myc-tag (Fig 20A). Transient transfection of these plasmids into cells results in the expression of proteins of the expected molecular mass (Fig 20B). To test for sub-cellular localization, C166 cells were transiently transfected with each of these vectors and stained to detect the ectopically expressed protein. The C166 cells were used because they are a relevant cell type; they are readily transfected; mShrm4 exhibits the correct localization in these cells; and mShrm4 expression induces the robust formation of the actin-based structures observed in primary cells. In C166 cells, both full-length Shrm4 (Fig 13B) and Shrm4-ΔPDZ (ΔPDZ, Fig 20C) exhibit the normal filamentous staining pattern observed for endogenous Shrm4. Deletion of the C-terminal ASD2 motif (ΔASD2, Fig 20D) does not eliminate localization but results in a much more diffuse distribution relative to that of either full-length Shrm4 or Shrm4-ΔPDZ. Proteins consisting of amino acids 1-522 (containing the PDZ domain) and 1180-1475 (ASD2) are completely cytoplasmic, indicating that neither of

these regions is sufficient for determining proper localization (Fig 20E and 20H, respectively). These results suggested that the central portion of Shrm4 targets the protein to actin-based structures. To test this, the central region of Shrm4 (amino acids 436-1180 or 436-966) was expressed and assayed for sub-cellular localization. These two Shrm4 proteins exhibit a localization pattern that is different from that of full-length Shrm4 in that they target to the cell periphery (Fig. 20F and 20G). Co-staining to detect 435-1180 or 436-966 and F-actin indicates that these proteins are localized primarily to the cortical actin cytoskeleton (Fig. 21A and 21B). These data suggest that the central region of Shrm4 regulates its localization to the cytoskeleton, but additional sequence motifs may function to define the specificity of this localization.

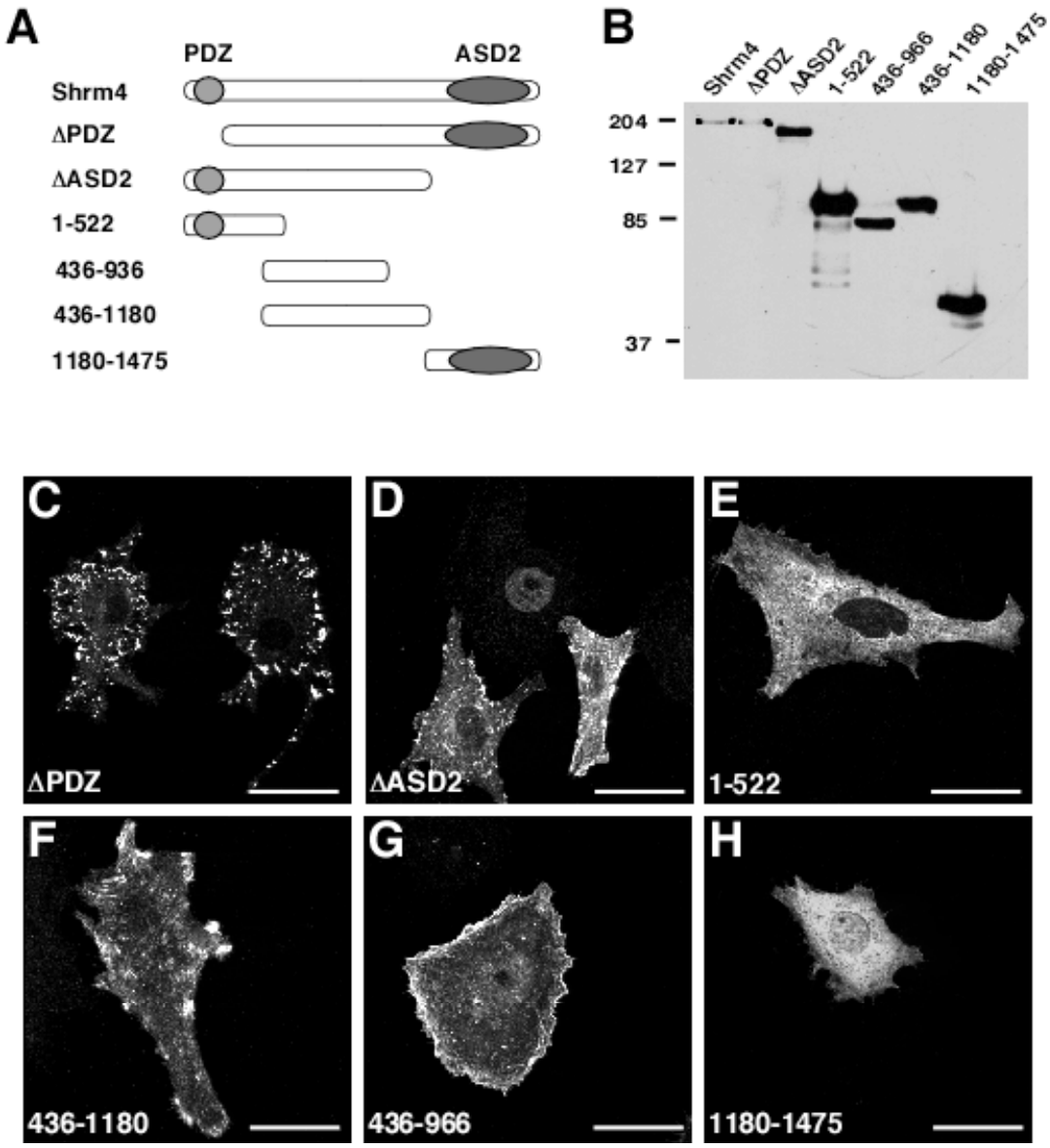

Figure 20: Mapping of the mShrm4 Targeting Sequence

Figure 20: Mapping of the Shrm4 Targeting Sequence.

(**A**) Schematic of the various proteins used to map the sequences required for proper localization of Shrm4. All constructs have an N-terminal myc tag.
(**B**) Western blot of lysates from MDCK cells expressing the constructs depicted in (**A**). Lysates were probed with the 9E10 anti-myc antibody.
(**C**-**H**) C166 cells were transiently transfected with expression vectors for the indicated Shrm4 variant and stained to detect Shrm4. Scale base equals 15 μm.

5.3 mSHRM4 IS AN ACTIN BINDING PROTEIN WITH BUNDLING CAPABILITIES

To determine if the localization of mShrm4 to the cytoskeleton is mediated by direct binding to F-actin, purified GST-mShrm4 fusion proteins consisting of amino acids 436-966 (arrowhead) or 436-1180 (arrow) were tested for the ability to co-sediment with purified F-actin (Fig 21C). In this experiment, increasing amounts of the purified GST-mShrm4 proteins were incubated with a fixed amount of F-actin (Fig 21C lanes 6-14, asterisk). GST-mShrm4 will not pellet independently at 100,000 x g (Fig 21C lanes 1,2), however F-actin will pellet at this speed (Fig 21C, lanes 4,5). As increasing amounts of GST-mShrm4 are added to the F-actin, increasing amounts of GST-mShrm4 are found in the pellet fraction after the centrifugation (Fig 21C, lanes 6-14, arrowhead and arrow). Since F-actin is the only species capable of pelleting in this reaction, GST-mShrm4 must be bound to the actin filaments in order to pellet at these speeds. Interestingly, there is a common degradation product found in the GST-mShrm4 protein sample (Fig 21C, oval) that does not retain the ability to bind to actin. Examination of this product could provide insight into the sequence requirements for actin binding.

Since the Shrm family displays a differential ability to bundle actin (Shrm3 can bundle F-actin whereas Shrm2 can not), mShrm4 was tested for the ability to induce higher-order actin structures. In these experiments, the actin-binding region of mShrm4 (amino acids 436-966 and 436-1180) was purified as N-terminal GST-fusion proteins (as described above) and used to test examine their ability to cross-link actin filaments. The actin bundling ability was observed via two methods. After the mShrm4 protein was incubated with F-actin, the sample was either labeled directly with TRITC-phalloidin (to label the actin fluorescently) or subjected to a low speed centrifugation to pellet the F-actin bundles. Indeed when mShrm4 is added to F-actin,

higher-order actin structures are observed when compared to the controls (Fig 22 D, E compared to 22 A-C). Similarly, mShrm4 and F-actin both pellet after low-speed centrifugation indicating that F-actin is assembling into some type of mShrm4 induced higher-order structure (Fig 22 F, lanes 6-13). For comparison, the F-actin without mShrm4 remains in the supernatant under low-speed conditions (Fig 22F, compare lanes 4 and 5 in the supernatant fraction to the pellet fraction).

These data indicate that mShrm4 binds directly to actin and can, in fact, induce the formation of higher-order actin structures. These properties of mShrm4 are surprising in light of the fact that mShrm4 does not have the conserved ASD1 motif. assumed to be required for actin association.

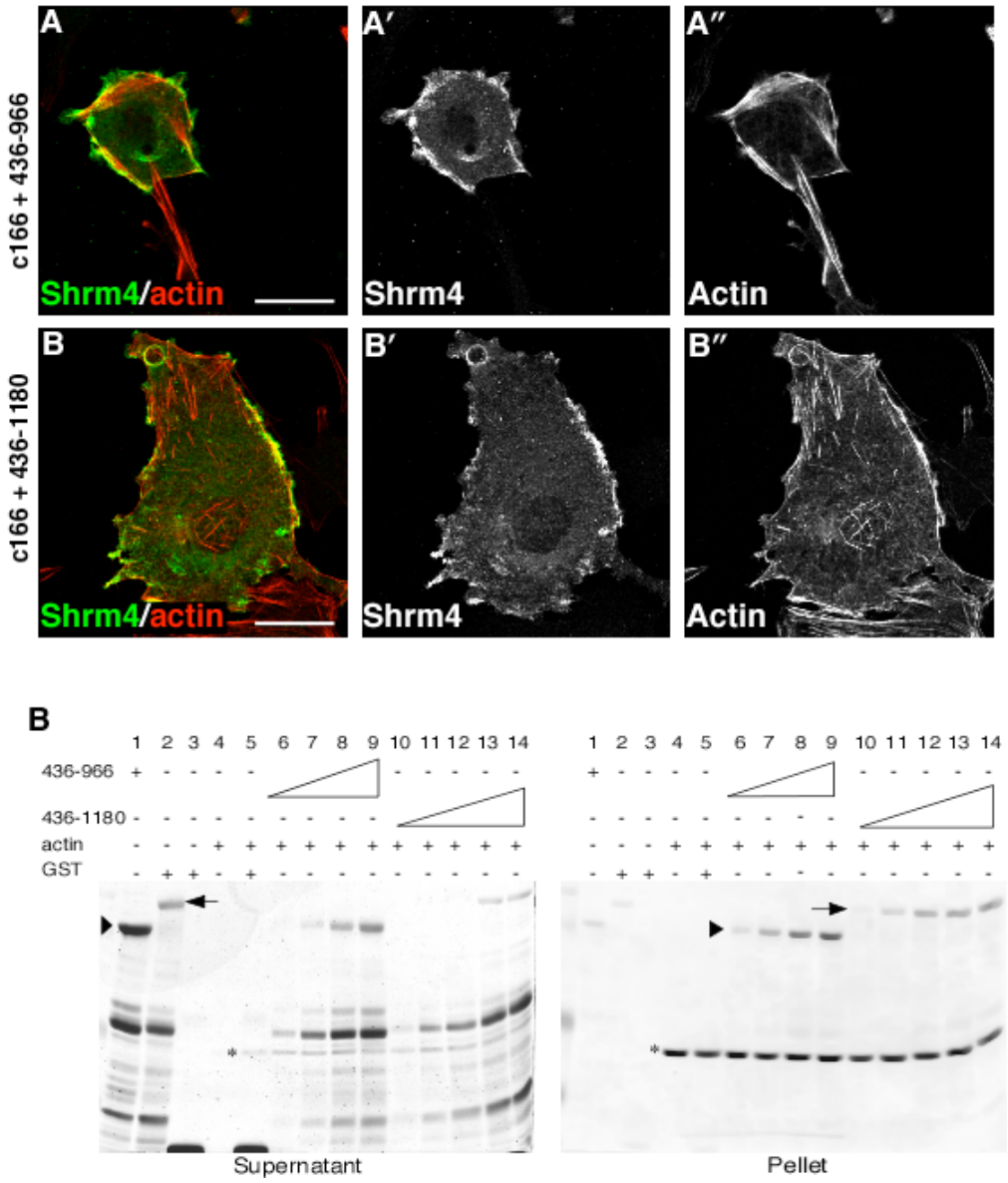

Figure 21: mShrm4 is an Actin Binding Protein

Figure 21: mShrm4 is an Actin Binding Protein

(**A** and **B**) C166 cells transiently transfected to express amino acids 436-966 (**A**) or 436-1180 (**B**) of mShrm4 were stained to detect mShrm4 and actin (green and red, respectively). Scale bar equal to 15 μm. (**C**) Amino acids 436-966 and 436-1180 of mShrm4 co-sediment with F-actin. Increasing amounts of purified GST-mShrm4 436-966 (arrowhead, lanes 1, 6-9) or 436-1180 (arrow, lanes 2, 10-14) were added to a fixed amount of F-actin (asterisk) and centrifuged at 100,000 x g. GST-mShrm4 fusion proteins do not pellet in the absence of F-actin (lanes 1 and 2). GST alone does not pellet in the absence or presence of F-actin (lanes 3 and 5 respectively). Contaminating breakdown products remain in the supernatant fraction (dot, lanes 6-14, supernatant fraction).

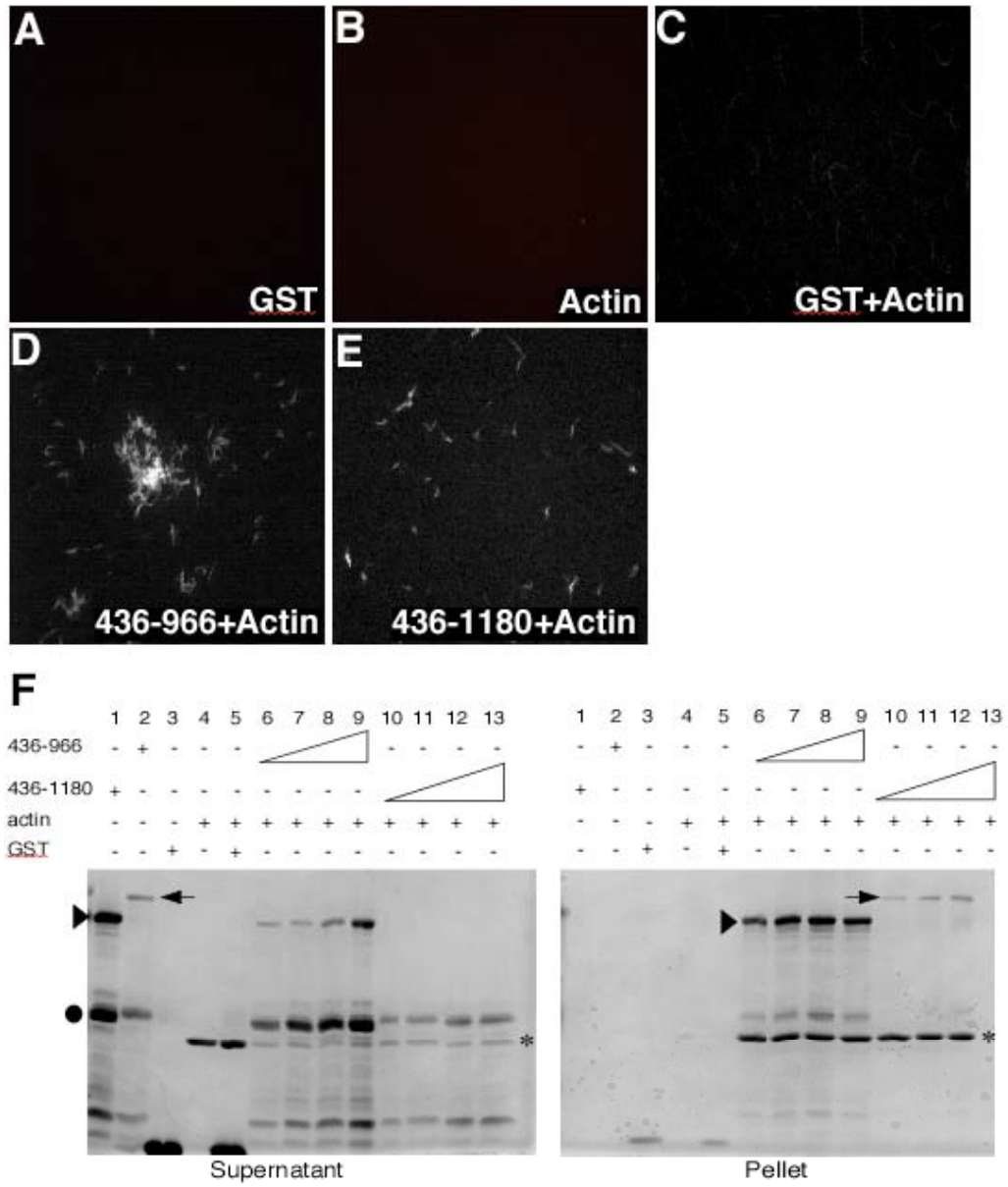

Figure 22: mShrm4 can Induce Higher Order F-actin Structures

Figure 22: mShrm4 can Induce Higher Order F-actin Structures

A-E. F-actin was polymerized and incubated with GST alone (**C**) or GST-mShrm4 fusion proteins of amino acids 436-966 (**D**) or 436-1180 (**E**) and placed on a coverslip for visualization. The GST only (**A**) and the actin only (**B**) controls show no F-actin structures. All samples were incubated with TRITC-phalloidin to fluorescently label the actin structures. **F.** Amino acids 436-966 and 436-1180 of mShrm4 induce higher-order actin structures. Increasing amounts of purified GST-mShrm4 436-966 (arrowhead, lanes 1, 6-9) or 436-1180 (arrow, lanes 2, 10-13) were added to a fixed amount of F-actin (asterisk) and centrifuged at 10,000 x g. GST-mShrm4 does not pellet in the absence of actin (lanes 1 and 2) and similarly actin does not pellet in the absence of GST-mShrm4 (lanes 4 and 5). GST alone does not pellet in the absence or presence of F-actin (lanes 3 and 5 respectively). Contaminating breakdown products remain in the supernatant fraction (dot, lanes 6-14, supernatant fraction).

5.4 DISCUSSION

The Shrm family of proteins is a well-known family of actin-binding proteins that facilitate various aspects of cytoskeletal organization. The ability to bind to actin resides in the ASD1 motif present in Shrm1, Shrm2, and Shrm3, but not in mShrm4. Actin-binding properties have been demonstrated for Shrm2 and Shrm3 [120, 135, 136], but until recently have not been observed for mShrm4 [149]. The data here show that mShrm4 can bind directly to actin and induce the formation of higher order structures. These properties are facilitated by a sequence that is not conserved among the Shrm family of proteins and in fact has not yet been described for any known protein.

The Shrm family of proteins provides an interesting case for studying actin-binding proteins. Shrm2, Shrm3, and Shrm4 all bind to actin, yet they all bind to different populations of actin. Shrm2 and Shrm3 use the same conserved actin-binding element to bind to these different populations, whereas mShrm4 uses a completely novel motif. These differences indicate that there is a series of regulatory elements inherent in the sequences surrounding the actin-binding motifs that regulate the actin-binding properties. In support of this hypothesis, amino acids 436-966 of mShrm4 are capable of binding actin, yet when expressed in cells and examined by IF, this construct targets to a different population of actin than the wild-type protein. Similarly, the ASD1 motif of Shrm3 can bind and bundle actin, whereas the ASD1 motif of Shrm2 can only bind (and not bundle) actin. In addition to regulatory elements inherent in the surrounding sequence, the populations of actin could be different in these locations. Perhaps the differential bundling abilities are a result of the conformation of the actin and not a function of the Shrm proteins having regulating sequences. These are interesting questions, whose answers will

certainly provide a better understanding of how the dozens of known actin-binding proteins decide which population of actin to bind.

The actin-binding ability of mShrm4 is quite clear, and the actin-binding motif is contained within amino acids 436-966. This motif also retains the ability to induce actin into higher-order structures. One caveat of these experiments is that GST has been shown to dimerize, so it is possible that the GST-fusion proteins in these experiments are forming artificial dimers. Based on scenario 2 in Fig 1B, this could falsely cause actin to cross-link due to mShrm4's ability to bind to actin. Experiments are being performed to address this issue.

Nonetheless, the data regarding the distribution of myosin II following mShrm4 expression and previous work showing that the ASD2 motif regulates the distribution of active myosin provide an interesting framework with which to speculate about mShrm4 function. Since mShrm4 can bind directly to actin, it is hypothesized that mShrm4 and myosin II work together to regulate the formation of the observed actin-based structures.

6.0 CONCLUSIONS AND FUTURE PROSPECTUS

6.1 mSHRM4 IS AN ACTIN-BINDING MEMBER OF THE SHRM FAMILY OF PROTEINS

This dissertation describes the cloning and characterization of *mShroom4*, a member of the Shroom family of genes and the mouse ortholog of the *SHRM4* gene in humans. The *mShrm4* protein product is 1475 amino acids in length and is 80% identical to the human protein (>90% to the PDZ and ASD2 domains) and shares two of the three conserved sequence elements that define the Shrm family: an N-terminal PDZ domain and a C-terminal ASD2 motif. However, mShrm4 lacks the ASD1 motif. The ASD2 domain is conserved in all known Shrm family members, while the PDZ and ASD1 elements are conserved in many, but not all, of the Shrm proteins. Functionally, the PDZ domain does not seem to be required for Shrm3 or Shrm4, as deletions of this domain do not affect the localization or the overall function of the proteins ([120] and this work). The PDZ domain does seem to enhance Shrm2 localization [135], indicating a potential regulatory mechanism for Shrm2 function. The ASD1 element is present in all vertebrate Shrm proteins, except for Shrm4, where it is required for targeting Shrm2 and Shrm3 to actin. The ASD2 element is required in Shrm3 to elicit apical constrictions in epithelial cells [136]. The ability to cause apical constrictions is a conserved function of ASD2, as the ASD2 motif from any one of the Shrm proteins can functionally replace the Shrm3 ASD2

motif in chimeric analysis [135]. Shrm4 possesses the conserved PDZ and ASD2 elements, but lacks an actin-binding ASD1 motif. However, mShrm4 does retain the conserved ability to bind directly to actin.

The differential actin-binding properties of the Shrm family are quite distinct. Shrm3 binds to actin stress fibers and bundles them, while Shrm2 is targeted to cortical actin [120, 135]. Based on these differences, it appears that the activities of the ASD1 motifs from Shrm2 and Shrm3 have diverged or are modified by surrounding sequences. Alternatively, mShrm4 binds and bundles F-actin with a completely different sequence. In fact, the amino acids 436-966 of mShrm4 represent, in part, a sequence that defines a novel actin-binding domain. While this domain cannot fully direct the localization of mShrm4, as it localizes to the cortical actin population, it can bind and cross-link actin filaments. Within the actin-binding motif lies a sequence element of 25 amino acids that is conserved among the Shrm4 orthologs (Fig 6A) across multiple species, with even greater identity between mShrm4 and xShrm4. It is interesting to speculate that this highly conserved sequence represents, at least in part, the novel actin-binding motif in Shrm4. However, since mShrm4 has been shown to facilitate the formation of higher-order actin structures, it is possible that another novel actin-binding motif exists in this region. The nature of the cross-linking ability has not been investigated in full, therefore it is not possible to determine if mShrm4 contains two actin-binding motifs or if it forms homomultimeric mShrm4 complexes that induce the structures. It is hypothesized that Shrm4 represents the most ancestral of the Shrm proteins, because the invertebrate Shrm homologs also lack the ASD1 motif and therefore most closely resemble the domain structure of Shrm4 [135]. While this could quite possibly be the case, the conserved Shrm4 sequence element has not been identified in the invertebrate Shrm proteins. It will be interesting to

investigate the nature of the actin-binding sequence motif and to potentially discover more proteins that utilize this element.

6.2 mSHRM4 REGULATES CYTOSKELETAL ARCHITECTURE

The sub-cellular localization of mShrm4 represents a unique distribution among known actin-binding proteins, as this restricted distribution has not been previously described. The short, dense filamentous structures are rich with a unique population of F-actin and NMMII, the two main components of a contractile actin network. In fact, the normal function of NMMII is required for maintaining the morphology of the filaments. These short, dense actin-based structures are not observed in cells, which do not express mShrm4. However, exogenous expression of mShrm4 in cells is enough to drive the formation of these structures. Additionally, endothelial cells that endogenously express Shrm4 have a large number of the actin-myosin rich structures. These structures seem to most closely resemble areas of rapid actin assembly or nucleation, like the 'cloud' structures observed in the formation of filopodia-like structures [21, 22]. These early filopodial clouds are induced by manipulating the system and using purified proteins, but nonetheless provide some insight into how actin-regulatory proteins can induce unique F-actin based structures. The mShrm4-based structures could provide an excellent system to better understand the assembly of actomyosin-based complexes in general. Thus far, the physiological purpose of the mShrm4-induced filaments has been elusive. Most cell types do not need Shrm4 for their normal function and, in fact, only a few cell types endogenously express mShrm4. To date, endothelial cells are the only cell types that have been identified as Shrm4 expressing cells. In support of this observation, the vasculature of developing mouse

embryos and some adult tissues (Fig 9 and 10) are greatly enriched for mShrm4 expression. These two observations, in addition to mShrm4's actin-binding ability, provide the basis for the hypothesis that Shrm4 functions to regulate the actin cyto-architecture in endothelial cells.

To date, research on mShrm4 has been focused on its sub-cellular mechanism of function. A model of mShrm4 function can be proposed which incorporates the current data into a number of predictive steps. Figure 23 represents the most plausible mechanism of action, which can be separated into two major hypotheses. Steps 1-3 address the modular actin-binding ability of mShrm4, while steps 4 and 5 focus on the assembly and organization of the mShrm4-actin based structures.

The first testable hypothesis addressed by the model is that mShrm4 binds to a specific population of actin through intrinsic regulatory motifs (Fig. 23, steps 1 and 2). This hypothesis stems from the observation that mShrm4 is only bound to a small population of actin in the cell while the actin-binding motif (amino acids 436-966) is directed to a cortical population of actin, but can still bind directly to F-actin in vitro. The in vitro experiments utilize only purified components (mShrm4 and F-actin), so it is possible that some accessory protein(s) bound either to F-actin or to mShrm4, could direct the in vivo specificity of mShrm4 binding to actin. In order to address the possibility of mShrm4 having intrinsic regulatory motifs, a number of experiments can be performed. These are discussed in section 6.4. Briefly, the minimal region of mShrm4 that localizes similar to wild type will be identified. Once defined, this 'regulatory' region will be tested for its ability to bind F-actin through co-sedimentation assays. Ideally, this region of mShrm4 will not promiscuously bind F-actin in vitro (no sedimentation), indicating that an external factor is necessary to specifically direct mShrm4 to actin or to perhaps modify a specific population of actin for mShrm4 to bind. If the specificity is regulated by external

factors, a couple of approaches can be employed that identify a potential interaction. Two-hybrid experiments or co-immunoprecipitation and mass spectrometry can be used to identify proteins that interact with mShrm4. However, these experiments do not address the possibility that the actin itself may be modified. Assays will need to be developed that would allow the specific isolation of the mShrm4-actin complex, so the actin can be more closely examined.

The second hypothesis that the model can address is that mShrm4 directs the de novo formation of actomyosin-based structures (Fig. 23, steps 3-5). This hypothesis is based on the observation that ectopic expression of mShrm4 in cells correlates with the appearance of the actin-based structures. Given that mShrm4 binds to a specific population of actin, how does this interaction facilitate the assembly of the short, dense actomyosin-rich structures? In order to address this question and test the hypothesis, an assay would need to be designed to enable the formation and assembly of the structures to be observed from start to finish. A few potential experiments to address this hypothesis are also described in section 6.4. Live video microscopy would need to be used with cells that express fluorescently labeled mShrm4, actin, and/or myosin II. Since one question addresses the hierarchy of assembly, the observations would ideally begin with all three proteins unbound to each other. This type of regulation could be attained if mShrm4 were under the control of an inducible promoter. Once induced, mShrm4 would conceivably bind to actin (steps 1 and 2), recruit myosin II (step 4), which in turn would help to cross-link the actin filaments and provide a mechanical force to create the dense structure (step 5). The key to this assay is to establish a cell line that expresses fluorescently labeled actin and myosin, which will likely lead to very high background since these are both abundant proteins. Once this cell line is established, effectors of actin dynamics and myosin II function could be used to help identify how the structures are formed and the role that myosin II plays in

their formation (also discussed in section 6.4). Since a function for the mShrm4-actin structures has been elusive thus far, the model is based on formation rather than function. Experiments designed to address function, such as creating Shrm4 knockout mice, are described in section 6.4. However, in order to determine how the mShrm4-actin structure is related to the overall actin cytoskeleton, high-resolution electron microscopy may be used to reveal this relationship. Similar to the work of Svitkina and Borisy in identifying filopodial precursors [21] [170], high resolution of the mShrm4-actin-based structure may provide insight into how it is linked to the cell or how it serves as a reservoir or an anchor for F-actin.

The proposed model in Figure 23 suggests a potential mechanism for how the mShrm4-actin rich structures form de novo. Based on the data, a few hypotheses can be proposed and tested based on the predictions of the model. The experiments described above and in section 6.4 are aimed at addressing these hypotheses and ultimately uncovering the function of mShrm4. By identifying how mShrm4 is directed to a specific population of actin, a greater understanding of how actin-binding proteins in general are specifically targeted to some populations of actin but not others.

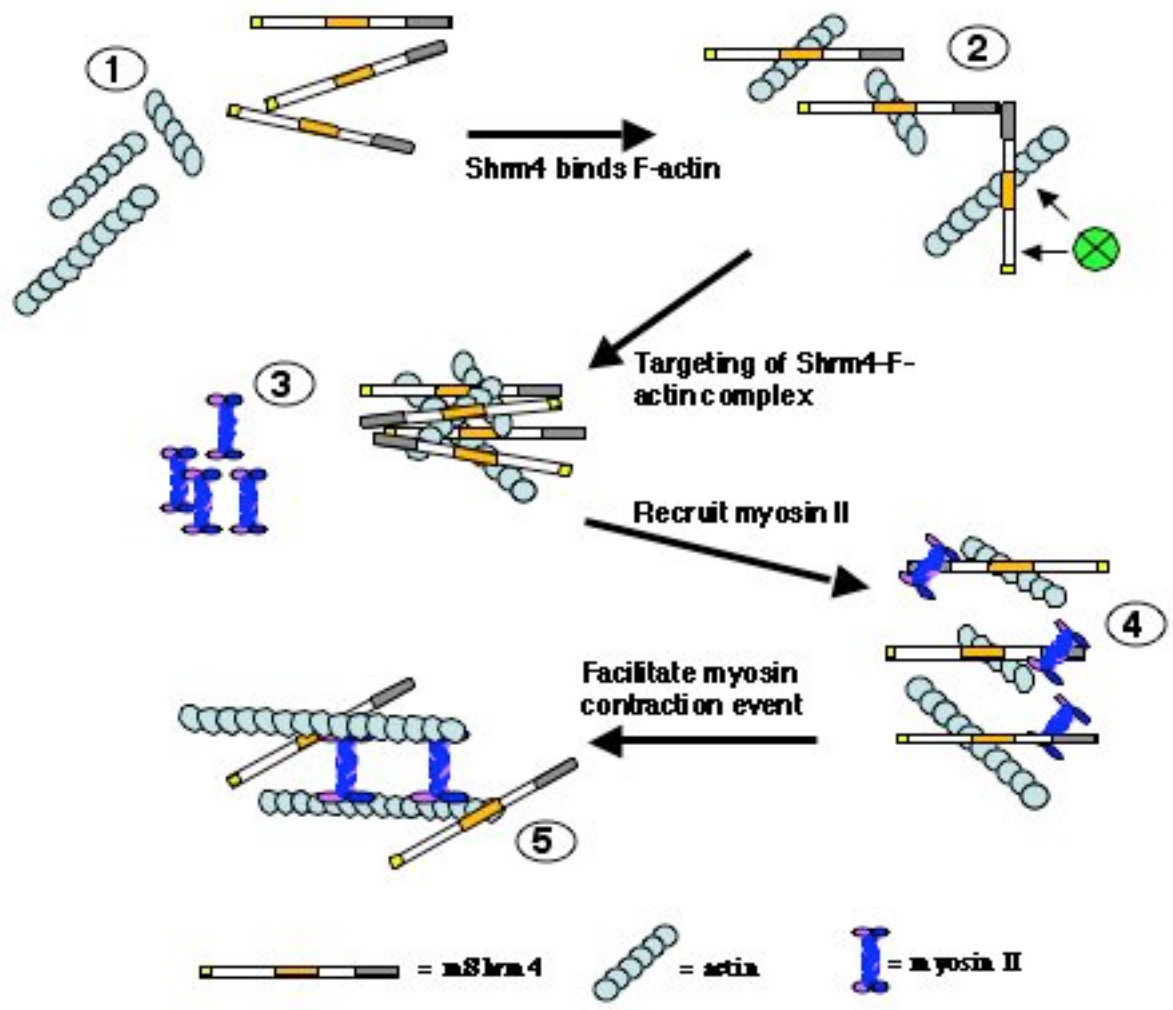

Figure 23: Model of mShrm4 Function

Figure 23: Model of mShrm4 Function

This model is based on the data for mShrm4 and for the established function of the ASD2 domain. The initial assumption is that mShrm4 and F-actin co-exist in the cell (**1**), where mShrm4 binds to a specific population of F-actin with the novel actin-binding motif (orange) (**2**). The mShrm4-F-actin complex is then targeted to a specific location within the cell (**3**), potentially with the assistance of a yet unidentified protein. Myosin II is then recruited to the mShrm4-actin structure and arranged within the actin complex under the direction of mShrm4 (via the ASD2 domain, gray) (**4**). Once all of the components are in place, mShrm4 facilitates a myosin-based constriction of the actin, thereby forming the dense mShrm4-actin structures (**5**).

6.3 mSHRM4 AND X-LINKED MENTAL RETARDATION

Mutations in various regions of *hSHRM4* have been associated with cases of XLMR [133]. Among the cases of *SHRM4* induced XLMR, only one has been identified as a missense mutation. This mutation is a serine (S) to leucine (L) exchange at a site that is not conserved between humans and mice (in mice the equivalent position is occupied by a proline (P) residue). This residue does not fall into any of the conserved regions, although it is located 10 amino acids N-terminal to the putative EHV1 binding site. The causal effects of the lesions in *SHRM4* in XLMR have not yet been elucidated and until the sub-cellular function of Shrm4 is better understood, this relationship will remain a mystery.

The tissue localization and sub-cellular distribution of mShrm4 place it in an interesting location for a role in XLMR. All developing tissues depend on the vasculature to provide nutrients and oxygen to enable normal growth and development. Perturbations in blood flow can have grave effects on developing tissues, especially the on the highly sensitive cells of the nervous system. As an example, mutations in the gene associated with Norrie disease, *NDP*, (Norrie Disease, pseudoglioma) perturb the normal development of the retinal and cochlear vasculature [171, 172]. mShrm4 is highly expressed in endothelial cells of the vasculature in both embryonic and adult tissues. Because mShrm4 represents a novel actin-binding protein that is able to direct the organization of contractile actomyosin structures by recruiting actin and NMMII, it is plausible that Shrm4 is necessary for proper vascular development. Shrm4 is also expressed in many adult tissues, indicating a continued function in adulthood. The adult vasculature also depends on the de novo formation of new vessels, so it is quite probable that Shrm4 functions to direct the development and maintenance of adult vasculature, also in an actin dependent manner. Both vasculogenesis and angiogenesis are processes that, much like the other

morphogenetic movements described in this dissertation, depend on the actomyosin network to facilitate cell shape changes. The endothelial cells in these systems require the coordinated functions of the downstream effectors of VEGFR-2 (vascular endothelial growth factor receptor) and of integrin signaling. Both of these pathways depend on the function of the RhoA-ROCK pathway [173]. Signaling through receptor tyrosine kinases, specifically VEGFR-2 (vascular endothelial growth factor receptor 2), can promote the activation of RhoA, which induces ROCK and promotes assembly and disassembly of stress fibers [174]. Additionally, the activated RhoA-ROCK complex is able to phosphorylate FAK, which then is able to promote focal adhesion turnover [175].

Alternatively, mShrm4 could be expressed in a population of neuronal cells at a critical stage of development. As described previously, the migration of neurons and the trafficking of synaptic vesicles are two processes that are fully dependent on a functional actin cytoskeleton (also regulated by the Rho-ROCK pathway). mShrm4 is observed at the apical and lateral portions of the neural epithelia and is also found in a punctate pattern throughout the neural tube (Fig 10). mShrm4 is certainly in the right place at the right time to affect neural development. Perhaps the localization of mShrm4 in specific neural cells is highly stage specific and those populations of mShrm4 expressing cells have not yet been observed.

In order to better test this hypothesis, it will be critical to establish an animal model to better understand the role of mShrm4 during early development.

6.4 FUTURE PROSPECTUS

The studies on mShrm4 described here provide a solid foundation to elucidate the function of Shrm4. The ability of mShrm4 to bind to actin coupled with its sub-cellular distribution provide an interesting source of speculation for how mShrm4 functions. In order to better understand mShrm4, a number of experiments are necessary, a few of which have already been undertaken and other that are in the planning stages.

Since *SHRM4* is implicated in XLMR, it is imperative to determine the developmental role of mShrm4. Two independent experiments are aimed at elucidating this function for Shrm4. The first is to create a *mSHRM4* null mouse using the genomic clone described in Fig 8. Regions of homology have been identified both 5′ and 3′ of exon 4 of *mShrm4* and are currently being used to design a targeting vector. The cloning of this region has thus far proven quite difficult, but the construction of this plasmid, and ultimately the *Shrm4* knockout mouse, is eminent. The fact that *mShrm4* is located on the X-chromosome, the generation of the knockout mice might prove to be a daunting experiment. Additionally, zebrafish can be used as an excellent model to examine the developmental role of Shrm4. In fact, other X-linked mammalian genes implicated in XLMR have been studied in zebrafish [176]. Since there is a Shrm4 ortholog in zebrafish, morpholino technology can be used to knockdown the endogenous protein and the ensuing defects can be visualized. The first step in this experiment is to determine the localization pattern for *zShrm4*. Using a riboprobe specific to *zShrm4*, whole mount *in situ* hybridizations were performed on various staged zebrafish embryos (Fig 24). At the 18-somite stage, *zShrm4* is localized to structures that resemble rhombomeres in the developing hindbrain (Fig 24A, arrow) [177]. In addition, *zShrm4* shows high expression in the tailbud (Fig 24A, arrowhead). At 44 hours past fertilization (hpf), the *zShrm4* staining is diffuse, yet specifically localized to the

anterior region (Fig 24B). Interestingly, at 72 hpf *zShrm4* becomes specifically localized to structures that resemble the developing vascular endothelium of the aortic arches (Fig 24C, box) [178]. More research is necessary to determine the identity of these structures and more importantly to determine the role of zShrm4. The next step will be to over-express mShrm4 to determine if there is an over-expression phenotype. Conversely, a construct consisting of the actin-binding motif (amino acids 436-966) will be expressed to examine the potential dominant-negative effects on zebrafish development. If the 436-966 construct does indeed represent a dominant negative, it is expected that the phenotype would be similar to a zShrm4 knockdown. The knockdown experiment will be conducted using morpholino probes designed from the known zShrm4 sequence. Finally, mShrm4 will be over-expressed to test its ability to rescue a zShrm4 knockdown. In all, the mouse and zebrafish experiments described above should provide crucial insights into Shrm4 function and how it could play a role in XLMR and in vascular development in general.

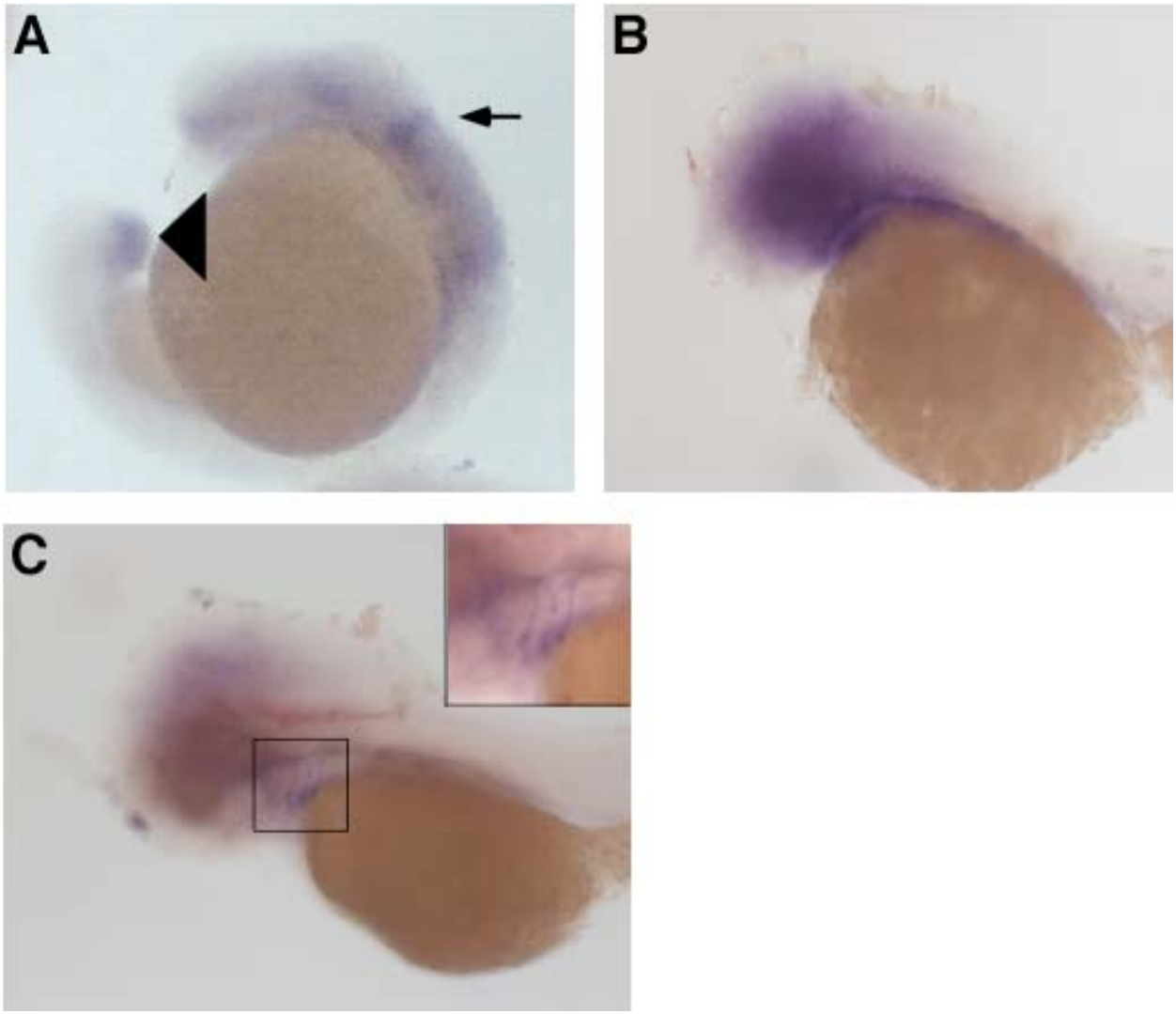

Figure 24: *zShrm4* Expression in a Developing Zebrafish Embryo

Figure 24: *zShrm4* Expression in a Developing Zebrafish Embryo

Zebrafish embryos were incubated with a digoxygenin-labeled riboprobe specific for *zShrm4* at the 18-somite stage (**A**), 44 hpf (**B**), and 72 hpf (**C**). At the 18-somite stage, *zShrm4* mRNA is localized to specific regions in the hindbrain (**A**, arrow) and in the tailbud (**A**, arrowhead). At 44 hpf, *zShrm4* is specifically localized in the head region but in a diffuse pattern (**B**). *zShrm4* is expressed in the aortic arches at 72 hp (**C** boxed region). Inset is a high magnification of the boxed region in **C.**

In order to better understand the sub-cellular function of Shrm4, a number of cytological tools can be employed. Perhaps the most interesting aspect to next examine for mShrm4, would be the assembly mechanism of the mShrm4-actin structures. In order to best understand this process, live-image microscopy can be used to observe the initial association of mShrm4 with actin and then their assembly into the structures previously observed only by IF on fixed samples. GFP-mShrm4 constructs have been created and transfected into C166 endothelial and RAT1 fibroblastic cells in order to generate cell lines that stably express GFP-mShrm4. These cells can be used to observe the formation of GFP-mShrm4 structures *de novo*. Additionally, these cells could be transfected with alternately labeled actin constructs (such as YFP- or RFP-actin), so the mShrm4-actin complex can be visualized. This system will also provide the means necessary to utilize the many inhibitors of actin and NMMII dynamics available to test the various steps of mShrm4-actin assembly. These data will provide valuable insight as to how mShrm4 is regulated *in vivo* and will determine if the Shrm family of proteins utilize the conserved mechanism for function.

Finally, the ability of mShrm4 to bind F-actin and induce higher-order F-actin structures is very intriguing. The novel actin-binding motif is poorly characterized and not understood. Experiments aimed at further refining the actin-binding motif of mShrm4 are of utmost importance in order to fully define this domain. Biochemistry will be used to empirically determine the minimal sequence necessary to bind to actin. Similarly, the sequence(s) necessary to bundle or cross-link F-actin can be determined in this fashion. The localization motif of mShrm4 extends beyond the actin-binding element, therefore these regulatory sequences should also be identified. A series of mShrm4 truncation or deletion constructs will be transfected into C166 cells to identify the minimal region of mShrm4 that still localizes to the actin-based

structures. In understanding the mechanism governing mShrm4 localization, a greater appreciation for the Shrm family in general can be gained.

6.5 SUMMARY

Shrm4 is an unusual member of the Shrm family of proteins. Unlike the other Shrm proteins, Shrm4 utilizes a novel sequence to bind to actin to facilitate the formation of unique F-actin based structures. The function of these structures has yet to be identified, but they clearly play a yet uncharacterized role in endothelial cell function. It will be interesting to discover this role, as data from zebrafish and mouse models will surely uncover. Even more importantly, the discovery of Shrm4 function will likely provide a better understanding of neural development in general with possible implications to human disease.

APPENDIX A

A.1 PROTEIN SEQUENCE OF *DANIO RERIO* SHROOM4

METVEQLVSFQHIHVQLNGGAPWGFTLKGGLEHGEPLIITKGTRPHQTLRQKLVCQPCIQ
HLGSTSRQNHSKVLFALREVPLEKVELLLKNDGVQTYIVGLVKNVMTSSLINEFIQRPNP
PRLVSVNVKYLIHLYGMQHCKIRLGSPFFTAAEAHAPYRLTGESFHAHTLNNMMDTRT
KLIPDALTQGVAEGLSALAASKIEEGGKAAQCKKLRVGDELVNINGSALYGSRQEALILI
KGSYRVLKIIVRRRSVPVIRPHSWHLAKLTEAPCTTGTGDPSDGPPAMQLHFTPLNVPW
HSGGDNSELSMQWGHISRHYSTDRSSSIGSMESLENPPNQGYYDSQLSPIDPVIFNNKRD
SAYSSFSASSNTSDYTVPVKPEETNSMDSLLQGLGSSCRYPDRGQHSAPTGHGNQLEEQ
EHSKVLSGRSEPKVRPSSYSCEEENCAPPQPPMRKESFRATRGQATDKRCVSAPVGIPNV
SSCTDEDQSQIQDVLTGRVYLNGIQDSEPERKGCSIQPYYTLNSDSGGDGKATDHEKEH
LEDYSKSVAAASSPPSQRNRDENFDTKLRPEHDLQSAMNRHSAPEKLLAAQLCMMDVS
ADKSDHSASPTCQWSQSPLYLSDSSQNTEQGKWGTSRCSTPGSVATSEVEDPRLEEESID
GQNPWGHPISLSGSPIGNGFSQNSENNGGEVMLDDGLGPKQPQKRQFRCSKSRRRSERF
ATNLRNEIQRKKAQLQKSRNPCGEETVEEEVGDFNTEVVAPPEYRQHKPQILPKPATNT
VPIQISKPATQKVFDATQTEEYSSARVVCFQTSQTQTQVPQHVRQVCVHVVEEVAPAGK
PRRWRWTPERKLELENESSESKRNETIGQMWSGKMESTRSRTSSSSGRLGRSDDCDILPF
ADRRRFFEETSRKLSQSVTNLSNLTSRSQRLDKPGRMNHPSSPEPHEIAAHLGRRRFSYQ

DVPYINSLDNGRQFISNQQDQEKTRQRLIEREQERERERQERLKEQEKLREQEREKERLRK

ERENEQQRLKDWEERQRLLQREQEWESVRESVHSEHNTSSDTVPHSLSHDVYRKQPPSP

QIPSSLQMSEPLYSSTTTNVQKPCSAFHPVTTQHNRYDGYQVNPPYNPRNYTPDELFCCL

KYSKYILQAQYVREQEKTRKINRNFSLTERDYPRSRRDFKTVDGANVPGFVRTGGNSITS

GRNEEHLFTPLRNRAMSENNLCVETQHYQNHSSSSGVSRMRSSTLSDLDENTVSVGEIK

KKKGPPPPRPPPPKWDQFHKRRASHHSLFSSTSHYSSPTASPPRPQPQTCTSRPPSVSEMS

RQRSYSLPPRDMSESLHCCRQEYSVAPSSPAFTRRAFKPVALPPREMDYNTRREVHQPEP

CTRIPIHPAAAPEEPRATHLKPTFPDHGVEWDRSSPHYPTHGTSRTPEVPSNSFSAGPLCP

ESYFSMNNFQLQPQQAGFPITAHKTQIIPSQGPEDGDLPLETDIDEICENEQAERKETVDR

MEMQGFARPVMVLETDIDHTPEEAAPSAISIRGPRGSLVDSILEDEYGVSRKELLGELFP

HSVNAEMSGDGWRGGYPISGGTLERFDNLIRQAFHFFEVLKPSRLNTTAPQVSRSTCYDI

SADNPQLLAKLREISERKEEDEELNYKKQLMESLRKKLTVLREAQRGLQEDIRANAQLG

DEVESLVLAICKPNEVDKYRMFIGDLDKVTSLLLSLSGRLIRVESALDCVDPETGHQERL

QLLEKKKQLLVQMGEAQELKEHVDRREQAVCRVLGCCLTPEQMRDYGHFVKMKAAL

LVEQRQLDDKIRLGEEQLRGLRESLGLGFGMAYGHY

A.2 PROTEIN SEQUENCE FOR *XENOPUS TROPICALUS* SHROOM4

MDPQPADGSSQCIHVQLQGGAPWGFTLKGGLEHGEPLIISKIENGGKASMCEKMEVGDE
LVNINGTPLYGSRQEALILIKGSYKILRMIVRRRNLSVIRPHSWHLAKLTEVHPDVASMQ
YPTDAFSLSWHSGCENSELPMQWNPLSRHCSTDKSSSIGSMESLDQPGHNYYEGTLSPID
PSMYQNKRDSAYSSFSASSNASDYTLSARAEESSQMDCVADSSKPCDGRYLHTGQGAIE
IQQETSSLSPSEHQQRPSSFPFDANHLSFIKSPPQPPIRRDSLRASKNQICHGERRRASAPG
DSLQISGMWSSENQQHKNSDTSQCKCGIEFCTVHLKNGLSSDQYYMLSSQTDGGNQST
DQLALSDREMPSCCTEMQSRWPRDGRNIKQTINKEMENSSYHSAQTVKTVKDSLSKHP
SCLHMKSSSLPQSEQEEVSVQTKFHRKEWRNTLLQENFQCQTSECNGISSQELKCNINRD
SEIYQVDTLTDTGDFSQLSGKNKEQSQHITTLERSVSEPNEVRESFPVLLPKHSVGGMRS
SCSSETLLEESHEQEESQGPTKKPGSSRHRSAQMRRRSDRFATNLRNEIQRRKAQLQKN
KGSSVLLCGEEPVEEREEPTESQSPPRPMPPPPPKNKSRLLELKRANAEQFHKGTDHQH
LEQNKQSLPSNRDTDKDNQKTEENSLNAVENVTRANDKSLISKSRLQNESNKSFVYDPR
QKDHQRVSSELNNESTMPSAENCREEWRTREPDFQRQHSKCLEQREPELGNVAESSATT
VRWNEVSRASPNPDVKVSHEMWRASSSLSINSVGSQTENFRGIGVESHSHYSGHKSLDA
DASFNGLLSSKEQLYSDGSSNDDWRISCLDNEEPMQRGREMMFSEPGRSSDHTSLFAAQ
WRSRHSSSDFEDPHVQQMPNGGRWKWSPEHKLLPHPQLSKGSPPDVSVVHAEGASLPN
RVASEENVLMPFADRRRFFENSSKVPNVSHIPLQIKSNKNNYCPSFPDPPLSQKVVSALR
RHSVDHTYHPSSPNRQDSALPYSDYCVNHTVDPLLCCSQGGHAAEYIHHPTGYGCRVH
ESCHCCSSDVCPALVKRNMPMSHLSCHFLHHHHHHHQWSRCGDYLCPAQHSTLEEGTS
LHGDPWHLQKPVLQEVPLKEWTQQLKIPNRKCSQSGSDLCHSNSGFHRAGPFRPCCDNS
EQDFPQCYRTVSSYDLSCEHSIRPELSSHHDEPSDQNLGRGRAYSVSQLNLDCLALRDK
KETSLSKLEEHMPSALAKKQKPPRPPPPNWDKYKERRASHQLTNSILSRHRENSVGSGH

SISMEAVRQRSQSLPMERILLKANENYHPSSECEHPQIRDCSPVPSDLRPAHQEPDTSADT

VSTTFEHARQEPFSDCRNSPRDASPPRTDHLVPSDPVTSSHHQTTDFYSPLEGGSIEEPSN

SVAEESEDGVIPPKSTHDHISPDIPEREHPANLLDGFDSGYRHYEDEWSTDRESEISIPERY

EFQPISPPPVCGAVSPTSCAAYYNTSAAKAELLNKMKELPGLQEEVGDQEEVEEEDELSL

KKVQLIESISRKVSVLHEAQQGLQEDINANTTLGCEMADLLKNLCKPNEYEKFRTFIGDL

EKVVNLLLSLSGRLARVESVLSSEDPEPSVDEKLNLLEKKKQLTEQLEDAKELRAHVTR

REHMVLESVSRYLNEEQLQDYHHYVKMTSALIVEQRELEDKIRLGEEQLRCLRESL

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