Identification of a Novel Formin-GAP Complex and Its Role in Macrophage Migration and Phagocytosis

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Essential and diverse biological processes such as cell division, morphogenesis and migration are regulated by a family of molecular switches called Rho GTPases. These proteins cycle between active, GTP-bound and inactive, GDP-bound states, and this cycle is regulated by families of proteins called Rho GEFs and GAPs. GAPs are proteins that stimulate the intrinsic GTPase activity of Rho-family proteins, potentiating the active to inactive transition. GAPs target specific spatiotemporal pools of GTPases by responding to cellular cues and utilizing protein-protein interactions. By dissecting these interactions and pathways, we can infer and then decipher the biological functions of these GAPs.

This work focuses on the characterization of a novel Rho-family GAP called srGAP2. In this study, we identify that srGAP2 is a Rac-specific GAP that binds a Formin-family member, Formin-like 1 (FMNL1). FMNL1 is activated by Rac and polymerizes, bundles and severs actin filaments. srGAP2 specifically inhibits actin severing by active FMNL1, and the assembly of an srGAP2-FMNL1 complex is regulated by Rac. Work on FMNL1 shows that it plays important roles in regulating phagocytosis and adhesion in macrophages. To learn more about srGAP2 and its role in regulating FMNL1, we studied macrophages isolated from an srGAP2 KO mouse we have recently generated. This work demonstrates that loss of srGAP2 decreases the
ability for macrophages to invade through extracellular matrix but increases phagocytosis. These results suggest that these two processes might be coordinated in vivo by srGAP2 and that srGAP2 might be a critical regulator of the innate immune system.
Dedication

To my wife and best friend, who makes me smile every day and loves me for the person I am.

To my parents, who have always supported me. I could never fully express my gratitude.
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List of Abbreviations

AF – AlexaFluor

ADP – adenosine di-phosphate

ATP – adenosine tri-phosphate

BAR - Bin-Amphiphysin-Rvsp

DH – Dbl homology

DHR – DOCK homology region

F-BAR – Fes/CIP4 - Bin-Amphiphysin-Rvsp

FMNL – Formin-like

GAP – GTPase activating protein

GEF – guanine nucleotide exchange factor

GDI – guanine nucleotide dissociation inhibitor

GDP – guanine di-phosphate

GTP – guanine tri-phosphate

IF-BAR – inverse Fes/CIP4 - Bin-Amphiphysin-Rvsp

PH – Pleckstrin homology

SH2 – Src-homology 2

SH3 – Src-homology 3

srGAP – Slit-Robo GTPase Activating Protein
WH2 – WASP homology 2
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1 Introduction

1.1 Rho-Family GTPases and Their Regulation

1.1.1 Rho-family GTPases

Approximately one percent of the human genome encodes proteins involved in Rho-family GTPase signaling (Jaffe and Hall, 2005). Rho GTPases, typified by Rho, Rac, and Cdc42, are a family of genes that share structural and biochemical homology with members of the Ras GTPase superfamily. The approximately 20 mammalian genes comprising the Rho family have homologues in yeast, C. elegans, and Drosophila. Rho GTPases are sophisticated molecular switches that cycle between an inactive and active state (Figure 1.1). When active, Rho GTPases bind GTP and, via a hydrophobic isoprenoid post-translational modification, are targeted to the membrane where they interact with downstream effectors. A magnesium ion is also present in the Rho protein and is essential for nucleotide binding. The GTP-binding induces a conformational change in Rho: the $\gamma$-phosphate of GTP creates hydrogen bonds with two portions of Rho called the Switch I and Switch II regions. The stabilization of these two regions by GTP allows for binding of downstream effectors such as kinases and actin binding and regulatory proteins.

A hallmark of Rho GTPase proteins is the hydrolysis of the $\gamma$-phosphate on GTP. This hydrolysis is key to the cycling of the active, GTP-bound protein to an inactive, GDP-bound protein. The hydrolysis is coordinated by a conserved glutamine and the $\gamma$-
phosphate transfers to a water molecule near the binding pocket (Rittinger et al., 1997b). The rate of hydrolysis by the Rho-family GTPase protein is actually quite slow in vitro (around 15-50 minutes) but is accelerated five orders of magnitude in the presence of a family of proteins called Rho GTPase activating proteins (Rho GAPs) (Lancaster et al., 1994; Self and Hall, 1995).

Rho GAPs are a family of 77 genes that bind to active Rho GTPases, stabilizing the Switch I and Switch II regions to induce an enzymatically active conformation; hydrolysis of GTP can then occur. This is induced by a conserved “arginine finger” that inserts into the GTP-binding site (Rittinger et al., 1997a). Once inactive, the GDP-bound Rho interacts with a family of proteins called RhoGDIs (Rho Guanine Nucleotide Dissociation Inhibitor), of which there are three. GDIs function to maintain a GDP-bound Rho-family GTPase, mask the prenylation group and hold the GTPase in the cytosol, and potentially even deliver the GTPase protein to a site where it can be activated (Dovas and Couchman, 2005).

When Rho-family GTPases are delivered back to the membrane, Rho GEFs activate the GTPases. To do this, the GEFs bind Rho-family proteins at the Switch I and Switch II regions and displace the magnesium ion in the GTP binding cleft. This disruption causes a loss of the nucleotide binding. The GTPase then binds GTP, as it is more abundant in the cytoplasm than GDP. The Rho-family GTPase protein is now active and bound to GTP at the membrane.
Figure 1.1: Activity cycle of Rho-Family GTPases

Rho-family GTPases, when active, are targeted to the membrane by a prenylation group and bind GTP. In this state, they can bind downstream effectors. GTPase Activating proteins (GAPs) bind the active GTPases and stabilize the enzymatically active form to allow for hydrolysis of the GTP. The inactive, GDP-bound GTPase is then held in the cytosol by Guanine nucleotide Dissociation Inhibitors (GDIs), which mask the prenylation group. The GTPases are then targeted to the membrane, where Guanine nucleotide Exchange Factors (GEFs) aid in the exchange of GDP for GTP.
As mentioned above, there are 20 mammalian genes encoding Rho-family GTPases that are divided into roughly 8 groups (Heasman and Ridley, 2008). Most of the work on Rho-family GTPases has focused on three family members: RhoA, Rac1, and Cdc42. A large body of the research has identified that these three GTPases have a profound effect on regulating actin cytoskeleton reorganization (Hall, 1998; Nobes and Hall, 1995a, b).

RhoA promotes actin stress fiber formation, focal adhesion formation, and myosin contractility. RhoA can stimulate actin polymerization through several formin family members (Lammers et al., 2008). It also signals through ROCK (Rho-associated protein kinase) to regulate myosin phosphorylation state, which in turn regulates its ability to contract actin fibers, and through LIMK to inhibit actin severing activity by Cofilin (Burridge and Wennerberg, 2004). These processes together regulate cell protrusion, adherence and migration in a coordinated fashion.

Rac1 is considered to promote actin polymerization, mainly through formins and a family of proteins called WAVEs. Rac1 stimulates the actin reorganization into a thin web-like sheet, forming a lamellipodia at the leading edge of a cell. Rac1 has also been implicated in the generation of phospholipids at the membrane (Tolias et al., 1995), regulation of phagocytosis and phagosome maturation (Hall et al., 2006; Wang et al., 2008), and also regulates signaling to myosin and cofilin (Giannone et al., 2004). Rac1 deficient (knock-out) mice are embryonically lethal at very early stages, owing to its
importance in development and numerous cellular processes. Rac1 and RhoA pathways have also been shown to functionally antagonize each other (Wildenberg et al., 2006).

Cdc42 generates actin polymerization, forming filopodia which act as protrusive forces to push the cell membrane outward (Pollard and Borisy, 2003). These tight actin bundles could potentially be involved in sensing extracellular cues (Jaffe and Hall, 2005; Wennerberg and Der, 2004). Cdc42 is known to activate the WASP and N-WASP proteins, activates several formins, and is important in generating polarity during a variety of cellular processes (Jaffe and Hall, 2005).

But while a plethora of work focuses on these GTPases, there are many Rho-family GTPase members that are largely uncharacterized. Some members like RhoB and RhoC are ubiquitously expressed and have been linked to cancer development and progression in mice (Clark et al., 2000; Liu et al., 2001; van Golen et al., 2000a; van Golen et al., 2000b). Rac1 has two highly homologous relatives called Rac2 and Rac3, and a third more distantly related homolog called RhoG. While Rac1 is widely expressed, Rac2 expression is restricted to hematopoietic cells where it performs a variety of functions, including regulation of phagocytosis and phagosome formation in macrophages and neutrophils (Wennerberg and Der, 2004). Rac3 is less well characterized, but has been implicated in neuronal development (Corbetta et al., 2009). Other families are the Cdc42-related GTPases (TCL, TC10, Wrch-1 and Wrch-2), the Rnd subfamily (Rnd1, -2, and -3)
that appear to be GTPase-deficient, RhoD and Rif, RhoH, RhoBTB (RhoBTB-1, and -2) (Wennerberg and Der, 2004).

Much is still unknown about these GTPases although they may be critical regulators of physiological processes. Recently Rif has been identified as an activator of mDia1 and mDia2 formins to form filopodia in cultured cells, demonstrating that RhoA might not be the only regulator of these formins and there is plenty to learn about the role of Rho GTPases at the biochemical, cellular and physiological levels (Goh et al., 2011; Pellegrin and Mellor, 2005).

1.1.2 Guanine nucleotide exchange factors

A critical step in the recycling of Rho GTPases is the exchange of GDP for GTP, which is potentiated by a large family of proteins called Guanine nucleotide Exchange Factors (GEFs). GEFs have GTPase binding domains that stabilize the switch regions of the GTPase in a conformation that displaces the magnesium ion, which is required for high affinity binding of the guanine nucleotides. Upon removal of Mg$^{2+}$, GDP is lost and GTP, because it is present in much higher cellular concentrations than GDP, enters the pocket. The GTPase is now “active.” There are two varieties of GEFs: the Dbl Homology-Plekstrin Homology (DH-PH) and the DOCK180 families. At a biochemical level, both “activate” the GTPases in a similar manner, but have different ways of doing so in a cellular context.
The DH-PH family, of which there are approximately 69 genes, utilize two paired domains to exchange GDP for GTP: DH and PH (Rossman et al., 2005). The Dbl Homology (DH) domain is named so because the first GEF identified was the Cdc42 GEF named Dbl (Hart et al., 1991). The DH domain is the domain that more directly binds to the GTPase in the switch regions. Unlike GAP domains, DH domains do not directly insert a side chain into the active site of the GTPases (Rossman et al., 2005). They induce a conformational change to disrupt the association of several conserved amino acids with the α-phosphate of GDP and Mg²⁺. The PH domain also plays a role in GEF targeting and GTPase recognition. The PH domains bind phospholipids and have been thought to play a regulatory role in activating GEFs. Surprisingly, very little evidence exists to support this hypothesis in vitro. However, phospholipids in the context of a cell membrane or lipid bilayer do appear to activate GEFs (Baumeister et al., 2003; Rossman et al., 2003). But data does suggest that PH domains do play an important role in targeting the protein to the membrane in cells. Additional evidence suggests that PH domains directly bind GTPases and modulate other protein-protein interactions, but the importance or function of these interactions is still largely unclear (Rossman et al., 2005).

The other family of Rho GEFs is called the DOCK family of proteins, of which there are eleven (Brugnera et al., 2002; Cote and Vuori, 2007). DOCK proteins have two main domains called DHR-1 and DHR-2. The DHR-2 domain binds nucleotide-free GTPases and promotes guanine nucleotide exchange in vitro and in vivo (Cote and Vuori,
The role of the DHR-1 domain is unclear, and while it is not involved in GTP-loading of Rac, mutations that block the function of DHR-1 domain disrupt Rac-dependent cell migration (Katoh and Negishi, 2003). Additionally, some DOCKs have SH3 domains that allow for binding to another family of proteins called ELMOs. The ELMOs enhance, albeit modestly, the exchange of GDP for GTP by DOCKs (Lu et al., 2004). And while they do regulate the activity of DOCKs in cells, potentially by stabilizing the DOCK-GTPase interaction or localizing DOCKs to proper cell locations, the exact mechanism of the DOCK-ELMO machine is still very unclear (Cote and Vuori, 2007).

Both types of GEFs display a large array of protein-protein and protein-lipid interaction domains, demonstrating their ability to regulate GTPase signaling in discrete pathways. GEFs contain SH3, SH2, kinase, PDZ and even RGS domains, which allow for integrations of heterotrimeric and small G-protein signaling. Interestingly, some genes such as Bcr and Abr act as GEFs and GAPs (Rossman et al., 2005). This indicates that it is imperative to have local coupling and balancing of Rho GTPase signaling via GEFs in vivo.

GEFs have been implicated in various disease states, such as hematopoietic development and cancer. Bcr, through chromosomal translocation with a tyrosine kinase called Abl, forms a gene fusion called Bcr-Abl, resulting in a constitutively active kinase that is expressed in human leukemias and can mediate oncogenesis (Advani and
Pendergast, 2002). Loss of another GEF subfamily called the Vavs (Vav1, -2, and -3) leads to defects in T-cell development and macrophage phagocytosis (Hall et al., 2006; Tybulewicz, 2005). And while the mammalian immune system may use GEFs to regulate immune cell function, bacteria, including strains of *Shigella, Salmonella* and *E. coli*, can hijack mammalian cells using GEF “mimetics” to allow for cell entry and other cytoskeletal changes (Bulgin et al., 2010).

While pathways and physiological roles for some GEFs are known, the family consists of 85 proteins, many of which are still unstudied. Additionally, most of the work on GEFs has focused on their role in regulation of Rho, Rac and Cdc42, despite their being an abundance of other Rho GTPases. And while some cellular mechanisms to activate GEFs have been identified, additional cellular cues that regulate GEF activation and inactivation remain unknown.

1.1.3 Rho GTPase activating proteins

As a molecular switch, Rho GTPases must transition between active and inactive states. And as discussed earlier, Rho GTPases have poor intrinsic hydrolytic activity for GTP. This hydrolysis is greatly enhanced by cellular factors called Rho GTPase Activating Proteins (GAPs). GAPs bind active Rho GTPases, stabilizing an enzymatically favorable conformation of the GTPase. To do this, GAPs insert a conserved arginine residue into the GTP binding pocket of GTPases (Nassar et al., 1998; Rittinger et al., 1997a; Rittinger et al., 1997b). This holds the switch regions of the GTPase in a stable
conformation, allowing the GTP to be hydrolyzed to GDP. The GDP-bound Rho GTPase is now in the inactive state. Thus, GAPs provide a crucial role for the recycling of these switches and allow for tight spatial and temporal signaling of Rho proteins.

There are currently believed to be over 70 Rho GAPs encoded for in the human genome (Bernards, 2003; Peck et al., 2002; Tcherkezian and Lamarche-Vane, 2007). These genes appear, at least at a primary protein sequence or structural level, to be divided into sub-families of two to four genes. These smaller families of proteins can even show remarkably different functions in vitro and in vivo. But while they may perform different functions on a biochemical or cellular level, the overarching role of Rho GAPs is highly conserved. The Drosophila melanogaster, Caenorhabditis elegans and Dictyostelium discoidium genomes each encode over 20 Rho GAPs, and Saccharomyces cerevisiae has at least 7 (Bernards, 2003; Tcherkezian and Lamarche-Vane, 2007). This conservation highlights the importance of Rho GAPs and their essential function as regulators of Rho GTPase signaling.

Despite having 70 Rho GAPs, the human genome only encodes around 20 Rho GTPases. This disproportionate ratio suggests several things. First, there must be a distinct expression profile for each Rho GAP throughout different organs, tissues, and cell types. One family of Rho GAPs, called the srGAP/WRP family, is comprised of four members. Three of these members, srGAP1, srGAP2, and WRP (also called srGAP3 and MEGAP), are all expressed highly in the CNS during development and throughout
adulthood (Carlson et al., 2011; Guerrier et al., 2009; Mattar et al., 2004; Wong et al., 2001). These genes appear to have overlapping expression patterns in the brain, including the hippocampus, olfactory bulb, and cerebellum. Interestingly, there do appear to be regions where the genes are uniquely expressed. For example, early in development, srGAP2 is expressed in the developing cortex (Guerrier et al., 2009), and WRP appears to be expressed along the subventricular zone (SVZ), potentially in a population of stem-like cells (Il-Hwan Kim, Duke University, manuscript in preparation). srGAP2 is also more broadly expressed in various organs such as the spleen and thymus, and cell types such as macrophages, dendritic cells and T-cells (Mason et al., 2011). The fourth member of the family, ARHGAP4 (also called RhoGAP C1 and RhoGAP p115), is expressed specifically in cells of hematopoietic lineage (Tribioli et al., 1996). These expression patterns support the idea that it is important to have myriad GAPs because they appear to be expressed in unique patterns throughout development and into adulthood.

But while some genes express in unique patterns, there does appear to be overlapping expression between many GAP genes. For example, macrophages express Bcr, Abr, and srGAP2, which are all Rac GAPs (Cho et al., 2007; Mason et al., 2011). Is it possible that they all regulate the same process? If so, do they act redundantly? Most likely, the answer is no, and that there are separate spatial pools of Rac that these GAPs
regulate. However, loss of Bcr and Abr indicate they might regulate migration and phagocytosis in macrophages in a combinatorial manner (Cho et al., 2007).

The disparate amount of GAPs and GTPases insinuates that a Rho GAP should be specific for one GTPase, meaning that one Rho GAP should turn off one of the 22 Rho GTPases. We know that this, however, is not the case. For example, ARHGAP9 (Rac and Cdc42) and TCGAP (Cdc42 and TC10) both show in vitro specificity for multiple Rho GTPases (Chiang et al., 2003; Furukawa et al., 2001). But while there are some examples of proteins that regulate two or more Rho GTPases, it appears that most Rho GAPs are specific for one GTPase, at least in vivo (Tcherkezian and Lamarche-Vane, 2007). It should be noted that most experiments to test GAP specificity focus on the three archetypal Rho GTPases: RhoA, Rac1, and Cdc42. A broader investigation might uncover that GAPs are less specific than we believe and could regulate signaling by several different GTPases. Additionally, not all identified Rho-family GAPs have actually been tested for their GAP specificity or activity. It is entirely possible that not all GAPs possess catalytic activity, but might act as signaling scaffolds by binding Rho GTPases. Despite the questions that remain about unstudied GAPs, our current understanding again suggests that there must be something intrinsic to the Rho-family GAPs for targeting these proteins to specific pools of active GTPases. It appears this specificity is determined by protein interaction domains contained within the GAPs.
GAPs have numerous domains used in many different combinations. As active Rho-family GTPases are localized at the cell membrane, targeting of GAPs to the membrane is an essential mechanism for regulating Rho GTPases. The ARAP family of GAPs has multiple Plextrin homology domains that bind phospholipids, while others such as MgcRACGAP, CYK-4 and the Chimaerins all have a C1-phorbol ester binding domain (Krugmann et al., 2004; Tcherkezian and Lamarche-Vane, 2007). Other proteins such as the srGAP family have IF-BAR domains that not only bind phospholipids at the membrane, but also induce membrane protrusions (Carlson et al., 2011; Guerrier et al., 2009). It should be noted that there is cross talk between some GTPases, lipids and membrane components, which would provide a feedback loop to ultimately propagate the GTPase activity cycle.

GAPs also contain domains that are important for protein-protein interaction, such as SH3 domains, which bind proline-rich regions, SH2 domains that bind phosphotyrosine and PDZ domains, which bind to specific C-terminal sequences in target proteins (Tcherkezian and Lamarche-Vane, 2007). These appear to serve as key recognition sequences for binding scaffolding proteins where Rho-family GTPases are present. For example, WRP is a Rac GAP that has an SH3 domain (Soderling et al., 2002; Soderling et al., 2007). This SH3 domain binds to the scaffolding protein WAVE-1, which is regulated by Rac activity to potentiate actin polymerization through the Arp2/3 complex (Lebensohn and Kirschner, 2009). With a mutant SH3 domain, WRP has a much
lower affinity for WAVE-1 and is less effective in regulating WAVE-1 activity (Soderling et al., 2002; Soderling et al., 2007). This, with the information above, suggests that targeting of GAPs to specific pools of Rho GTPases is mediated by membrane binding domains and protein-protein interaction domains.

As highlighted here, there are many things we presently know about Rho-family GAPs, but only half of the genes have been studied in a meaningful way. With approximately thirty GAPs still uncharacterized, there is still so much more for us to learn. Also, most of the Rho GAPs investigated are studied in terms of their regulation of RhoA, Rac1, and Cdc42. With so many Rho GTPases and so many Rho GAPs, many possibilities and questions still exist.

One question that is much more difficult to answer is how are these GAPs regulated in vivo? What are the physiological cues that stimulate GAP activation? Do growth factors, which have been shown on many occasions to activate Rho GEFs, also activate Rho GAPs? Are cycles of activating Rho GAPs paired with activating of Rac GEFs, to shift the GTPase balance? More work must be done to study these Rho GAPs in vivo. Only then will we have a chance to understand the complicated and sophisticated signaling mechanisms regulating GAPs and their GTPases.
1.2 Actin and Actin Remodeling Proteins

1.2.1 Actin

A major role of Rho GTPases is to regulate remodeling of the actin cytoskeleton. Actin is highly conserved and present in almost all eukaryotes (Pollard and Cooper, 2009). Monomeric, globular actin (G-actin) is a 42kDa ATP-binding protein that, at physiological concentrations, can undergo spontaneous self-polymerization into helical, filamentous actin (F-actin). After this polymerization, the ATPase activity of actin hydrolyzes the γ-phosphate of ATP and ADP-bound actin filaments are indicative of older filaments.

Structurally, F-actin has an intrinsic polarity: a fast growing barbed end, where subunits are rapidly added, and a much less dynamic pointed end. Filaments are stabilized, bundled or cross-linked by a number of actin binding proteins along the length of the filament or at the ends. Additionally proteins can polymerize actin filaments: some from the barbed end and some from the pointed end. Filamentous actin performs many critical functions within a cell: providing mechanical structure and integrity at focal points, allowing for intracellular transport of molecules and organelles, and generating force for movement and migration, among many others (Pollard and Borisy, 2003; Pollard and Cooper, 2009).

While actin is extremely abundant in a cell and important for a variety of processes, the amount of total actin and the ratios of polymerized and unpolymerized
Figure 1.2: Actin and Actin Polymerizing Proteins

A. Monomeric actin (Globular or G-Actin) is an ATPase, and exists in two states: ATP- and ADP-bound states. B. Filamentous actin (F-Actin) has polarity. Monomer addition occurs at the fast growing barbed end. As the filament ages, actin hydrolyses ATP. The slower growing end, where monomer addition is minimal, is called the pointed end. C. Arp2/3 complex binds the side of existing actin filaments and, with the help of WAVE or WASP, nucleates a branch at 70° angle. D. Formins are autoinhibited by intramolecular binding between the DAD and DID domains. They become active by binding of active Rho GTPases, which releases the autoinhibition. E. Formins dimerize when active and can associate with barbed ends via the FH2 domains. F. Alternatively, Formins can associate with filament sides by the FH2 domains. G. Spire nucleates actin polymerization by utilizing four Wasp Homology 2 (WH2) domains. H. Cordon-bleu utilizes three WH2 domains and a linker domain to form an actin trimer to nucleate elongation.
actin varies depending on the organism or cell type (Blikstad et al., 1978; Koestler et al., 2009; Pollard et al., 2000). And even though the overall levels of actin are stable, a large amount of work has shown that local actin levels are extremely dynamic: by localized enrichment, treadmilling or rapid cycles of polymerization and depolymerization (Ponti et al., 2004; Wu and Pollard, 2005). In lamellipodia for example, roughly fifty percent of the actin is filamentous (Koestler et al., 2009). This is a substantial pool of the monomeric actin, and as discussed above, monomeric actin can spontaneously polymerize. To counteract this, G-actin is sequestered in the cytoplasm by two proteins: thymosin-β4 and profilin. Thymosin-β4 strongly binds monomeric, ATP-actin and prevents it from polymerizing (Pollard et al., 2000). Profilin can compete with thymosin-β4 for actin binding and is the major protein that binds G-actin in the cell (Pollard et al., 2000). Profilin has a low affinity for actin filaments (Pollard et al., 2000; Schutt et al., 1993). Profilin can slow down barbed-end elongation and spontaneous nucleation of filaments, but these events still occur. Profilin has been thought to slightly lower the critical concentration for actin, and recent work shows that profilin is essential for potent actin filament elongation by formins (Kovar et al., 2006; Paul and Pollard, 2008). This work and more demonstrates that profilin is important for sequestering cytoplasmic actin and provides an efficient delivery system to locally concentrate actin to polymerize into filaments.
Several different types of proteins polymerize actin filaments in eukaryotic cells. The WASP/WAVE family of proteins nucleate actin polymerization via the Arp2/3 complex. Formins can nucleate actin polymerization and elongate filaments from existing barbed ends. A host of other cellular factors exist to regulate actin polymerization. Meanwhile, proteins such as ADF/cofilin, gelsolin and others also play a crucial role in the disassembly of actin filaments and their networks. The interplay between the assembly and disassembly by these cellular factors is absolutely fascinating and new mechanisms and pathways are constantly being discovered. Additionally how this balance is regulated by signaling through upstream factors, like Rho GTPases, is particularly interesting and still somewhat unclear.

1.2.2 Arp2/3 and Arp2/3-activating proteins

Arp2/3 complex was the first major actin nucleator to be identified. The complex binds the side of existing actin filaments to induce actin polymerization at a 70° angle. It is a complex of seven proteins: ARPC1-5, Arp2 and Arp3. Arp2 and Arp3 subunits are similar to actin, both at the sequence and structural levels, and form a heterodimer. Nucleation promoting factors (NPFs) like WASP and WAVE, discussed below, bind monomeric actin and deliver actin to the Arp2 and Arp3 subunits. This forms a trimer, mimicking an actin seed necessary to stimulate polymerization (Goley and Welch, 2006; Kelleher et al., 1995; Robinson et al., 2001). Arp2 and Arp3 are ATP binding proteins that
bind the G-actin delivered from a NFP, which initiates nucleation. The ARPC subunits provide additional structural functions, discussed below.

On its own, the Arp2/3 complex is incapable of stimulating actin polymerization. However, factors such as WAVE and WASP family proteins are regulated by Rac and Cdc42, respectively, and activate the Arp2/3 complex to induce nucleation (Campellone and Welch, 2010; Lebensohn and Kirschner, 2009). WAVEs (three family members) and WASPs (two family members) possess three C-terminal domains that are sufficient for Arp2/3 activation: 1) WASP homology 2 (WH2) or verprolin domain which binds G-actin; 2) Connecting region that potentially induces an activating conformational change; 3) Acidic region that binds Arp2/3 (Goley and Welch, 2006). The WCA/VCA regions of WASPs/WAVEs bind to the Arp2, Arp3 and nearby ARPC1 and ARPC3 subunits (Weaver et al., 2002; Zalevsky et al., 2001). Other Arp2/3 complex subunits ARPC2 and ARPC4 make contacts with the mother actin filaments (Goley and Welch, 2006). It appears that the Arp2/3 complex prefers to bind to the sides of ATP-actin filaments, which suggests that it may nucleate closer towards the barbed end of the mother filaments (Ichetovkin et al., 2002). ATP-binding to Arp2 and Arp3 subunits is also essential for effective activation of the Arp2/3 complex and hydrolysis of ATP is important in branch disassembly (Martin et al., 2006; Martin et al., 2005).

To promote actin branch initiation, cortactin can form a complex with the Arp2/3 complex and the WAVE/WASP proteins (Soderling, 2009). As a branch is formed,
WAVE/WASPs dissociate from the branch and cortactin can stabilize the Arp2/3 complex at the branches to inhibit dissociation (Weaver et al., 2002). Cortactin has been shown to activate Arp2/3 complex-mediated actin nucleation but this activity is much less potent that the WAVE/WASP proteins. Cortactin also competes with Coronin 1B for Arp2/3 complex binding. Coronin 1B displaces Arp2/3 complex from branch points and can accelerate actin filament turnover (Cai et al., 2008).

While Coronin 1B has been shown to regulate turnover of Arp2/3, very few mechanisms are understood for turnover of the activity of the nucleation promoting factors, WAVE and WASP. One possibility for regulating turnover is to regulate the activating Rho GTPase signal. One protein, WAVE-associated Rac-GAP Protein (WRP), has been identified as terminating the Rac signal at WAVE-1 (Soderling et al., 2002). Physiologically, the consequence of regulating Rac signaling through a WRP-WAVE complex is important for regulating hippocampal spine density, learning and memory in mice (Soderling et al., 2007). One interesting question that remains is how WRP affects the biochemical ability of WAVE to activate Arp2/3 to induce actin nucleation and branching and the possibility of WRP-induced turnover of WAVE-1.

Branched actin induces membrane protrusions, is found in a variety of cellular processes, including dendritic spines, laemellipodia, phagocytic and endocytic cups, and appears to be essential for life (Goley and Welch, 2006; Pollard and Borisy, 2003). Loss of Arp2/3 complex by genetic methods is embryonically lethal very early on in a number of
developmental systems. On a cellular level, RNAi knockdown of ARPC3 in HeLa cells is also lethal (Harborth et al., 2001). Loss of nucleation promoting factors such as WASP and WAVE results in a variety of disease states in mice and humans, including immunodeficiency and mental retardation (Soderling et al., 2007; Soderling et al., 2003; Thrasher and Burns, 2010; Yamazaki et al., 2003; Yan et al., 2003). These studies highlight the essential role of branched actin initiation, maintenance and turnover.

1.2.3 Formins

Formins are a large family of actin binding proteins that, unlike the Arp2/3 complex, polymerize unbranched actin filaments. Conserved from yeast to mammals, the 15 mammalian Formin genes are divided into 7 groups to make up this diverse family. Formin-1 was originally identified in 1990 as a gene mutated during limb deformity. Since then the family of formins have been characterized as binding to the barbed end and sides of F-actin to elongate, bundle and sever actin filaments (Harris and Higgs, 2006; Harris et al., 2004; Kovar, 2006; Mass et al., 1990; Woychik et al., 1990). Formins are modular proteins with several key domains. The N-terminal Rho GTPase binding domain (GBD) is responsible for initiating activation by Rho GTPases. The dimerization domain allows for homo-dimerization, and while it is believed the functional unit of the formin is a dimer, recent data may contradict that (Nezami et al., 2010; Otomo et al., 2010; Xu et al., 2004). Formins also possess two unique domains called the Diaphanous inhibitory domain (DID) and Diaphanous autoregulatory domain
(DAD), which bind each other and hold the protein in an autoinhibited state (Li and Higgs, 2003).

Two hallmark domains of formins are the Formin-homology domain 1 and 2 (FH1 and FH2). The FH1 domain is a proline-rich region that is responsible for binding profilin-sequestered G-actin. Profilin is essential for efficient barbed-end actin elongation by formins (Kovar et al., 2006). The FH1 domain increases the local concentration of profilin-actin and the increase in profilin binding sites is proportional to polymerization rates (Paul and Pollard, 2008). The FH2 domain, which also has dimerization sequences, binds the barbed end of actin filaments, adds monomers to the barbed end, and can also associate with actin filament sides (Harris and Higgs, 2006; Harris et al., 2004). It is still unclear how actin delivered to the FH1 domain via profilin is incorporated into the growing actin filament.

Of the 15 mammalian formins, not all have been characterized in a meaningful way. The most well studied genes are the mouse Diaphanous sub-family: mDia1, mDia2, and mDia3. mDia1 is activated by Rho and has potent actin nucleation and elongation activities (Kovar et al., 2006; Lammers et al., 2005). mDia1 activity is inhibited by binding to phospholipids or a cellular factor called Diaphanous interacting protein (DIP) but the exact mechanisms for their regulation is still unclear (Eisenmann et al., 2007a; Ramalingam et al., 2010). One interesting study also shows that mDia1 could be regulated by another formin called INF2, which demonstrates there is dynamic
regulation of formins not only by other protein families but by each other (Sun et al., 2011). mDia1 knockout exhibits multiple hematopoietic phenotypes, including myelodysplastic syndrome and lymphopenia, and T-cells lacking mDia1 poorly adhere to extracellular matrix and do not migrate in response to stimuli (Eisenmann et al., 2007b; Peng et al., 2007). mDia2 and mDia3 are less well characterized, but it appears their activities are also regulated by Rho, and also the GTPases Rac, Cdc42 and Rif (Lammers et al., 2008; Pellegrin and Mellor, 2005; Peng et al., 2003). mDia2 induces filopodia formation downstream of Rif and its activity can be inhibited by WAVE2, demonstrating that Formins and Arp2/3 promoting factors can antagonize each other (Beli et al., 2008). This data suggests that the equilibrium between branched and unbranched actin is important in a cellular context and that direct binding of these actin regulatory proteins regulates this balance.

Another formin sub-family that has been investigated is the formin-like (FMNL) genes: FMNL1, FMNL2 and FMNL3. FMNL1 (also called FRL1 and FRLα) is activated by Rac1 and is unique among formins because it can not only polymerize actin but also bundle and sever actin filaments by binding filament sides (Harris and Higgs, 2006; Harris et al., 2004; Seth et al., 2006). Expressed highly in hematopoietic cells, FMNL1 regulates migration in macrophages and knockdown of FMNL1 reduced efficiency of phagocytosis by roughly fifty percent (Seth et al., 2006; Yayoshi-Yamamoto et al., 2000).
In T-cells, activation and cytotoxic cell killing is also impaired during FMNL1 knockdowns (Gomez et al., 2007).

While a number of other formins exist, they are largely uncharacterized. More importantly, cellular roles of most formins are still unknown. Also, it appears that several formins signal together to regulate the same cellular process, which can complicate our understanding of unique formin signaling (Gomez et al., 2007; Sun et al., 2011). One way we can dissect cellular functions of formins is to identify formin binding proteins and the signaling pathways they regulate. Additionally, few mechanisms exist to regulate Formin inactivation (Chesarone et al., 2009; Eisenmann et al., 2007a; Quinlan et al., 2007). Identification of new regulatory proteins will give new insight into the in vivo functions of formins.

1.2.4 WH2-containing proteins

New classes of actin polymerizing proteins have been recently identified. The WH2-domain containing proteins have multiple WASP Homology 2 (WH2) domains in tandem that bind G-actin to induce unbranched actin polymerization. They do this by bringing multiple actin subunits together to nucleate a filament (Campellone and Welch, 2010). Spire, Cordon-bleu (COBL), and leiomodin are three mammalian factors that make up this new family.

Spire was originally identified in D. melanogaster, and subsequently, two orthologues have been identified in mammals (Quinlan et al., 2005). Spire contains a C-
terminal FYVE domain and four WH2 domains. These WH2 domains, along with a G-actin-binding linker region, bind four actin monomers and can nucleate actin polymerization (Bosch et al., 2007; Quinlan et al., 2005). The precise mechanism for nucleation is still unclear, but it does appear that Spire can nucleate filaments that have free barbed ends. Conflicting data exists as to whether Spire binds barbed or pointed ends of actin filaments, but data does suggest that Spire can sever actin filaments and inhibit profilin-actin dependent polymerization (Bosch et al., 2007; Quinlan et al., 2005). Additionally, Spire can cooperate with formins to stimulate potent actin assembly. Spire-binding inhibits nucleation by formins while potentiating nucleation by Spire (Quinlan et al., 2007). Spire can also potentiate actin polymerization by mDia1, which utilizes profilin-actin (Bosch et al., 2007). These data suggest that Spire is able to nucleate polymerization in complex with formins, and subsequently allow formins to processively elongate the filament utilizing profilin-actin (Campellone and Welch, 2010).

Cobl is a newly-identified vertebrate-specific gene that contains three WH2 domains (Ahuja et al., 2007). Cobl binds barbed ends of actin filaments to prevent disassembly. Two WH2 domains appear to hold two actin monomers in a dimer and the third, more distant WH2 domain promotes lateral interactions with a third monomer to nucleate polymerization. Recent work suggests that Cobl regulates actin cap formation in ciliated epithelial cells and loss of Cobl leads to a deficit in motile cilia formation in zebrafish tissues (Ravanelli and Klingensmith, 2011).
Leiomodins have been identified as regulators of actin polymerization in skeletal and cardiac muscle (Chereau et al., 2008; Conley, 2001). Leiomodin 2 nucleates actin filaments and does not affect elongation rates (Chereau et al., 2008). A precise mechanism for leiomodin-dependent nucleation is still unclear, but it is predicted that the three G-actin binding domains stabilize an actin trimer and the lone WH2 domain reorients the third monomer to allow for barbed end elongation (Campellone and Welch, 2010).

1.2.5 Actin severing proteins

Just as numerous protein families enhance actin assembly, proteins also regulate actin turnover by stimulating disassembly. Several protein families are known to perform this function and this discussion will focus on two of these: cofilin/ADF and the gelsolin superfamily.

The cofilin/ADF family is composed of small, single domain proteins and has three members: Actin depolymerizing factor (ADF), cofilin-1, and cofilin-2. Cofilin-2 expression is restricted to developed muscle tissue. ADF message is present postnatally in epithelial and endothelial cells at low levels. Cofilin-1 is the predominate actin severing protein of this family, as it is ubiquitously expressed at the highest levels and, unlike other members of the family, knockout of cofilin-1 is embryonically lethal (Gurniak et al., 2005; Van Troys et al., 2008). Based on cryo-electron microscopy, cofilin binds along the length of actin filaments and inserts between the binding cleft of two
actin monomers (Van Troys et al., 2008). Additionally, cofilins have a higher affinity for older filaments containing ADP-actin and it has been shown to accelerate the release of inorganic phosphate from ADP-Pi subunits (Van Troys et al., 2008).

Cofilins behave differently at different ratios of actin:cofilin, as observed using TIRF microscopy (Andrianantoandro and Pollard, 2006). Low concentrations of cofilin effectively severe actin filaments, while moderate concentrations of cofilin stabilize filaments. High concentrations of cofilin actually appear to nucleate polymerization of actin filaments by stabilizing small seed filaments. This work provides an interesting model in which a concentration gradient will shift the balance from severing to nucleating (Van Troys et al., 2008). Additionally data shows that slightly raising levels of cellular levels of cofilin enhances motility and but overexpression of cofilin decreases migration, indicating that tight regulation in cells is important (Bamburg and Bernstein, 2010).

Phosphorylation of Serine3 inactivates cofilin. When this phosphorylation is removed by the phosphatase Slingshot, cofilin can then bind and sever filaments (Huang et al., 2006). It is believed that cofilin severs filaments using a “cooperative strand separation” whereby it weakens the inter-filament strand interactions and removes single-stranded actin (Bamburg and Bernstein, 2010).

The gelsolin protein family is comprised of seven members: gelsolin, adseverin, villin, capG, advillin, supervillin, and flightless I (Silacci et al., 2004). All members
contain up to six gelsolin-like repeat domains and some members have additional
domains, such as nuclear localization signals or leucine-rich repeats (Khurana and
George, 2008). Ca$^{2+}$, pH, phosphoinositides and phosphorylation regulate the activity of
these proteins. Gelsolin is normally autoinhibited and Ca$^{2+}$ binding induces a
conformational change, allowing for association with actin filaments and severing
activity. Also, it appears that phosphorylation potentiates the severing capability
(Kumar and Khurana, 2004). Villin has been shown to also nucleate and bundle actin
filaments (Khurana and George, 2008). As gelsolin-family members sever actin
filaments, they stay associated with the barbed end to cap the filament. Phosphoinositide binding displaces them from filaments. Loss of villin or gelsolin
decreases cell migration and overexpression has the opposite effect (Khurana and
George, 2008). Additionally, loss of gelsolin in mice results in a thickening of bones and
inability to form osteoclast podosomes, an adhesion structure that is responsible for
degrading and remodeling the extracellular matrix (Chellaiah et al., 2000).

1.3 srGAP (WRP) Family of Rho GAPs

The srGAP family of proteins is the focus of study in our lab. There are four
members: srGAP1, srGAP2 (also called FNBP2), WRP (also called srGAP3 and MEGAP),
and ARHGAP4 (also called RhoGAP C1 and p115 Rho GAP). All four genes encode for
an N-terminal Inverse F-BAR domain (IF-BAR), a GAP domain (discussed above), and a
C-Terminal SH3 domain. These domains coordinate to localize the Rho GAPs to the
Figure 1.3: srGAP Family of Proteins

All members of the family have N-terminal IF-BAR, GAP, and SH3 domains. The IF-BAR binds the membrane. The GAP domain binds Rho GTPases to stimulate hydrolysis of GTP. SH3 domains bind poly-proline ligands in downstream effectors. The table above indicates GAP and SH3 binding specificity.
membrane via the IF-BAR domain where they can inactivate specific pools of Rho GTPases. The SH3 domains bind downstream effectors that are activated by or signal to Rho GTPases. The srGAPs provide an interesting and excellent area of focus: little is known about their biochemical and cellular functions. Also, at the organismal level, only WRP has been studied in a mouse model. The discussions below focus on what is known about these GAPs and their domains.

1.3.1 F-BAR and IF-BAR domains

Targeting proteins to the membrane can be an essential mechanism to initiate their activation. Additionally, subcellular targeting plays a role in regulating distinct cellular processes at the membrane, such as filopodia formation, endocytosis and exocytosis. Certain proteins have developed sophisticated membrane binding domains that not only bind specific phospholipids, thereby accumulating in specific subcellular spaces, but also function to deform the membrane. One such protein family is called the F-BAR domain-containing family.

Originally identified in yeast as having homology to the Bin-Amphiphysin-Rvsp (BAR) proteins, the first member of the Fes/CIP-4 homology-BAR (F-BAR) domain family is Cdc15 (Nurse et al., 1976). Subsequently, work on Cdc15 mutants demonstrated that it is critical for actomyosin ring formation during cytokinesis (Fankhauser et al., 1995). It was the first work linking F-BAR proteins to actin, a now well-recognized signaling connection. Most F-BAR containing proteins have SH3
domains (described below), and it appears that F-BAR domains can be inhibited by intramolecular interactions with C-terminal SH3 domains (Aspenstrom, 2009; Rao et al., 2010). While not all have SH3 domains, F-BAR-containing proteins can also have Rho GTPase binding, Rho GAP, SH2, tyrosine kinase, C1, and WW domains. F-BAR domain modules have been identified in yeast, Dictyostelium, C. elegans, Drosophila, and mammals.

F-BAR domains form a banana-shaped structure, bind phospholipids at the membrane and induce membrane protrusions. Some F-BAR proteins like FBP17 induce inward membrane tubulation as seen during endocytosis, while proteins of the srGAP family induce outward membrane protrusions, similar to filopodia. These outward-inducing F-BAR proteins share functional homology with Inverse-BAR domains (I-BAR) but share more structural homology with the F-BAR proteins. We have termed these domains Inverse F-BARs or IF-BARs (Carlson and Soderling, 2009; Carlson et al., 2011). These evaginations can contain actin, but actin is not necessary for their formation (Aspenstrom, 1997; Guerrier et al., 2009; Kamioka et al., 2004). These domains are also responsible for dimerization and it has also been suggested that the F-BAR domains are responsible for oligmerization, leading to tubule formation (Aspenstrom, 2009; Fricke et al., 2010).

Two of the most well characterized F-BAR proteins are FBP17 and Toca-1. These proteins bind N-WASP using their SH3 domains and mediate N-WASP activation by
recruiting N-WASP to curved membranes (Takano et al., 2008). This work indicates that in addition to deforming the membrane, F-BAR domain-containing proteins provide additional functions in the cell by regulating actin signaling pathways.

1.3.2 SH3 domains

Protein recognition modules are important for a number of reasons, one of which is to convey specificity to protein-protein interactions. Specificity is determined by the structure of two factors: a binding factor and its ligand. The most abundant ligand in metazoans is the proline-rich motif (Castagnoli et al., 2004). These are partially hydrophobic sequences enriched in the amino acid proline, which if strung together as a repetitive proline motif, lacks secondary structure and is capable of breaking an $\alpha$-helix or $\beta$-sheet (Li, 2005). As a sequence that changes secondary structure, it is typically exposed on the surface of a protein. This unique and exposed sequence provides a wonderful protein recognition sequence, and since it can lack structure, also allows for some promiscuity among binding partners. Thus proline-rich regions are ligands for multiple protein-protein interaction domains.

The major proline-rich binding domains are called SH3 and WW domains. SH3 domains are the most prominent proline-binding domain in eukaryotes and will be the focus of this discussion (Zarrinpar et al., 2003). SH3 domains are globular domains containing around sixty amino acids with a narrow binding pocket that can typically recognize eight amino acids of the ligand. At the center of the eight residues is typically
a PXXP sequence, where P is a proline and X is any amino acid. Residues outside of the PXXP motif also contribute to binding specificity of the SH3 domain. This is particularly true for the case of one SH3 domain that was recently crystallized: srGAP1.

The srGAP1 SH3 domain shares over 90% sequence homology with srGAP2 and WRP SH3 domain and it has several atypical residues around the ligand binding pocket (Li et al., 2006). This contributes to a narrower and shallower pocket, which means that the srGAPs tend to bind more proline-rich peptides (five or more continuous prolines). This is exactly what is seen in the case of WRP – WAVE-1 and srGAP1 – Robo binding: both WAVE-1 and Robo have stretches of many prolines that the SH3 domains recognize (Li et al., 2006; Soderling et al., 2002; Wong et al., 2001). Additionally, it was determined that since the binding pocket is so tight, the srGAP SH3 domains are particularly sensitive to the amino acids adjacent to the prolines and prefer acidic amino acids (Li et al., 2006).

srGAP1 and WRP were identified by screens for Robo-1 and WAVE-1 binding proteins (Soderling et al., 2002; Wong et al., 2001). These interactions were both mediated by the SH3 domains. By identifying the SH3 specificity of these proteins, the functions of these proteins were characterized and placed into a cellular pathway. This suggests that further characterizations of this protein family should be initiated by determining SH3 domain binding specificity.
1.3.3 srGAP1

srGAP1 was the first member of the srGAP family to be characterized and is predominantly expressed in the lungs and central nervous system (Wong et al., 2001). srGAP1 was identified as a Robo1-binding protein through a yeast two-hybrid approach. Robo1 is a single-pass transmembrane receptor important in cell guidance. Slit ligand binds the Robo receptor, acting as a chemorepulsive cue and causing the cell to migrate away from the ligand (Dickson and Gilestro, 2006). In this ligand-bound state, Robo1 undergoes a conformational change that allows for srGAP1 to bind using the C-terminal SH3 domain (Wong et al., 2001). Upon binding, the GAP domain can stimulate Cdc42 GTPase activity, turning off actin polymerization to mediate cell repulsion from the Slit gradient. These data suggest that upstream signaling regulates the binding of srGAP SH3 domains to ligands and that regulation of Rho GTPases by srGAPs is spatially and temporally coordinated.

1.3.4 WRP

WAVE-associated Rac GAP (WRP) was identified as a WAVE-1 binding protein via an immunoaffinity purification – mass spectrometry approach (Soderling et al., 2002). WRP, via its SH3 domain, binds the proline-rich region (PRR) of WAVE-1. WAVE, is a scaffolding protein, which is activated by Rac and PI3 to stimulate Arp2/3-dependent actin polymerization (Lebensohn and Kirschner, 2009). WRP inactivates the Rac signal through the GAP domain. This Rac-GAP activity of WRP is stimulated by the
binding of PIP2 to the IF-BAR domain (Unpublished data, Soderling Lab, Duke University). It is unclear if WRP-binding to the PRR directly regulates Arp2/3-mediated polymerization by WAVE-1, and a precise mechanism and consequence for the regulation of Rac by WRP in this system is still unknown.

In vivo models of WRP and WAVE demonstrate that this complex regulates important processes during neuronal development, as both are highly expressed in the brain, particularly in the hippocampus. Loss of WRP or WAVE-1 in mice leads to deficits in learning and memory (Carlson et al., 2011; Soderling et al., 2007; Soderling et al., 2003). Additionally, a knockout mouse model of WRP shows decreased dendritic spine density in primary cultures and in vivo. Rescue and structure-functional experiments linked this phenotype to the ability of the WRP IF-BAR domain to induce filopodia-like protrusions in dendrites (Carlson et al., 2011). This loss of dendritic filopodia and the subsequent deficit in spine density is associated with mental retardation in the WRP mouse. Corroborating research demonstrates that humans lacking the WRP gene present with a severe form of mental retardation called 3p- syndrome (Endris et al., 2002; Gunnarsson and Foyn Bruun, 2010).

1.3.5 srGAP2

srGAP2 has been identified as a regulator of migration and neurite branching in the developing cortex of mice (Guerrier et al., 2009). Expression of srGAP2 in the brain is enriched during cortical migration of neurons from the sub-ventricular zone and peaks
at post-natal day 1. Loss of srGAP2 using shRNA in culture and \textit{in vivo} decreases branching of leading processes and neurites, but increases cell migration rates. Overexpression of srGAP2 increases branching and impedes migration. Results also show that this effect is mediated directly by the IF-BAR domain, as expression is sufficient to induce filopodia-like structures in non-neuronal cells and neurite branching in cortical neurons. However, no work was done to discern the role of the SH3 domain in this process.
2 Materials and Methods

2.1 Cell Culture

HEK293T cells were cultured in DMEM supplemented with 10% FBS. HeLa cells were cultured in MEM supplemented with 10% FBS, non-essential amino acids and sodium pyruvate. RAW264.7 cells were obtained from Duke Cell Culture Facility and cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin and supplemented with 10% RAW264.7 conditioned media. Culture and maintenance for primary macrophages are discussed below. Transfections were performed using calcium phosphate for HEK293T cells, and Lipofectamine 2000 (Invitrogen) as per manufacturer’s protocol for HeLa cells.

2.2 Plasmids and Constructs

A Formin-like 1 (H. sapiens, Uniprot O95466) construct was kindly provided by Daniel Billadeau Lab (Mayo Clinic). A portion of the gene was cloned from I.M.A.G.E. clone 5729432 into the FMNL1 construct. This resulting full-length cDNA (a.a. 1-1100) product was subsequently cloned into pcDNA3.1D-TOPO-V5His (Invitrogen). N-terminal GFP fusion was made by subcloning GFP into pcDNA3.1D-FMNL1-V5His. M. musculus (Uniprot Q9JL26) FMNL1 WT (a.a. 1-1094)-GFP, FMNL1 L1062D-GFP and FMNL1 N-terminus (a.a. 1-450)-GFP in pAS were generously given by Michael Rosen Lab (UT Southwestern). Mutations in srGAP2 (H. sapiens, Uniprot O75044) were made
using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) as per manufacturer’s protocol.

2.3 Antibodies for Staining and Immunoblots

For immunoblots, immunoprecipitations, and immunostaining, antibodies were used against FlagM2 (1:1000-1:2000) (Sigma; F3165), GST conjugated-horseradish peroxidase (1:10,000) (HRP) (Bethyl; A190-122P), V5 (1:500-1:1000) (Invitrogen; 46-0705), GFP (1:1000-1:2000) (Abcam; ab13970, or Invitrogen; A11122), Actin (1:10,000) (Sigma; A5441), Rac1 (1:1000) (BD Transduction; R56220), FMNL1 D14 (1:500) (Santa Cruz). FMNL1 and FMNL3 antibodies generously provided by Dr. Daniel Billadeau (1:1000-1:2500) (Mayo Clinic) (All concentrations listed are for immunoblots). A rabbit srGAP2 antibody was raised and purified against an SH3-domain fusion protein. HRP-conjugated secondary was purchased from GE Life Sciences and fluorescent secondary antibodies were purchased from Invitrogen. All western blots are representative images from at least three different experiments.

2.4 Immunoprecipitations and Pulldowns

HEK293T were transfected with constructs described above using calcium phosphate for 12-48 hours. Cells were rinsed with PBS and lysed with ice cold Lysis Buffer (25mM HEPES, pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% TritonX-100) with AEBSF, leupeptin, and pepstatin (RPI Corp). Lysate was precleared by centrifugation and antibodies were added to supernatant with Protein A or G agarose beads (Millipore)
or V5-antibody agarose (Sigma) at 4°C. For GST pulldowns, cells were lysed, precleared
by centrifugation and GST proteins were added with glutathione sepharose (GE Life
Sciences). After incubation, beads and associated proteins were washed three times with
Lysis Buffer with 1M NaCl and one additional wash with Lysis Buffer at 4°C. Sample
buffer is added to beads and western blots are performed. For PDGF-stimulated cells,
HEK293T cells were transfected with calcium phosphate overnight. Cells were rinsed
with PBS and placed in serum free DMEM overnight. Cells were rinsed with PBS and
PDGF-BB (Chemicon) in PBS was added at 10ng/mL for 15 minutes at 37°C. Cells that
were un-stimulated remained in PBS. Cells were lysed and immunoprecipitations were
performed as described above.

2.5 Immunostaining

Cells (excluding invasion assasys) were prepared for microscopy by fixation in
4% PFA with 4% sucrose in PBS for 10-20 minutes at room temperature. Cells were
permeabilized with 1xPBS with 0.2% TritonX-100 for three minutes at room
temperature. Cells were rinsed twice with 1xPBS. Cells were blocked with 5% Goat
Serum in TBS with 0.2% TritonX-100 for 30 minutes to one hour. Antibodies in blocking
buffer were incubated on coverslips for 30 minutes to one hour. Coverslips were washed
three times for five to ten minutes with 1X TBS with 0.2% TritonX-100. Secondary
antibodies (all AlexaFluor-conjugated secondaries from Invitrogen) were diluted 1:500
in blocking buffer and incubated for 30 minutes while covered. Coverslips were washed
three times 10 minutes with 1x TBS with 0.2% Triton X-100. DAPI was added to the first wash. Coverslips are rinsed in water, blotted dry, then mounted with FluorSave Reagent (Calbiochem, San Diego, CA). Images were taken on a Zeiss LSM 710 or 510. All images were acquired using a 40X/1.3 oil immersion objective, 63X/1.4 NA oil immersion objective for the 710 and 10x/0.30 420340-9901: EC Plan-Neofluar, NA: 0.3, air, WD: 5.2mm dry objective for the 510. Maximum image projections were also made in ImageJ and Metamorph from confocal z-series images. Additional image preparation was done using Adobe Photoshop.

2.6 Yeast Two-Hybrid

The SH3 domain of srGAP2 was cloned into pLexNA vector and this vector was transformed into the L40 yeast strain. A mouse embryonic (Day 9.5-10.5) cDNA library was screened for SH3 binding partners using the yeast two-hybrid assay as described (Hollenberg et al., 1995). Positive colonies were cured of the pLexNA-SH3 vector and retransformed with various baits described below to determine specificity of interaction using 3-aminotriazole and β-galactosidase activity. The clones with the strongest and most specific activity for srGAP2 were then sequenced.

2.7 Protein Production and Purification

GST and 6xHistidine tagged proteins were purified from BL21 E. coli as previously described (Westphal et al., 2000). Full-length srGAP2-V5 for in vitro GAP assays was expressed in HEK293T. Cells were lysed with Lysis Buffer, lysate was
precleared and srGAP2-V5 was purified using anti-V5-conjugated agarose beads (Sigma) as previously described (Soderling et al., 2002). FMNL1-C (M. musculus amino acids 449-1094) was purified as previously described (Harris et al., 2004). Briefly, FMNL1-C in pGEX-KT was expressed in BL21-DE3 E. coli, and after induction with isopropyl-1-thio-β-D-galactopyranoside, protein was extracted via sonication. Supernatant after ultracentrifugation was loaded onto glutathione-sepharose 4B (Amersham Biosciences) column, washed, and then cleaved with thrombin (Sigma) for one hour. The eluted protein was further purified via FPLC using a SourceS15 chromatography column (Amersham Biosciences). FMNL1-C containing fractions were pooled and dialyzed into 50mM KCl, 1mM MgCl₂, 1mM EGTA, 10mM Imidazole pH 7.0, 1mM DTT and 0.01% sodium azide. The protein was snap frozen using liquid nitrogen and stored at -80°C. Actin was purified from rabbit skeletal muscle (Spudich and Watt, 1971) and gel filtered on S200 gel filtration column (Amersham Biosciences) (Harris et al., 2004). AlexaFluor488-labeled actin was purchased from Invitrogen.

## 2.8 Production and Purification of Profilin

A poly-L-proline column was prepared as follows. 1g CNBr-activated sepharose (Sigma) was added to 50mL 1mM HCl and incubated at room temperature for one hour. 12mg of poly-L-proline was dissolved in 5mL Coupling buffer (500mM NaCl, 100mM NaHCO₃, pH 8.0). Transfer resin solution to bottle top vacuum filter and wash with 200mL of 1mM HCl. Wash with 200mL Coupling buffer. Transfer resin to 50mL conical
using spatula or resuspend resin in a small volume with the vacuum off and transfer using a pipette. Pellet resin and resuspend in poly-L-proline solution. Incubate overnight at 4°C. Add Blocking buffer (200mM Glycine, pH 8.0), and incubate for 2 hours at room temperature. Transfer slurry to column and drain. Wash with 20mL Coupling Buffer. Wash with 20mL PBST. Store at 4°C in PBST with 0.02% NaN₃. 

Profilin in pDest17-6xHis vector in BL21 E. coli was grown in LBamp at 37°C overnight. Dilute the next day to 1% in new LBamp and grow at 37°C until OD600 between 0.7 and 0.9. Add IPTG to 0.5mM. Grow for another 2 hours and spin down. Store pellet in -80°C. Thaw pellet at 37°C and resuspend in lysis buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 2mM DTT, and protease inhibitors). Sonicate. Spin lysate at 18,000rpm in SS34 at 4°C for 30 minutes. Place supernatant over poly-L-proline column. Wash with 50mL wash buffer (10mM Tris-HCl, 1mM EDTA, 100mM NaCl, 2mM DTT). Wash with 50mL wash buffer + 250mM Urea. Elute with 20mL wash buffer with 2M Urea. Pool samples that have protein and dialyze into storage buffer (10mM Tris-HCl pH8.0, 150mM NaCl, 1mM EDTA, 1mM DTT).

2.9 Actin Severing Assays

Recombinant 6xHis-srGAP2 SH3 was purified from E. coli (BL21) using Nickel Nitrilo-Triacetic Acid (NTA) agarose (Qiagen, Valencia, CA) at 4°C. Purified protein was dialyzed into 1xKMEI (50mM KCl, 1mM MgCl2, 1mM EGTA, 10mM Imidazole pH 7.0) with 1mM DTT. Assays were performed as previously described (Harris et al., 2004).
Briefly, actin was polymerized for 1 hour in 1xKMEI in G-Mg Buffer (2mM Tris pH 8.0, 0.5mM DTT, 0.2mM ATP, 0.1mM MgCl₂, 0.01% sodium azide). FMNL1-C and srGAP2 SH3 were diluted in 1xKMEI in G-Mg Buffer with 0.2mM nonaethylene glycol monododecyl ether (Thesit)(Sigma). Actin filaments were incubated with FMNL1-C and srGAP2 SH3. The reaction was stopped by adding rhodamine phalloidin (Invitrogen) and diluted into Dilution Buffer (25mM Imidazole pH 7.0, 25mM KCl, 4mM MgCl₂, 1mM EGTA, 0.5% methylcellulose) supplemented with 250mM NaCl, 100mM DTT, 3mg/mL glucose, 100mg/mL glucose oxidase, and 18mg/mL catalase. The solution was placed onto a 18mm coverslip coated with poly-L-lysine and imaged on a Leica DMAR2 microscope. 10-15 images per coverslip were taken of random fields. Filament lengths were quantified using MetaMorph software (Molecular Devices). Percent severing was calculated for each experiment from fractions of filaments greater than 9 microns \(1 - \frac{F_{FMNL1}-F_{FMNL1}}{F_{actin}-F_{FMNL1}}\)*100, and from fractions of filaments less than 3 microns \(\frac{F_{actin}-F_{FMNL1}}{F_{FMNL1}-F_{actin}}\)*100; where \(F=\)fraction of filaments, \(F_{FMNL1}=\) Average fraction of filaments with FMNL1 alone, \(F_{actin}=\) fraction of filaments with actin alone. Data was plotted using Prism software (GraphPad).

For TIRF imaging of actin severing, assays were performed as previously described (Cai et al., 2008). Briefly, imaging chambers were made using glass coverslips attached to glass slides with Parafilm strips. NEM-inactivated myosin is wicked through the chamber, followed by Superblock (Pierce), then 1xKMEI. 30% AlexaFluor 488-
labeled actin was polymerized in 1xKMEI/G-Mg for 30 minutes at room temperature and placed on ice. FMNL1-C was diluted to working concentrations in Imaging Buffer (16mM Imidazole pH 7.0, 40mM KCl, 0.8mM EGTA, 1.6mM MgCl₂, 200mM DTT, 6mg/mL dextrose, 5mg/mL methylcellulose, 40µg/mL glucose oxidase, 20µg/mL catalase, 0.4mM ATP). Actin filaments were diluted in Imaging Buffer to concentrations described below and loaded into imaging chamber using a cut pipette tip. After 5 minutes, FMNL1-C was added and images were acquired on Leica AM TIRF MC. Image were processed using ImageJ, version 1.42q.

2.10 Preparation of NEM-Treated Myosin

Dialyze 400µL of chicken skeletal muscle myosin (Sigma, M-7266 at 7.9mg/mL or 18µM assuming MW of 430kDa) for 2 hours against 10mM Imidazole pH 7.0, 500mM KCl, 10mM EDTA pH8.0 at 4°C. Remove protein from dialysis and place into microfuge tube. Add 5µL of 100mM N-ethylmaleimide (NEM, Sigma, 04260, MW 125.13), which has been diluted in dialysis buffer, DMF, or DMSO. Incubate on ice for one hour. Add DTT to 1mM. Incubate on ice for one hour. Dialyze overnight with 50% glycerol in 10mM imidazole pH 7.0, 500mM KCl, 10mM EDTA, 1mM DTT. Remove dialyzed protein and, if necessary, add some of the dialysis buffer to increase the volume and dilute the protein. Determine concentration by BCA or Bradford assay. Store small aliquots at -20°C. When using the NEM-inactivated myosin in assays, you would like to
use it at final concentrations of 0.2µM but optimal concentration and dilution will have to be determined empirically.

### 2.11 Actin Filament Binding and Bundling Assays

Actin was polymerized in 1x NaMEI buffer (50mM NaCl, 1mM MgCl2, 1mM EGTA, 10mM Imidazole pH 7.0, 0.2mM Thesit) in G-Mg Buffer at room temperature for one hour and stabilized with unlabeled phalloidin (Invitrogen). FMNL1-C, srGAP2 SH3, and actin filaments were added to concentrations described below in 1xNaMEI/G-Mg and spun at 80,000 rpm in TLA100 rotor for 20 minutes at four degrees for high speed actin filament binding assays. For low speed actin bundling assays, proteins were added to 1.5mL tube and spun at max speed in tabletop centrifuge for fifteen minutes. Supernatant was removed, concentrated using Speed-Vac, and resuspended in sample buffer. Pelleted proteins were resuspended in sample buffer. Samples were subjected to SDS-PAGE. The pelleted proteins and concentrated supernatant samples were run and separate gels, with the supernatant gels having 10X NaMEI in sample run in empty lanes. Gels were stained as per manufacturer’s protocol using Flamingo Stain (Bio-Rad). Fluorescent gels were scanned using Typhoon 9400 phosphoimager (GE Healthcare, Piscataway, NJ).
2.12 Pyrene Actin Assays

Protein was prepared as described above. Each condition was set up in triplicate, meaning three wells for each condition. Each experiment was run three times. Each well contains a final volume of 120µL.

For bulk polymerization assays, mix up actin at 7.5µm, 5% pyrene in 8mL of G-Buffer or whatever amount needed for experiment. If 7.5µm stock is prepared, when diluted 40µL is diluted into 80µL, the final concentration will be 2µm actin (see below). If using profilin, add to actin stock in 1:1 ratio. All other proteins, GST-srGAP2 SH3, 6xHis srGAP2 SH3 and FMNL-C, are diluted to 1.5x what the final concentration should be and brought up to volume with Dilution Buffer (1.5xKMEI in G-Mg Buffer with 0.5mM Thesit). This means that a 1.2µm stock of FMNL1-C should be prepared so the final concentration will be 800nm. Serial dilutions should be made from this stock. If using srGAP2 and FMNL1-C in same well, pre-mix proteins in stock. 80µL of each stock solution (or DB or G-Buffer) was added in triplicate to wells of black-bottomed 96 well plate (Costar).

Combine 1360µL pyrene actin + 170µL G-Mg Buffer + 170µL 10mM EGTA, 1mM MgCl2 (10E/1M), mix and add 40µL immediately to each well. Start recording using plate reader (Tecan Microplate Reader in Higgs Lab or Optima Plate Reader in Wright Lab). Excitation = 365nm; Bandwidth 10. Emission = 410nm, Bandwith 20. Make sure to optimize Z-position and Gain. For Tecan it is possible to autoinject solution into wells,
but for Optima Plate Reader you may need to add using multichannel pipette. Make sure that the program is already set up and ready to go so that once you add the actin, you can start acquiring data as soon as possible.

For actin elongation assays, polymerize 5mL of 10µM actin for at least 30 minutes in 5 TLA120 tubes at RT or equivalent tubes to get that approximate volume. Add G-Mg, dilute 10xKMEI to 1x, and add unlabeled actin and incubate for 30 minutes. After this incubation, add G-Mg, 10x KMEI to 1x, and 2µL unlabeled phalloidin (5mM in EtOH, Sigma). Centrifuge at TLA120 for 20 minutes at 100K rpm at 4°C. Carefully remove supernatant and extremely gently wash pellet with 1mL of 3xK50MEI/G-Mg. Resuspend each pellet in 800µL of 3xK50MEI/G-Mg by pipetting up and down with P200 tip. Pool samples and shear with 2 passages through 27 gauge needle. During pipetting and shearing, try to avoid bubbles. Store 1.25µM actin filaments overnight at room temperature.

In the morning, set up pyrene actin stock: 1.5µM, 10% pyrene in G-Buffer (Final concentration will be 0.75µM). Dilute Formin and other proteins in 4x stocks (400nM stock gives 100nM final concentration). Add 37.5µL solution of Formin in DB (Same as above except supplement with NaCl so final concentration in well is 250mM), Formin+SH3 in DB, or SH3 alone in DB. Add 37.5µL of preformed filaments using cut pipette tip. Avoid bubbles. Mix 1618µL pyrene actin stock with 82µL 20E/2M. If not using autoinjector, make up less actin stock. Dispense 75µL of actin stock into wells.
2.13 GAP Assays and PAK Pulldowns

*In vitro* GAP assays were performed as previously published (Soderling et al., 2002). Briefly, 300ng of purified recombinant GST-RhoA, -Rac1, or -Cdc42 was loaded with 10mCi of [γ-32P]-GTP in 20mM Tris-HCl (pH 7.6), 0.1mM DTT, 25mM NaCl, and 4mM EDTA. Rac and [γ-32P]-GTP were incubated at 30°C for 15 minutes. [γ-32P]-GTP loaded Rac was stabilized with 17mM MgCl₂ and placed on ice. 3μl of [γ-32P]-GTP loaded Rac mixture was added to sample containing partially purified srGAP2-V5 protein from HEK293T cells on V5-agarose beads in 20mM Tris-HCl (pH 7.6), 0.1mM DTT, 1mM GTP, and 1mg/ml BSA and incubated at 30°C. After 2 minutes, the reaction was stopped with 1ml of ice-cold stop buffer containing 50mM Tris-HCl (pH 7.6), 5mM NaCl, and 5mM MgCl₂. The solution was filtered through a 0.45μm nitrocellulose filter (VWR International, West Chester, PA), and washed three times with 10ml ice-cold stop buffer. Samples were read in a Tri-Carb 2100TR liquid scintillation analyzer (Canberra, Meriden, CT).

Cellular Rac activity assays in HEK293T cells were performed as previously published (Soderling et al., 2002). Briefly, cells were transfected with pcDNA3.1D-srGAP2-V5His using calcium phosphate. After 48 hours, cells were rinsed with PBS and lysed with MLB (25mM HEPES pH 7.4, 150mM NaCl, 1% NP-40
Substitute, 10mM MgCl₂, 1mM EDTA, 2% glycerol). Lysate was incubated with recombinant GST-PAK and glutathione beads for 1 hour at 4°C. Pulldown was washed twice with MLB. Sample buffer was added to protein beads and western blots were performed.

2.14 Induction and Isolation of Peritoneal Macrophages

Make 4% Thioglycollate broth from powder (Sigma). To do this, add enough thioglycollate powder to make an 8% solution in ddH₂O and stir. You can use a low heat to help the powder get in solution. Autoclave. Add sterile 2xPBS to 8% solution once it has cooled. Store in aliquots at 4°C and protect from light. Inject mouse, age 6 weeks or older (typically between 8 and 12 weeks), with 0.5mL 4% Thioglycollate Broth. After 72 hours, euthanize mouse and harvest macrophages, as described below.

Using 27 gauge needle, inject 5mL cold 30% sucrose solution in PBS into peritoneum. Inject at a 45° angle to the skin, just off midline. The peritoneum should inflate in a uniform manner. If not, the solution has been injected to the stomach, intestines or some other organ. Remove the syringe and re-inject 5mL again, making sure to inject directly into the peritoneum. Palpate or gently agitate the belly, sides and back areas to make sure the solution has reached all areas of the peritoneum and that the sucrose solution is homogenous. Using a 5mL syringe with a 20-gauge needle, aim slightly off midline at a 45° angle and pierce the skin, aiming away from organs. Tilt the mouse slightly to the side where the needle has been introduced. Gently aspirate. As
you aspirate, most likely fat will clog the syringe and you will not be able to aspirate any more. Gently push the plunger in and carefully move the needle around in the peritoneum to avoid the fat. If done well, you should be able to aspirate all of the solution containing peritoneum exudate cells (PECs), which after 72 hours should be predominately macrophages.

If you have been unsuccessful with the syringe method, you may return the mouse to its back. Carefully cut a tiny incision through the skin and open the peritoneum. The opening should be small enough to fit a 5mL pipette and no larger. It also helps to elevate the mouse where the incision is made, to retain solution in the peritoneum. If left on its back, the solution will leach out of the hole and you will lose your cells. Use a 5mL pipette to aspirate the rest of the solution from the peritoneum.

Keep isolated cells on ice. Centrifuge for 5-10 minutes at 1500 rpm. Wash once with 10 mL PBS. Resuspend in 10mL RPMI containing 10% FBS and 20% L929-conditioned media. Plate into 10cm dish and store at 37°C with 5% CO₂. Macrophages should adhere in as little as 2-4 hours.

To passage or transfer macrophages, rinse plate with PBS and add 2 mL PBS to plate and place in the refrigerator for 10 minutes. Then use a rubber scraper to gently dislodge the cells from the plate. Centrifuge, resuspend in media and use as needed.

**2.15 2D Matrix Degradation by Macrophages**

Peritoneal macrophages should be isolated one day prior to assay as described.
AlexaFluor 488-conjugated gelatin was prepared using Alexa Fluor 488 Protein Labeling Kit (Invitrogen, A10235). Provided protocol was followed. To summarize, Pig Skin Gelatin (Sigma, G1890) was resuspended at 2mg/mL in 1xPBS, while rocking at 37°C for 2 hours or until in solution. Filter using 0.2µm Steriflip or similar product. 50μL of 1M sodium bicarbonate (pH 9.0, provided with kit) was added to 0.5mL of 2mg/mL gelatin solution. Warm a vial of AF488 dye to room temperature and add the 0.55mL protein solution to the dye tube. Stir while covered at room temperature for 1 hour. Assemble column as described in manual and load resin into column until it reaches the top. Add protein solution when finished mixing, allow it to load onto column then start washing with 1X Elution buffer (provided, 1xPBS + sodium azide). Collect protein as it is eluted (the first fluorescent band). Store AF488-labeled gelatin at 4°C for up to 2-3 weeks. Labeled protein can also be frozen as aliquots in -80°C, but that was not done for these studies.

Rinse cleaned 12mm glass coverslips with PBS twice in 24 well plate. Add poly-L-lysine solution (250µL of 1mg/mL in 0.1M Borate Buffer, pH 8.5) to coverslips and rock for 20 minutes at room temperature. Wash three times with PBS, five minutes each. Add 500µL 0.5% gluteraldehyde (Sigma, G7526) in 1xPBS to coverslips and rock for 15 minutes at room temperature. Wash 3 times with PBS, 5 minutes each. Dilute AF488-labeled gelatin 1:7 into fresh, filtered gelatin solution prepared as described above. The protein solution will now be 1/8 AF488-labeled gelatin. Place a 40µL spot of the gelatin
solution onto parafilm and invert the glass coverslip onto the gelatin such that the poly-L-lysine treated side is touching the gelatin. Cover. Incubate at room temperature for 15 minutes. Invert coverslip back into 24-well plate and wash three times with PBS for five minutes. Add 500µL of a 5mg/mL solution of Sodium Borate in PBS to each well. Cover and rock for 15 minutes at room temperature. Wash coverslips three times with PBS for five minutes. Add 1mL 70% ethanol to each well to sterilize coverslips and incubate for 3 minutes. Remove ethanol and let well dry out for a few minutes. Rinse out well and coverslip with media. Add fresh media and store in incubator until ready to use.

Add 50,000-100,000 peritoneal macrophages to coverslips in RPMI + 10%FBS + 20% L929-conditioned media. After 24 hours, fix with 4% PFA, 4% sucrose in PBS for 10 minutes at room temperature. Permeabilize with 0.2% Triton X-100 in 1xPBS for 3 minutes. Block for 30 minutes to 1 hour with 5% Goat Serum in TBS + 0.2% Triton X-100 (TBST) (Blocking buffer). Add primary antibody in blocking buffer for 30 minutes at room temperature. Wash three times 5 minutes with TBST. If only using phalloidin, omit the primary antibody step and dilute 1:2000 for Rhodamine Phalloidin or 1:50 for AF647 Phalloidin (Invitrogen) into blocking buffer. Incubate same as primary antibody. Wash three times 5 minutes with TBST, then rinse with water and mount on slide with Fluor Save Reagent. If using secondary antibody add at 1:500 for all Alexa Fluor conjugated secondaries in 5% Goat Serum in TBST for 30 minutes at room temperature. Phalloidin can also be added in this step. Wash once with TBST containing DAPI for 5 minutes.
Wash three times with TBST for 10 minutes each. Rinse coverslips with water and mount using FluorSave Reagent.

2.16 Preparation of Radiolabeled Protein for Peptide Array Overlay

Prepare and purify protein as needed. Thaw ATP. Wash desalting column (Pierce, 20439) with 10mL TBST. Mix 2µL 10X CAK Buffer (500mM MOPS, 500mM NaCl, 20mM MgCl2, 10mM DTT, pH 6.8 and stored as aliquots in -20°C) with 1µL PKA catalytic subunit, 4µg of protein of interest, 5µL P32γ with water to 20µL. Incubate at 37°C for 1 hour. Add reaction to desalting column and wash with 1.5 mL TBST. Collect approximately 100µL fractions and dilute 1µL of each sample in scintillation fluid and measure using the scintillation counter. Pool fractions that are over 50,000cpm. Highly labeled protein fractions should be well above this. Dilute radiolabeled protein into 1% Blotto (5% milk, 1% BSA in TBST with 0.02% NaN3) and incubate with pre-blocked peptide array overnight at room temperature. Wash blot four times with TBST for 10 minutes and continue as you would a western blot.

2.17 Isolation of Bone Marrow Cells (Macrophages)

Euthanize mouse and spray thoroughly with 70% ethanol. Cut skin around ankle and then up length of leg. Pull skin to expose muscle. Carefully trim muscle up entire length of leg and remove leg (femur and tibia) being extremely careful not to cut off top of femur. If top of femur is removed, the marrow is no longer sterile and should not be
used for cell culture, but could still be processed for immediate use, such as FACS. Carefully trim off remaining muscle and tendons using scissors and cheesecloth wetted with 70% ethanol. Be firm in removing muscle but be mindful to not break bones. Place cleaned bones in PBS or RPMI on ice; a 6 well plate works well for this purpose.

When all bones have been removed, move to tissue culture hood. Remove PBS or RPMI in wells, and replace with 70% ethanol. Incubate for 2-3 minutes while preparing equipment for dissections. Exchange 70% ethanol with PBS twice. Move bones for one mouse into a new petri dish with 5-7mLs fresh PBS. Hold bone using forceps/tweezers and carefully separate femur from tibia by cutting towards end of femur. Then, still using forceps, hold the femur with bone marrow exposed over a 50mL conical tube. Take a 10mL syringe filled with warmed RPMI+10%FBS with a 27-guage needle and place needle into marrow. Flush out bone with media. Reverse orientation of bone and repeat until fairly clean. You should see the marrow, which is red, come out of the bone, which should now be clear (white) and hollow. Repeat for the tibia.

Centrifuge marrow cells for 10 minutes at a low speed. Remove supernatant and resuspend pellet with 2-3 mLs of RPMI +10% FBS with a 20-guage needle and syringe. Plate cells evenly into two to three plates of RPMI + 10% FBS + 20% L-929 conditioned media. Replace media every 2-3 days. When replacing media, leave 1mL plate and do not use the vacuum. Always use pipette to remove or add media. Cells should be ready
to use after 5-7 days. If splitting cells is necessary, very gently scrape cells and centrifuge and re-plate at density needed.

If interested in isolating dendritic cells, use the protocol same as above except culture cells in GM-CSF instead of L929-conditioned media, which contains M-CSF.

2.18 3D-Invasion of Bone Marrow Macrophages

Isolate bone marrow macrophages as described above.

Chill all pipette tips at 4°C. Thaw Matrigel (BD) on ice until liquefied, approximately one hour or up to overnight in an ice bucket at 4°C. Add 150µL 1mg/mL poly-L-lysine in 0.1M Borate Buffer (pH8.5) each well of BD Culture Slide. Incubate at room temperature for at least twenty minutes. Dilute Matrigel 1:1 in FBS and store on ice. To make nutrient-rich matrix, add components in the following ratio: 1µL of 2.5mg/mL M-CSF (R&D), 1µL 10mg/mL AF488 monomers in 1xPBS solution (Invitrogen) and 8µL Matrigel:FBS solution on ice. Prepare one or two extra wells worth of matrix solution. Add 6µL to each well of 8-well chamber slide (BD) on ice. Use pipette tip to spread even level of matrix across each well, taking precaution to not introduce bubbles. If bubbles do occur, remove by aspiration. Place matrix and slide into incubator for one hour. After one hour, remove slides from incubator and add 24µL of Matrigel containing 1mg/mL AF488 monomers final concentration to the top of the adherent, nutrient-rich matrix. Place in incubator and solidify for thirty minutes to one hour. After incubation, add phenol red-free RPMI to each well and return to incubator for one hour.
Prepare bone marrow macrophages and 10,000 cells in 350µL into each well in RPMI + 0.5% FBS. Incubate four to five days, which allows enough time for invasion into matrix.

For subsequent steps use 300-400µL solution per well and use caution and patience when removing solution from each well. Fix with 4% PFA, 4% sucrose in 1xPBS for 20 minutes at room temperature. Rinse with PBS and proceed with staining or store in fresh PBS at 4°C, wrapped tightly. Permeabilize cells with TBS with 0.5% TritonX-100 for 10 minutes at room temperature. Rinse wells three times with PBS/Glycine buffer (130mM NaCl, 7mM Na₂HPO₄, 3.5mM NaH₂PO₄, 100mM Glycine) for 10 minutes each. Incubate each well with IF Buffer (130mM NaCl, 7mM Na₂HPO₄, 3.5mM NaH₂PO₄, 7.7mM NaN₃, 0.1% BSA, 10% Goat serum, 0.2% TritonX-100, 0.05% Tween-20) for 45–60 minutes. Add Rhodamine Phalloidin in IF Buffer (1:500) and incubate, covered, at room temperature overnight. Wash once with IF Buffer for twenty minutes and three times with PBS for ten minutes. Add DAPI in PBS and incubate for 15 minutes. Rinse once with PBS for five minutes. Carefully remove plastic wells from slide using apparatus provided. Be extremely careful to not remove any matrix that may be attached to the wells.

Very gently dip the slide into water to rinse and tilt onto a Kimwipe to dry. A Kimwipe can be used to carefully dry between wells but do not use a vacuum. Add one drop of pre-warmed FluorSave Reagent (15 minutes in 37°C water bath) to each well,
even wells that are empty. Gently place cover glass onto slide without introducing bubbles. Use vacuum to remove extra FluorSave that may leak out. Store at room temperature to dry out for one to two days.

2.19 Phagocytosis

Prepare beads for phagocytosis, modifying protocols previously described (Chow et al., 2004; Yates and Russell, 2008). Add 60µL (60µg) 3µm diameter latex beads (Sigma) and 3mg (60µl) Human IgG (Sigma) into 3mLs 1xPBS. Incubate 1 hour rocking at 37°C. Rinse three times with cold 1xPBS. If not using fluorescently labeled beads, stop here and store beads in 1mL PBS + 5µL of 5% NaN₃ solution and store at 4°C. If labeling beads, add 10µL of a 5mg/mL of AF594-SE (AlexaFluor594 carboxylic acid, succinimidyl ester (mixed isomers)) in DMSO. Make sure to open the tube of AF594 only after it has completely thawed. Incubate, while rocking, at room temperature. Wash three times with PBS and store in 1mL PBS + 10µL of 2% NaN₃ solution at 4°C.

Once isolating peritoneal macrophages as described above, plate 250,000 cells onto 12mm glass coverslips in RPMI + 10% FBS + 20% L929-conditioned media. Incubate overnight. Wash cells once with PBS and replace solution with cold RPMI + 10% FBS + 20% L929-conditioned media containing. Place cells in fridge and incubate for 10 minutes. Spin plate in centrifuge at 4°C for 10 minutes at 750xg.

Remove cold media, and replace with warmed RPMI + 10% FBS and 20% L929-conditioned media. Fix now for time zero or place in incubator to allow for later time
points. At designated time to terminate assay, rinse once with ice cold PBS and fix with cold 4% PFA, 4% sucrose in 1xPBS for thirty minutes. Rinse with PBS and do not fix cells. Block using Superblock (Pierce) or another buffer without detergent. Stain using αHuman IgG-AF488 (1:100) (Invitrogen) in Superblock for one hour at room temperature. Wash three times with PBS for ten minutes each. Mount coverslips using FluorSave Reagent.
3 Bi-modal regulation of a formin by srGAP2

3.1 Introduction

Remodeling of the actin cytoskeleton is a tightly controlled process that directs cellular functions including cell migration, adhesion, polarity, and membrane trafficking. Many studies suggest that the regulation of actin filament assembly and disassembly must be coordinated by a complex interplay between multiple cellular signaling pathways (Chesarone and Goode, 2009; Pollard and Borisy, 2003; Soderling, 2009; Weaver et al., 2003). One signaling pathway that plays a prominent role in regulating actin is the Rho-family GTPase pathway, which is typified by Rho, Rac, and Cdc42 (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). Rho-family GTPase signaling pathways are inactivated by Rho GTPase activating proteins (GAPs) and activated by Rho Guanine nucleotide exchange factors (GEFs). Once activated by GEFs, Rho-family GTPases bind to and modulate the action of actin binding proteins such as the formins. The mammalian formin family is composed of 15 different members, which suggests that they have widespread roles for regulating distinct actin processes (Chesarone and Goode; Higgs, 2005; Kovar, 2006). However, there is some commonality to the molecular mechanisms regulating the Diaphanous-related Formins (DRFs) (Goode and Eck, 2007; Higgs, 2005; Kovar, 2006). DRFs are autoinhibited by an intra-molecular interaction between an amino-terminal Dia-inhibitory domain (DID) and a carboxy-terminal Dia-autoregulatory domain (DAD). Activated Rho-family GTPases disrupt this
autoinhibition by binding to the GTPase-binding domain (GBD) and DID region (Otomo et al., 2005; Rose et al., 2005). This induces the release of the DID-DAD interaction, opening the formin so that the Formin Homology 2 (FH2) domain can associate with the barbed end of actin filaments to protect them from capping proteins and allow processive elongation. In addition to actin filament elongation, some formins, such as Formin-like 1 (FMNL1, also called FRL1 and FRLα), have unique activities, including severing and bundling actin filaments by associating with filament sides (Esue et al., 2008; Harris et al., 2004). Thus, members of the formin family have multiple actin remodeling activities.

In vitro, formins polymerize actin filaments that are much longer than those found in vivo or sever filaments into very small fragments. Because formin activity appears to be exaggerated in vitro compared to their activity in vivo, it is clear that formin activation must be tightly regulated. Additionally, it is estimated that some formin activity cycles last less than 5 seconds in vivo (Chesarone et al., 2009). In vitro, however, formin activity can persist much longer (Kovar et al., 2006). This suggests that cellular mechanisms must exist to turn "off" formin activity, to counterbalance Rho-family GTPase-induced activation of formins. Along these lines, several factors have been identified that appear to inhibit formin activity. These include Bud14p in yeast, the Drosophila Spire, and the mammalian DIP/WISH; however, none of these regulates
formin activity at the level of the Rho-family GTPases (Chesarone et al., 2009; Eisenmann et al., 2007a; Quinlan et al., 2007).

Here, we report an interaction between the formin FMNL1 and the RhoGAP family member srGAP2 (Slit-Robo GAP family member 2). This complex forms via binding between the FH1 domain of FMNL1 and the SH3 domain of srGAP2. This binding is temporally regulated by the Rac-mediated activation of FMNL1. Additionally, srGAP2 functions as a selective Rac GAP when compared to Cdc42 or RhoA. Finally, actin filament severing assays show that the srGAP2 SH3 domain also directly inhibits FMNL1 actin severing activity. Together, our data suggest two novel mechanisms for srGAP2-mediated regulation of FMNL1, including GAP domain-mediated regulation of local Rac signaling to FMNL1 and steric/allostERIC inhibition of actin severing by FMNL1.

3.2 Results

3.2.1 srGAP2 binds Formin-like 1, Formin-like 3, and Afadin

The srGAP family consists of srGAP1, srGAP2, WRP, and ArhGAP4, all of which contain a conserved N-terminal Inverse F-BAR domain, a central Rho-family GAP domain and a C-terminal SH3 domain (Guerrier et al., 2009; Soderling et al., 2002; Wong et al., 2001). The srGAP1 SH3 domain binds the Robo1 receptor in response to Slit binding and down-regulates Cdc42 activity (Wong et al., 2001). The WRP SH3 domain binds the proline-rich region of WAVE-1. WAVE-1 is activated by Rac and binds Arp2/3
to induce branched actin polymerization. The activation of WAVE-1 by Rac can be terminated by WRP, a Rac-selective GAP (Soderling et al., 2002). This interaction is important for the development of dendritic spines, synaptic plasticity, and memory retention (Soderling et al., 2007). Together, these findings suggest that the srGAPs coordinately regulate Rho-family GTPase complexes and pathways using a common mechanism: SH3 domain binding to proline-rich ligands in downstream effectors. To identify ligands for SH3 domains of other srGAP family members, we performed a yeast two-hybrid screen of a mouse embryonic day 9.5-10.5 cDNA library using the SH3 domain of srGAP2. This initial screen identified 58 clones, which were sequenced for identification. Of these, one clone represented the intracellular tail of Robo-2, confirming previous findings that srGAP2 can bind members of the Robo receptor family (Wong et al., 2001). Two clones corresponded to proline-rich regions of the nectin- and actin-binding protein Afadin (AF-6). Another clone corresponded to a portion of the proline-rich FH1 domain of Formin-like 3 (FMNL3) (Figure 3.1C). Co-IPs confirmed the interactions of full-length srGAP2 with FMNL3 and srGAP2 with AF6 (Figure 3.1A). Yeast two-hybrid analysis shows that this fragment of FMNL3 specifically interacts with srGAP2 SH3 domain (Figure 3.1B). We were particularly interested in the srGAP2-FMNL3 interaction because Formins have numerous actin signaling properties and are regulated by Rho GTPases, which are subsequently regulated by GAPs.
FMNL3 belongs to a subset of three highly homologous Diaphanous-related formins (DRFs), FMNL1, -2, and -3, that are activated downstream of Rho-family GTPases (Higgs and Peterson, 2005). This clone was re-tested for interactions with empty vector (negative control), the srGAP2 SH3 domain, the closely related WRP SH3 domain, and the unrelated SH3 domain of Nck (Figure 3.1B). Profilin was used as a positive control, as it binds the proline-rich FH1 domain of many formins. Of the SH3 domains tested, only srGAP2 interacted with FMNL3. To further analyze this interaction and to assess specificity amongst other formins, we overlaid radiolabeled srGAP2 SH3 domain onto a peptide array of 20mer peptides spanning the FH1 domains of FMNL1, -2, -3 and mDia1, -2, -3, which are variable in length (Figure 3.2A). The results showed that, although the srGAP2 SH3 domain could bind to FMNL3, it preferentially bound peptides within the FH1 domain of FMNL1. No binding was detected between srGAP2 and FMNL2 or members of the mDia family. Western blots were used to determine the tissue distribution of FMNL1, FMNL3 and srGAP2. All three are expressed in similar tissues, but srGAP2 (which often migrates as two distinct molecular masses on SDS-PAGE, presumably due to proteolysis) and FMNL1 are preferentially co-expressed within the brain and thymus (Figure 3.2B). To confirm the initial two-hybrid results, co-immunoprecipitations were performed between recombinant full-length proteins expressed in HEK293T cells (Figure 3.2C). Compared to a negative control (Figure 3.2C, lane 1), srGAP2 readily co-immunoprecipitated with FMNL1 (Figure 3.2C, lane 2),
Figure 3.1: srGAP2 binds FMNL3 and Afadin.

Fragments of FMNL3 and Afadin (AF-6) were identified in a yeast two-hybrid with the srGAP2 SH3 domain as bait. A. Full length GFP-FMNL3 and Afadin-GFP interact with full length Flag-srGAP2 via coimmunoprecipitation experiments. B. Specificity of the srGAP2 and FMNL3 interaction was verified in the two-hybrid assay. Yeast containing clone 24 and either empty vector (negative control), srGAP2 SH3, WRP SH3, Nck SH3, or profilin (positive control) were plated onto plates lacking Uracil, Tryptophan, and Leucine (-UWL), or also Histidine and Lysine (-WHULK). While all yeast could grow on the -UWL plate, indicating the yeast contain both bait and prey vectors, only the srGAP2 and profilin containing yeast grew on the -WHULK plates, confirming an interaction. Yeast were also assayed for β-galactosidase activity (LacZ), which further confirmed srGAP2 SH3 is specific for FMNL3. C. Schematic showing the fragment of FMNL3 identified in the library screen using the srGAP2 SH3 domain bait. Amino acid positions are indicated for each.
Figure 3.2: srGAP2 Forms a Complex With FMNL1.

A. Peptide array analysis of the binding specificity of the srGAP2 SH3 domain for the FH1 domains of several formins. The beginning and ending amino acid positions indicate regions synthesized as 20mer peptides offset every 3 amino acids. Binding was detected by overlay of [γ-32P]-radiolabeled GST-srGAP2 SH3. Results suggested the srGAP2 SH3 domain prefers peptides within the FMNL1 FH1 domain. B. Expression distribution of srGAP2, FMNL1, and FMNL3 as determined by western blot analysis using specific antibodies. Actin was used as a control for loading. Each panel is labeled to the right, and each tissue or cell line is labeled above each panel. RAW cells are a macrophage-derived cell line. C. Full length srGAP2 and FMNL1 interact by co-IP. Cells were transfected with GFP-FMNL1 alone (lane 1) or with Flag-srGAP2 (lane 2) or Flag-srGAP2 with a point mutation in the SH3 domain (Tryptophan 765 to Alanine; lane 3). Immunoprecipitation of FMNL1 co-precipitated wildtype srGAP2 (lane 2, top panel), but GFP alone or the SH3 mutant of srGAP2 did not (lanes 1 and 2, top panel). Immunoblot analysis of extracts indicated equal levels of srGAP2 in all three lysates (middle panel) and similar levels of FMNL1 (bottom panel). D. Endogenous FMNL1 and srGAP2 form a complex in cells. Immunoprecipitation of FMNL1 co-precipitates srGAP2 from Hela cells (lane 1), whereas α-rabbit IgG (negative control) does not (lane 2).
srGAP2 readily co-immunoprecipitated with FMNL1 (Figure 3.2C, lane 2), confirming the interaction between the full-length proteins. This interaction depended on a conserved tryptophan within the SH3 domain required for poly-proline peptide recognition (Erpel et al., 1995). Mutation of this tryptophan to alanine completely abolished the interaction (Figure 3.2C, lane 3). The binding between srGAP2 and FMNL1 was further confirmed by co-immunoprecipitation of the endogenous proteins (Figure 3.2D). Immunoprecipitation of FMNL1, using an FMNL1-specific peptide antibody, co-precipitated srGAP2 from HeLa cell extracts (Figure 3.2D, lane 1), while negative control rabbit IgG did not (Figure 3.2D, lane 2). Collectively, these results show that srGAP2, via its SH3 domain, forms a complex with FMNL1, binding specific proline-rich peptides within the formin FH1 domain.

3.2.2 srGAP2 regulates signaling between Rac and FMNL1

Because srGAP2 is a GTPase-activating protein, we tested the GAP specificity of full-length srGAP2 towards the prototypic Rho-family GTPases (Figure 3.3A-C). We tested the binding preference of srGAP2 with all three GTPases in a GST-pulldown assay (Figure 3A). Lysates from cells expressing recombinant srGAP2 were incubated with constitutively active GST-Rho, -Rac, or -Cdc42 bound to beads. Western blot analysis of pelleted proteins showed that srGAP2 preferentially bound to Rac, with some interaction also evident for Cdc42, but not RhoA or GST alone (Figure 3.3A, Top panel). Coomassie staining of each pulldown verified equivalent amounts of each
Figure 3.3: Regulation of the Rac-srGAP2-FMNL1 Pathway.

A) GTPase specificity pulldown assay for srGAP2. Lysate from cells expressing srGAP2 (Top panel, input) were incubated with GST-fused constitutively active Rho, Rac, or Cdc42 on glutathione beads (Bottom panel, Coomassie stain). After centrifugation, bead fractions were assayed for bound srGAP2 by western blot analysis (Top panel). srGAP2 specifically associated with Rac, but did not interact with Rho or Cdc42. B) In vitro GTPase assay for srGAP2 GAP specificity. 300 ng of purified Rho, Rac, and Cdc42 were loaded with radiolabeled GTP and incubated with increasing amounts of full-length srGAP2. srGAP2 exhibited greater GAP activity toward Rac when compared to Rho or Cdc42. C) Cellular assay for srGAP2 Rac-GAP activity. Cells were transfected with empty vector or srGAP2 (bottom panel) and levels of Rac-GTP were analyzed (top panel) compared to total Rac (middle panel) by the PAK pulldown assay. Cells expressing srGAP2 had lower levels of Rac-GTP, confirming Rac GAP activity in situ. D) Activity dependent interaction of FMNL1 with Rac. Lysates (input) from cells expressing the FMNL1 GTPase binding domain (FMNL1 GBD; a.a. 1-450) were subjected to a pulldown assay using wildtype Rac or constitutively active Rac (RacCA) bound to beads as GST fusion (bottom panel). The FMNL1 GBD domain preferentially interacted with active Rac. E) Regulation of membrane targeting of FMNL1 by Rac. Cells were co-transfected with either cherry fluorescent protein (ChFP) and FMNL1-GFP (Top panels) or ChFP-Rac CA and FMNL1-GFP (bottom panels). Without active Rac, FMNL1 was predominately cytosolic, whereas with
constitutively Rac, FMNL1 was enriched in membrane ruffles where it co-localized with Rac. Scale bar represents 15 microns.

GTPase were used in the assay (Figure 3.3A, bottom panel). In *vitro* GTPase activity measurements were performed using partially purified full-length recombinant srGAP2 and purified wild-type GTPases (Figure 3.3B). Recombinant srGAP2 preferentially stimulated the intrinsic GTPase activity of Rac rather than RhoA or Cdc42. Together with the pulldown assays, this indicated srGAP2 is a Rac-GAP. To test the Rac-GAP activity of srGAP2 in cells, a PAK1 pull-down assay for cellular Rac-GTP was performed (Figure 3.3C). Lysates from HEK293T cells transfected with vector alone (Figure 3.3C, lane 1) contained more Rac-GTP than lysates from cells transfected with srGAP2 (Figure 3.3C, lane 2), confirming srGAP2 can function as a Rac-GAP *in vitro* and *in situ*.

Previous *in vitro* studies have suggested FMNL1 can bind to Rho-family GTPases, including both Rac and Cdc42, and that endogenous FMNL1 may be associated with Rac (Gomez et al., 2007; Seth et al., 2006; Yayoshi-Yamamoto et al., 2000). To confirm the Rac interaction, we performed GTPase pulldown assays with recombinant wild-type or constitutively active Rac (RacQ61L) and an N-terminal fragment containing the GTPase binding domain of FMNL1 expressed in HEK293T cells (Figure 3.3D). These results verified previous studies, indicating that FMNL1 preferentially interacts with the activated form of Rac. Prior studies have also shown that Cdc42 translocates FMNL1 to the plasma membrane, suggesting that membrane
Localization is one feature of FMNL1 activation (Seth et al., 2006). Therefore, we analyzed the localization of full length FMNL1-GFP when co-transfected with either soluble cherry fluorescent protein (ChFP) or constitutive active Rac (ChFP-Rac CA). When expressed with ChFP, FMNL1 was predominantly cytosolic (Figure 3.3D, top panels). In contrast, co-expression of Rac CA resulted in a marked translocation of FMNL1 to the membrane and dorsal ruffles, suggesting Rac can indeed interact with and activate FMNL1 (Figure 3.3D, bottom panels). Together, these results suggest that the srGAP2/FMNL1 complex can be functionally linked to Rac signaling.

### 3.2.3 Dynamic formation of the srGAP2/FMNL1 complex

The above results suggest that srGAP2 complexes with FMNL1 for one of two potential reasons: (1) to prevent Rac from activating FMNL1 (Figure 3.4A); or (2) to turn off Rac signaling after FMNL1 has been activated (Figure 3.4B). If srGAP2 impedes the activation of FMNL1 by Rac, it should constitutively bind the formin or preferentially bind the inactive conformation. Conversely, if srGAP2 functions to terminate Rac-mediated activation of FMNL1, we would predict srGAP2 preferentially binds FMNL1 that has been activated by Rac. To distinguish between these two possibilities, we assayed the interaction between the srGAP2 SH3 domain and FMNL1 in the presence of increasing amounts of constitutively active Rac (Rac CA) (Figure 3.4C). Because these experiments utilized the isolated SH3 domain, we could rule out any confounding effects of the GAP domain associating with Rac in the pulldown assay. GST-fused
**Figure 3.4: Dynamic Assembly of the srGAP2/FMNL1 Complex.**

**A-B.** Schematic of possible roles for srGAP2 in regulating GTPase signaling to FMNL1.  
**A)** In this model srGAP2 is bound to FMNL1 in the inactive state and limits the ability of Rac to activate FMNL1. This could favor the specificity of FMNL1 activation towards other GTPases such as Cdc42.  
**B)** In this model srGAP2 only binds to activated FMNL1, where it functions to turn off Rac after activation is achieved.

**C.** GST srGAP2 SH3 (bottom panel) pulldown from cells expressing FMNL1 (2nd panel from bottom) and increasing amounts of constitutively active Rac (Rac CA; 3rd panel from bottom; lanes 2-4). Whereas very little FMNL1 associated with the srGAP2 SH3 domain in the absence of active Rac (lane 1), increasing amounts of FMNL1 co-associated with the SH3 domain in the presence of increasing levels of active Rac (lanes 2-4).

**D.** Association of the srGAP2/FMNL1 complex was analyzed by co-immunoprecipitation without (lane 1) or with (lane 2) PDGF stimulation from cells co-transfected with srGAP2 (middle panel) and FMNL1 (bottom panel). Increased complex association was observed following PDGF stimulation (top panel).

**E.** srGAP2 preferentially associates with the active form of FMNL1 in cells. Cells were co-transfected with srGAP2 and either GFP tagged wildtype FMNL1 (top panels) or GFP tagged constitutive active FMNL1 (bottom panels; FMNL1 CA). Immunostaining for srGAP2 showed cytosolic and membrane staining that co-localized with FMNL1 CA (bottom panels). Inset shows higher magnification images of the corresponding boxed regions. Scale bar represents 15 microns.
srGAP2 SH3 domain pulled down very little FMNL1 from cell lysates co-expressing GFP alone (Figure 3.4C, lane 1). In contrast, the srGAP2 SH3 domain pulled down increasing amounts of FMNL1 from lysates expressing GFP-Rac CA in a dose-dependent manner (Figure 3.4C, lanes 2-4). These data suggested the srGAP2/FMNL1 complex is dynamically regulated by the Rac-mediated activation of FMNL1. To confirm this with full-length proteins, HEK293T cells were co-transfected with srGAP2 and FMNL1 and either serum starved (unstimulated) or treated with PDGF to activate endogenous Rac (Ouyang et al., 2008; Takahashi et al., 2008) (Figure 3.4D). Western blots of co-immunoprecipitations demonstrated that assembly of the srGAP2/FMNL1 complex was enhanced by PDGF treatment (Figure 3.4D, lane 2). To visualize this activation-dependent interaction, HeLa cells expressing srGAP2 were transfected with either wildtype FMNL1 or FMNL1 that is rendered constitutively active (FMNL1 CA) by a point mutation in the DAD domain (L1062D) (Seth et al., 2006). When co-expressed, srGAP2 was predominantly at the membrane while wild-type FMNL1 was largely cytosolic (Figure 3.4E, top panels). In contrast, both srGAP2 and FMNL1 CA co-localized at the membrane (Figure 3.4E, bottom panels), further supporting the idea that srGAP2 preferentially interacts with FMNL1 when it is activated. Together, the results indicate that the srGAP2/FMNL1 complex is formed in response to Rac-mediated activation of FMNL1 in vivo. The cumulative evidence is consistent with a model whereby srGAP2 acts to turn off Rac-mediated activation of FMNL1 (Figure 3.4B).
3.2.4 Both srGAP2 and FMNL1 co-localize with F-actin during Fc-γ receptor phagocytosis

srGAP2 has been characterized primarily as a neuronal GAP, where it may regulate membrane protrusions during cortical neuron migration (Guerrier et al., 2009). In contrast, FMNL1 is expressed in cells of hematopoetic lineage, where it can function to regulate either synapse formation in T-cells or phagocytosis in macrophages (Gomez et al., 2007; Seth et al., 2006; Yayoshi-Yamamoto et al., 2000). Thus, it was important to examine whether endogenous FMNL1 and srGAP2 co-localize during either of these events. Based on our finding that both proteins are expressed in the macrophage-derived line RAW264.7 (Figure 3.2B), and the known role of Rac and FMNL1 signaling during Fc-γ receptor (Fc-γR)-mediated phagocytosis (Caron and Hall, 1998; Seth et al., 2006), we focused on the potential role of the FMNL1/srGAP2 complex in these cells. One of the earliest events during phagocytosis is the formation of extensive membrane evaginations that engulf Fc-coated particles. Recent work on the membrane binding Inverse F-BAR (IF-BAR) domain has shown that the srGAP family of GAPs can be efficiently recruited to outward membrane protrusions (Guerrier et al., 2009). Thus, the possibility that endogenous srGAP2 may co-localize with FMNL1 and actin within membrane extensions during phagocytosis was tested (Figure 3.5). In cellular regions where phagocytosis was not occurring, FMNL1 and srGAP2 were mostly cytosolic and neither co-localized with actin very well (Figure 3.5A-D, 3.5F-I). However, upon stimulation of Fc-γR-phagocytosis using Fc-coated beads, FMNL1 and srGAP2 co-
localized with actin at beads undergoing phagocytosis (Figure 5A&F, closed arrowheads; B-D and G-I, boxed regions and insets). This was clearly evident in z-projections (Figure 5E&J) of the periphagosomal regions, where both FMNL1 and srGAP2 co-localized with actin in the phagocytic membranes as they surrounded the bead (dashed circle). These data indicate that one function of the FMNL1/srGAP2 complex may be to regulate actin dynamics during phagocytic cup formation, which involves both membrane evagination and actin-driven internalization downstream of Rac signaling.

3.2.5 Direct regulation of FMNL1 actin severing, but not bundling or severing by the srGAP2 SH3 domain

Our data demonstrate that upon FMNL1 activation, srGAP2 binds the FH1 domain. The C-terminus of FMNL1, including the FH1 and FH2 domains, is tightly associated with actin filaments and possesses actin severing activity (Harris et al., 2004). The binding of srGAP2 might modulate this FMNL1 activity. To test this hypothesis, we assayed the ability of the srGAP2 SH3 domain to inhibit severing by the FMNL1 C-terminus (FMNL1-C, a.a. 449-1094). First, we visualized FMNL1-C severing actin in a live, single filament assay of using TIRF microscopy (Figure 6A). In this assay, the severing of filaments by FMNL1-C was directly observed in time-lapse every ten seconds. Within one minute, most filaments were severed at multiple sites (Figure 6A, yellow arrow heads indicate new breaks within each panel), demonstrating that FMNL1
Figure 3.5: Localization of FMNL1 and srGAP2 to the Actin-Rich Phagocytic Cup During Phagocytosis

(A-E) Co-localization of FMNL1 or (F-J) srGAP2 with F-actin during phagocytosis. A, F) Differential contrast image (DIC) of a RAW cell incubated with Fc-coated beads. Closed arrowheads indicate a bead undergoing phagocytosis, open arrowhead indicates a nearby bead that is not being phagocytosed. Maximum projection images depicting (B) immuno-localization of endogenous FMNL1, (G) srGAP2, (C,H) phallodin-stained actin, or (D,I) composite images. Insets correspond to boxed regions. (E,J) Images depict composite boxed regions from (D,I). The central image, surrounded by blue, in (E,J) is a 0.25 micron section from the Z-stack. The images below and to the side of this section are orthogonal projections of the phagocytic cup. The images to the bottom (surrounded by green) represent the image projected where the green line bisects the stack. The images to the right (surrounded by red) indicate the image projected where the red line bisects the stack. The blue lines seen in the images to the bottom and right are the Z-position of the central image section. Scale bar represents 15 microns.
Figure 3.6: Regulation of FMNL1-Mediated Actin Filament Severing by the srGAP2 SH3 Domain.

A. Time-lapse images of polymerized actin filaments every ten seconds in the presence of purified active FMNL1-C (a.a. 449-1094). Numbers in the upper right corner of each frame indicates time. Extensive severing is observed during the 1 minute time-lapse period. Yellow arrowheads mark the locations of new filament severing events in each frame. B-G. Image of actin filaments (2µM) either (B) alone, (C) with FMNL1-C (400nM), or with FMNL1-C and increasing concentrations of the purified srGAP2 SH3 domain (D-G). Scale bar represents 15 microns. H-J. Quantification of filaments lengths from three independent severing assays showing the fraction of filaments (H) longer than 9 microns or (I) shorter than 3 microns for each condition. J. Percent severing as calculated from H&I for increasing concentrations of the srGAP2 SH3 domain. Scale bars represent 15 microns. K. Polymerized actin filaments (250nM, Lane 1), stabilized by phalloidin, are pelleted by ultracentrifugation in the presence of FMNL1-C (100nM) without and with srGAP2 SH3 (200nM and 400nM, Lanes 4 and 5 respectively). In the absence of actin, FMNL1-C does not co-pellet with actin filaments (Lane 2). With actin filaments, FMNL1-C does co-pellet (Lane 3) and the srGAP2 SH3 domain does not disrupt this interaction (Lane 4 and 5).
possessed potent severing activity in this assay (Harris et al., 2004). We next tested whether the binding of the srGAP2 SH3 domain modulated this FMNL1 activity in a quantitative actin severing assay (Harris et al., 2004) (Figure 3.6B-G). In this assay, actin was polymerized and incubated for five minutes with FMNL1-C, without and with increasing concentrations of the srGAP2 SH3. The reaction was stopped using rhodamine-labeled phalloidin, which inhibits FMNL1-mediated actin severing (Harris et al., 2004). The resulting filaments were imaged and lengths were quantified. F-actin alone showed filaments of varied lengths (Figure 3.6B). In contrast, FMNL1-C severed actin into predominantly shorter filaments (Figure 3.6C). This potent severing activity was inhibited by the srGAP2 SH3 domain (Figure 3.6D-G). Furthermore, the srGAP2 SH3 domain did not affect actin filaments in the absence of FMNL1, suggesting that the effects were mediated by inhibition of FMNL1 and not a direct effect on actin. To quantify severing, we calculated the fraction of filaments that were either long (greater than 9 microns, which decrease with severing) or short (less than 3 microns, which increase with severing). This data is graphically represented in Figure 6H-J. Percent of actin severing at each concentration of the srGAP2 SH3 domain was calculated by normalizing to the effect of FMNL1 alone. This demonstrated a dose-dependent inhibition by srGAP2 SH3 on FMNL1 (Figure 3.6J).

The effect of the srGAP2 SH3 domain could be mediated by either dissociating FMNL1 from the sides of actin filaments or by an allosteric or steric hindrance effect on
Figure 3.7: srGAP2 Does Not Inhibit Actin Polymerization or Elongation by FMNL1

A. 400nM FMNL1-C polymerizes 2µM Actin (5% pyrene). Increasing concentrations of srGAP2 SH3 domain do inhibit this polymerization. B. 2µM Profilin slows 2µM Actin (5% pyrene) polymerization. FMNL1-C increases polymerization from Profilin-Actin complexes. Increasing concentrations of srGAP SH3 domain do not inhibit this polymerization. C. 0.75µM Actin (10% pyrene) do not spontaneously polymerize actin under these conditions. Addition of 0.3125µM pre-formed filaments induced elongation at barbed end. FMNL1-C inhibits this elongation. This inhibition is released by addition of 0.75µM Profilin. The srGAP2 SH3 domain does not inhibit this elongation.
Figure 3.8: srGAP2 Does Not Inhibit FMNL1 Bundling of Actin Filaments.

Actin filaments are polymerized and stabilized with phalloidin to prevent actin severing. Spinning on a low speed, these actin filaments do not pellet unless bundled by addition of FMNL1-C. Addition of srGAP2 SH3 domain does not inhibit this bundling ability.
severing. The srGAP2 SH3 domain did not alter the ability of the FMNL1-C to bind actin filaments in an actin filament co-sedimenation assay (Figure 3.6K).

We performed pyrene actin assay to determine if srGAP2 may regulate actin polymerization by FMNL1-C. Pyrene-labeled actin has decreased fluorescence in the monomeric state, but when incorporated into a filament, the fluorescence increases by up to 20-fold. By using a spectrophotometer, actin polymerization can be monitored in real time to observe actin assembly. We see that FMNL1-C polymerizes actin from monomers in a bulk polymerization assay (Figure 3.7A). srGAP2 SH3 is unable to inhibit this polymerization. Profilin is capable of binding proline-rich regions of FMNL1 (Harris et al., 2004), and increased profilin binding sites is directly proportional to increased polymerization rates. We were interested in determining srGAP2 SH3 binds the same FMNL1 sequences as profilin. Indeed they do and binding is competitive (Figure 3.9). We tested to see if srGAP2 might be able to inhibit profilin-actin complexes from increasing FMNL1-mediated actin polymerization and slow polymerization rates (Figure 3.7B). We see that srGAP2 SH3 is unable to inhibit bulk polymerization by FMNL1-C utilizing profilin-actin. To determine if srGAP2 SH3 domain inhibits specifically elongation at the barbed ends, we utilized an experiment where actin polymerization requires the presence of actin filaments (Figure 3.7C). We see that srGAP2 is unable to inhibit barbed end elongation by FMNL1-C.
Figure 3.9: srGAP2 and Profilin Bind Identical Sequences in FMNL1 FH1 Domain.

Peptides from FMNL1 FH1 domain are synthesized onto nitrocellulose membranes. srGAP2 SH3 domain (100mM) or 6xHis Profilin (100mM) are overlaid onto peptides and western blots are performed. Sequences of peptides bound are highlighted in blue and red boxes. Equimolar profilin and srGAP2 SH3 (100mM each) are added to array and blotted for presence of profilin. srGAP2 can inhibit profilin binding to proline-rich peptides in vitro.
Additionally, FMNL1 has been shown to be able to bundle actin filaments (Esue et al., 2008; Harris and Higgs, 2006; Harris et al., 2004). To see if srGAP2 inhibits actin bundling, we mixed srGAP2 SH3, FMNL1 and preformed actin filaments stabilized with phalloidin to inhibit severing. Normally actin filaments do not pellet when spun at a low speed, but addition of an actin bundling protein like FMNL1 causes filaments to pellet (Figure 3.8). Addition of srGAP2 SH3 did not affect this bundling activity.

Thus, these results show that while FMNL1 can sever actin filaments, the binding of srGAP2 interferes with this activity, most likely by an allosteric or steric-hindrance mechanism. However, srGAP2 is unable to inhibit polymerization or bundling by FMNL1.

### 3.3 Discussion

In this study, we have identified a complex between srGAP2 and FMNL1 that is temporally regulated by Rac signaling and appears to regulate FMNL1 activity by two mechanisms (Figure 3.10). This complex forms by a direct interaction between the SH3 domain of srGAP2 and the FH1 domain of FMNL1. Interestingly, our data suggest the accessibility of the FMNL1 binding site is dependent on activation by Rac. While we found that srGAP2 is selective for Rac-GTPase activity and that FMNL1 could also interact with Rac, it should be noted that FMNL1 might also be regulated by Cdc42 (Seth et al., 2006). Our data show that srGAP2 likely regulates Rac signaling to FMNL1, by
A. FMNL1 is basally inhibited and cytosolic. Upon activation by Rac (B) FMNL1 translocates to the membrane and the active form of FMNL1 can bind and sever actin filaments. This potentially regulates Fc-γ receptor-mediated phagocytosis in macrophages. C. In this active state, srGAP2 can bind FMNL1 at the membrane to (1) inactivate the Rac signal and (2) inhibit the severing activity of FMNL1. This would return FMNL1 to its quiescent, inactive state. This recycling mechanism could be responsible for rapid turnover of actin filaments and Rac signaling during phagocytosis.
stimulating the cycling of Rac to its inactive state. To our knowledge this is the first description of a mechanism to terminate Rho-family GTPase-dependent activation of a formin, which has been hypothesized to be a key step in regulating formins (Goode and Eck, 2007). Furthermore, it is quite likely that GAP-mediated regulation of formins is a conserved mechanism (unpublished observations).

A second mechanism by which srGAP2 regulates FMNL1 is through direct and specific inhibition of its actin severing activity, but not polymerization, elongation or bundling. An active form of FMNL1 (FMNL1-C) containing the C-terminal FH1-FH2 domains severs and bundles preformed actin filaments, in addition to enhancing polymerization at barbed ends using profilin and actin (Figure 6 and (Harris et al., 2004)). These diverse activities elicit many questions about the function of FMNL1 in vivo, and how these activities might be regulated. For example, our work and the work of others show that actin filaments in vitro can be elongated to lengths well over 40µm, and FMNL1-C can sever these filaments into sub-micron lengths (Harris et al., 2004). Neither of these scenarios is overtly physiological, so there must be a balance established between these two; a regulatory factor must exist in vivo. Few inhibitors (DIP/WISH, SPIRE, and Bud14p) and even fewer mechanisms have been identified to inhibit formins (Chesarone et al., 2009; Eisenmann et al., 2007a; Quinlan et al., 2007). In one recent example, Bud14p has been shown to displace a yeast formin, Bnr1p, from the barbed end of actin filaments (Chesarone et al., 2009). Bnr1p is a potent elongator of actin
filaments, so displacing this protein from the barbed end provides an elegant and practical mechanism for inhibiting formins: physically displacing them from the filaments they polymerize. Interestingly, yeast genetic data suggest Bud14p does not simply oppose Bnr1p activity, but instead regulates the duration of Bnr1p polymerization.

srGAP2 is the fourth biochemical inhibitor of formin activity identified. Our data show that srGAP2 functions differently from Bud14p in that it does not displace FMNL1 from the sides of actin filaments (Figure 6K). Interestingly, it seems the FH1 domain might play an important role in regulating the adjacent FH2 domain, acting as a gas or brake pedal to the FH2 engine. For example, profilin-actin complexes accelerate elongation of filaments by formins (Kovar et al., 2006; Paul and Pollard, 2008). This effect is directly mediated by binding to the FH1 domain, acting as a gas pedal to elevate polymerization rates at the barbed end. We hypothesize that the srGAP2 SH3 domain acts as a brake, binding the FH1 domain of FMNL1 to oppose severing, through an allosteric or steric mechanism. To our knowledge, this is also the first example of a GTPase activating protein that may also modify the actin cytoskeleton architecture in a GAP-independent manner, utilizing only the SH3 domain. These data suggest that factors that bind the FH1 domain may play a critical role in regulating formin biochemistry and signaling in vivo, warranting future work to identify and characterize their functions.
The cellular mechanisms and functions for many of the mammalian formins are still unknown. FMNL1 is critical for T-cell polarization and subsequent activation, as well as Fc-γ receptor-mediated phagocytosis in macrophages, a Rac-dependent process (Gomez et al., 2007; Hall et al., 2006; Seth et al., 2006; Wang et al., 2008). Our data show that both FMNL1 and srGAP2 localize to the actin-rich Fc-γ receptor phagocytic cup in macrophages. Phagocytosis of foreign particles can occur in less than one minute, indicating that rapid polymerization and turnover of actin is essential for driving the Fc-γ receptor-dependent process (Diakonova et al., 2002). Because rapid actin polymerization is necessary, a large pool of free barbed ends of actin filaments are likely required. Elongation of these filaments may also be coordinated with Arp2/3-dependent branching, an essential driving force during the phagocytic process (Lorenzi et al., 2000; May et al., 2000). Actin severing mediated by FMNL1 could provide a mechanism for generating new barbed ends for abundant filament growth. Essential to this amplification of new filaments may also be a mechanism for the coordinated regulation of Rac activity and FMNL1 actin severing by srGAP2. Analogous to observations on the Bud14p regulation of Bnr1p in yeast, where Bud14p may promote Bnr1p activity cycling (Chesarone et al., 2009), the Rac-FMNL1-srGAP2 complex may provide a succinct mechanism to coordinately recycle FMNL1 and Rac for rapid actin turnover in the phagocytic cup.
4 The Role of srGAP2 in Macrophages

4.1 Introduction

Macrophages are indispensable members of the innate immune system and provide one of the first lines of defense in mammals. In this role, they have several critical functions. Macrophages recognize and phagocytose foreign particles, such as bacteria, clearing and then degrading these microorganisms. To do this, macrophages receive signals to migrate to an area of infection and degrade extracellular matrix to invade into tissues where the infection or area of inflammation resides. Alternatively, macrophages can also reside in a variety of tissues, such as the lungs. Once their phagocytic function is performed, they must also be able to exit the resident tissue, via degradation of the extracellular matrix, and migrate to secondary lymphoid organs such as the spleen and thymus to potentiate an inflammatory response. Thus, to function properly, macrophages must coordinate phagocytosis, adhesion, migration, and invasion. For invasion, in particular, it is essential that a macrophage be able to pass through basement membrane and the extracellular matrix.

To accomplish invasion, macrophages secrete matrix metalloproteases, such as MT1-MMP and MMP2 (Cougoule et al., 2010), through a specialized organelle called the podosome. Podosomes are actin-dense membrane protrusions that bind to extracellular matrices via integrins and allow for adhesion and focused secretion of proteases. Macrophage podosomes are functionally analogous to podosomes found in osteoclasts.
or invadopodia studied in cancer cells. However, it appears that the actin in macrophage podosomes are more dynamic than cancer cell invadopodia in culture (Evans and Matsudaira, 2006). Despite this difference, invadopodia and podosomes are comprised of many identical cellular factors that coordinate adhesion, regulate structure, and focus degradation (Albiges-Rizo et al., 2009; Linder and Aepfelbacher, 2003).

In culture, β1 integrin localizes to the core of podosomes, and β2 and β3 integrins are present at the periphery (Gimona et al., 2008; Linder and Aepfelbacher, 2003; Linder and Kopp, 2005). These receptors link extracellular matrix binding to cellular actin. Filamentous actin is oriented perpendicular to the extracellular substrate, such as gelatin, fibronectin or glass (Trotter, 1981). In addition, a diffuse “actin cloud” is present at the dorsal side or apex of the filaments, and podosomes are connected to each other by actin filaments that run parallel to the substrate (Albiges-Rizo et al., 2009; Evans et al., 2003; Linder and Aepfelbacher, 2003). The actin-nucleation promoting factors mDia2 and WASP and the Arp2/3 complex are also enriched in podosomes and are essential for their formation and maintenance (Baldassarre et al., 2006; Destaing et al., 2005; Linder et al., 1999; Yamaguchi et al., 2005). Rho, Rac and Cdc42 play a role in podosome formation as well, as constitutively active forms of these proteins induce formation of actin-rich podosomes; however, not all are capable of inducing degradation (Linder and Kopp, 2005; Tatin et al., 2006). Cdc42 mediates podosome formation through WASP (Linder et al., 2000; Linder et al., 1999), which induces branched actin formation along with
cortactin, an essential component of invadopodium (Artym et al., 2006; Weaver et al., 2002). The roles of Rac and Rho are unclear, but it is possible that Rho could signal to regulate myosin, which is essential for podosome formation (Collin et al., 2008).

In addition to actin polymerization, there is dynamic actin turnover at podosomes, particularly in macrophages (Albiges-Rizo et al., 2009; Evans et al., 2003). The half-life of actin is approximately twenty seconds (Evans et al., 2003), and actin severing proteins provide the machinery for actin turnover. One such protein, gelsolin, is present in osteoclast podosomes and is responsible for resorption of bone. Loss of gelsolin results in cortical bone thickening (Chellaiah et al., 2000). Cofilin, another well-known actin severing protein, is present in invadopodia and is essential for actin turnover (Linder and Aepfelbacher, 2003; Yamaguchi et al., 2005). A third actin binding and severing protein, Formin-like 1 (FMNL1), has also been shown to be present in podosomes (Mersich et al., 2010). Furthermore, loss of FMNL1 causes a reduction in the number of actin foci present at the adherent surface of the macrophage, but the average size of the foci is increased.

Another essential function of macrophages is the process of phagocytosis by which foreign particles are recognized and engulfed. Phagocytosis is characterized by recognition of an antigen by cell surface receptors on macrophages and membrane protrusions, such as lamellopodia and filopodia, being formed to engulf and internalize the particle. After internalization, the endosome containing the particle (called the
phagosome) is loaded with hydrolytic enzymes and the pH is lowered to kill the bacteria. Inducing membrane curvature and utilizing actin polymerization and rapid actin turnover are hallmarks of this process. Similar proteins that regulate podosome formation are also important in phagocytosis. Rac and Cdc42 are involved in generating membrane protrusions, initiating and maintaining the engulfment by activating WASP, an actin polymerizing protein, and FMNL1, an actin severing protein (Hall et al., 2006; Hoppe and Swanson, 2004; Seth et al., 2006; Thrasher and Burns, 2010). FMNL1 is important for regulation of phagocytosis, as knock-down of FMNL1 resulted in a 45% decrease in Fcγ-mediated phagocytosis. FMNL1 is thought to play a role in phagocytosis through the regulation of actin turnover during this process (Hall et al., 2006; Hoppe and Swanson, 2004; Seth et al., 2006; Wang et al., 2008). Interestingly, both Rho family GTPases and FMNL1 have been linked to the two macrophage processes of phagocytosis and adhesion. These findings, that multiple proteins are involved in the regulation of both podosome formation and phagocytosis, suggest that coordination of these two processes might be important for cellular function.

Our work has demonstrated that FMNL1 is regulated by the Rac GAP, srGAP2. srGAP2 binds FMNL1 through its SH3 domain, and this inhibits FMNL1-mediated actin severing. Additionally, Rac regulates this binding: srGAP2 prefers to bind to FMNL1 after it has been activated by Rac. We have recently generated a knockout mouse of srGAP2 to study its in vivo functions. Noting that FMNL1 has been studied in
macrophages and that losses in FMNL1 reduce Fcγ-mediated phagocytosis and induce alterations in actin foci that resemble podosomes, we investigated the role of srGAP2 in phagocytosis and podosome formation in primary macrophages.

Here, we show that loss of srGAP2 increases phagocytosis in macrophages. These results agree with recent work in *C. elegans* demonstrating that loss of SRGP-1, a homologous IF-BAR-containing Rac GAP, also increases clearance of apoptotic bodies via phagocytosis (Neukomm et al., 2011). Additionally, loss of srGAP2 has a dramatic effect in reducing podosome formation in primary macrophages. While it appears that srGAP2-deficient macrophages can respond to chemoattractant, adhere to extracellular matrix and form actin foci, these structures are much less efficient at matrix degradation. To our knowledge, this work is one of the first mouse models to exhibit loss of macrophage podosome formation and matrix degradation. This study suggests that regulation of actin severing by a Rac-FMNL1-srGAP2 pathway may be vitally important for the body’s ability to fight infection.

### 4.2 Results

#### 4.2.1 Generation of an srGAP2 knock-out mouse

To more directly determine the role of srGAP2 in phagocytosis, we have made an srGAP2 knockout mouse using the Duke BAC Recombineering Core Facility. Our strategy was to knock-in TdToma fluorescent protein (TmFP) cDNA into the third
Figure 4.1: Generation of an srGAP2 Knock-out Mouse.

A. “Knock-in” strategy for srGAP2 KO mouse. Tomato fluorescent protein (TdTomato) cDNA with a Stop Codon is inserted into the third exon of the srGAP2 gene. B. Western blots from WT (+/+) spleen and thymus organ lysates show expression of srGAP2 protein and this is decreased in the heterozygous mice (+/-) and gone in the knock-out (−/−). C, D. srGAP2 is expressed in both bone marrow-derived macrophages and peritoneal macrophages and expression is lost in the KO animals.
exon of the srGAP2 gene (Figure 4.1A). This represents the analogous exon we successfully targeted when making the WRP transgenic mouse, which was recently characterized by our lab (Carlson et al., 2011). The TmFP cDNA also encodes a stop codon at the 3’ end, which should result in translation of a very small portion of the srGAP2 gene (only 87 amino acids), followed by TmFP then a stop, essentially knocking out gene expression. We had hoped this TmFP would also be a useful tool to track expression of srGAP2 in heterozygous mice. However, it appears that TmFP expression is too low to detect in these mice (data not shown). It is also possible that the srGAP2-TmFP fusion is not very stable. While we are unable to monitor TmFP expression, srGAP2 expression is lost in the knockout (KO) mice (Figure 4.1B-D). srGAP2 KO mice are viable, live to adulthood and have no apparent gross phenotypic abnormalities.

4.2.2 srGAP2 negatively regulates phagocytosis

Knowing that srGAP2 is expressed in macrophages (Figure 4.1) and is present at the phagocytic cup (Figure 3.5), we investigated if srGAP2 might be playing a role in regulating phagocytosis in macrophages using our srGAP2 KO mouse.

To study phagocytosis, we utilized a previously described method (Chow et al., 2004; Yates et al., 2009; Yates and Russell, 2008). In this assay, 3µm latex beads are coated with human IgG so they will be phagocytosed through the stereotypic Fcγ-receptor-mediated phagocytic pathway. Beads are also coated with a fluorescent AF594 dye, so
Figure 4.2: Loss of srGAP2 Increases Phagocytosis.

A. Latex beads are coated with IgG and AF594 dye (Beads, Red) and incubated with peritoneal macrophages. Cells are fixed and stained for the IgG using αHuman IgG-AF488 secondary (External, Green) to identify beads that have not been internalized. Scale bar = 15µm. B. Quantification of phagocytosis using WT and srGAP2 KO macrophages after one hour. Three animals were used for each genotype. 489 total beads quantified for WT and 679 beads for KO. Two-tailed t-test; P=0.0096.
they can be easily visualized via fluorescent microscopy. The labeled beads are introduced to the macrophages, and after a period of time, fixed and immunostained using an α-human IgG-AF488 conjugated secondary antibody. Only external beads are immunolabeled (Figure 4.2A). This provides an easy and efficient way to determine phagocytosed versus non-phagocytosed beads.

Wild-type (WT) and srGAP2 KO macrophages were isolated from mice and subjected to the phagocytosis assay. We found that loss of srGAP2 increased the fraction of phagocytosed beads (Figure 4.2B). Initially, we were surprised by this result because we hypothesized that the IF-BAR domain of srGAP2 might be responsible for inducing membrane protrusions at the phagocytic cup. Therefore, loss of srGAP2 would result in fewer protrusions and less efficient phagocytosis. On the contrary, srGAP2 KO macrophages demonstrated increased phagocytosis.

4.2.3 Loss of srGAP2 correlates with loss of podosomes in macrophages

In addition to phagocytosis, one critical process for macrophages is to migrate through membranes. To do this, macrophages use podosomes, a cellular process that degrades the extracellular matrix with a dense actin network. This actin structure is dependent on signaling by Rho GTPases through numerous proteins, including FMNL1 and Rac. Knowing that these srGAP2 binding proteins are critical for this outward protruding process, we examined whether srGAP2 localizes to this structure.
To set up this assay, fluorescently labeled gelatin was coated onto glass coverslips (Baldassarre et al., 2003; Bowden et al., 2001), and peritoneal macrophages were seeded on top of the gelatin-coated coverslips (Figure 4.3). The absence of fluorescence correlates with matrix degradation by podosomes.

Using this assay, we were able to observe that while actin was present at the periphery of the cell, it was also localized at the center of the cell in a defined focus (Figure 4.3, 4.4). srGAP2 co-localized with the actin-rich structure that degraded the gelatin (Figure 4.4). Orthogonal views demonstrated that srGAP2 was present throughout the length of F-actin structures at the podosome. This localization is similar to the previously published localization of FMNL1 at actin puncta resembling podosomes (Mersich et al., 2010). This previous study demonstrated that FMNL1 localized around the podosomes, however it did not determine if the podosomes were functional (i.e. if the podosomes degraded matrix).

To determine if srGAP2 was necessary for matrix degradation, we isolated macrophages from WT and srGAP2 KO mice and performed matrix degradation assays. WT macrophages formed actin-rich podosomes that correlated with matrix degradation (Figure 4.3). In contrast, srGAP2 KO macrophages were much less efficient at forming podosomes (Figure 4.5). Interestingly, it appeared that srGAP2 KO macrophages were able to form actin puncta; however, in most instances, these foci were unable to degrade the gelatin.
Figure 4.3: Macrophages Degrade Gelatin on Coverslips Using Actin-Rich Podosomes.

AF488-labeled gelatin is coated onto coverslips and peritoneal macrophages are plated on top. Cells are incubated for 24 hours, then fixed and stained with phalloidin to visualize F-Actin. These images are maximum image projections of z-series. A. AF488-labeled gelatin (Green) and loss of fluorescence indicates degradation. B. Inverse of image (A) where degradation is visualized as colored puncta. C. Rhodamine Phalloidin (Red) stained cells. D. Composite of (B) and (C) with DAPI (Blue). E-G. Enlargement of cell boxed in (D). H, I. Highlight of two podosomes present in cell from (D-G). Scale bar is 15µm.
Figure 4.4: srGAP2 Localizes to Podosomes.

A. Maximum image projections from z-series of peritoneal macrophages plated onto AF-647-labeled gelatin coverslips show actin (Red, Rhodamine-Phalloidin) co-localizes with srGAP2 (Green, AF-488) and degraded matrix (Purple, AF-647, absence of color indicates degradation). Merge demonstrates that Actin and srGAP2 are present at the site of degradation. DAPI is blue in the merge. B. Orthogonal view of this z-series shows again that srGAP2 and actin localize to the site of degradation and that srGAP2 is present throughout the actin foci. Scale bar is 10µm.
Figure 4.5: srGAP2 KO Macrophages are Less Efficient at Degrading Gelatin.

A. Green puncta are areas of matrix degradation. B. Rhodamine Phalloidin staining to visualize F-Actin. C. Composite image of (A) and (B) with DAPI. Arrowhead highlights cell that actin puncta that do not correlate with matrix degradation. D. Enlargement of cell that is highlighted by box in (C). Colors are same as seen in (C). Scale bar is 15µm.
As seen in the images above, srGAP2 KO macrophages appeared to be deficient in forming podosomes (compare 4.3B and 4.5A). Therefore, we quantified differences in matrix degradation between WT and srGAP2 KO macrophages. For this analysis, we defined a podosome as an actin-rich focus associated with gelatin degradation. We quantified the number of cells with podosomes, number of podosomes per cell, and area of degradation per podosome (Figure 4.6). The mean fraction of cells with podosomes was dramatically decreased in srGAP2 KO macrophages, from 0.53±0.05 in WT macrophages to 0.15±0.02 in KO cells (Figure 4.6A). In addition to having fewer cells with functional podosomes, srGAP2 KO cells that contained podosomes averaged fewer podosomes per cell than WT macrophages (1.44±0.09 vs. 1.18±0.06) (Figure 4.6B). More srGAP2 KO macrophages had only one podosome when compared to WT macrophages, which more frequently had two or more podosomes per cell (Figure 4.6D).

Furthermore, srGAP2 KO podosomes were less capable of degrading extracellular matrix (Figure 4.6C,E). WT macrophage podosomes were capable of degrading an average area of 10.86±1.24µm² while KO podosomes, on average, degraded only 6.55±1.08µm² (Figure 4.6C). Additionally, as we dissected this data further and examined WT and KO macrophages that only had one podosome, we found an even greater difference between WT and KO macrophages regarding the amount of
Peritoneal macrophages were isolated and plated on AF488-gelatin coated coverslips. Podosomes for these analyses are defined as actin-foci that co-localize with matrix degradation. All experiments are from cells isolated from three animals of each genotype. A. Fraction of macrophages with podosomes (WT 0.53±0.05 vs. KO 0.15±0.02, P<0.001). B. Average number of podosomes per macrophage (WT 1.44±0.09 vs. KO 1.18±0.06, P=0.0335). C. Average area of degradation per podosome (WT 10.86±1.24µm², N=145 vs. 6.55±1.08µm², N=54, P=0.452). D. Fraction of cells with 1, 2, or 3 podosomes (WT 1 podosome 0.60±0.08 vs. KO 0.85±0.06, P=0.02). E. Area of degradation per podosome, when cell has 1, 2, or 3 podosomes (WT 1 podosome 13.73±2.28µm², N=72 vs. 6.72±1.52µm², N=34, P=0.047). Data is presented as Mean±SEM, P values determined by two-tailed t-test.
matrix degraded (Figure 4.6E). Finally, we noted a discrepancy between the pattern of matrix degradation seen in WT and srGAP2 KO macrophages. We found that WT macrophages with one podosome typically demonstrated one large area of degradation, and the size of this area of degradation decreased as podosome number increased. In contrast, the area of matrix degradation seen with srGAP2 KO macrophages do not vary depending on podosome number, but rather remained relatively constant (Figure 4.6E).

4.2.4 Deficiency in podosome formation leads to defects in invasion

As macrophages utilize podosomes to invade in and out of tissues, we tested to see if loss of srGAP2 would affect the ability of macrophages to invade through extracellular matrix in culture. To do this, we used a similar approach that has been previously described (Van Goethem et al., 2011). In this assay, nutrient and growth factor-rich Matrigel was plated onto a glass slide. Matrigel lacking nutrients was then layered on top. After the gel solidified, macrophages in media containing low FBS (0.5%) were plated outside the matrix. Cells will normally invade through the top nutrient-deficient Matrigel to reach the bottom nutrient-rich layer. After a period of time, cells were fixed, stained, and imaged. We used fluorescently-labeled Matrigel and stained cells using phalloidin and DAPI, and determined whether the loss of srGAP2 affected macrophage invasion using confocal microscopy.

Our results showed that srGAP2 KO macrophages were deficient in their ability invade (Figure 4.7A-G). Approximately 50% of WT Macrophages were inside the matrix,
Figure 4.7 srGAP2 Macrophages (Mφ) Do Not Invade as Well as WT Macrophages.

Bone marrow-derived macrophages are plated outside of matrix, and must invade to get nutrients. A. WT macrophages are stained with Phalloidin (red) and DAPI (blue) are used to label cells. B. AF488-containing Matrigel. C. Merge of (A) and (B) demonstrates that WT macrophages have ability to invade into matrix. D. srGAP2 KO macrophages are less able to invade into matrix. Phalloidin (red), DAPI (blue) and Matrigel (Green) are shown. Scale bars = 100µm. E. & F. Cells were quantified as either inside or outside of the matrix. E. Fraction of cells inside: WT 0.51±0.026, 5 animals. KO 0.18±0.04, 4 animals, P=0.0002. F. Fraction of cells outside matrix: WT
0.49±0.026, 5 animals. KO 0.81±0.04, 4 animals, P=0.0002. G. Distance (µm) from the edge of the matrix. Distance was quantified as distance from the nucleus to nearest edge of matrix. Distances were binned into 20µm intervals and plotted as fraction of cells in each bin. Negative values indicate cells that have migrated into the matrix. Data represents mean±SEM.

while only 18% of srGAP2 KO macrophages invaded into the matrix (Figure 4E,F). The distance that cells migrated from the edge of the matrix was also measured (Figure 4G). Positive distance values corresponded to cells outside the matrix and negative values indicated how far a cell invaded into the labeled Matrigel. These data demonstrate that the ability of cells to invade Matrigel depends on the presence of srGAP2.

4.3 Discussion

We have previously shown that srGAP2 regulates the activity of the actin severing protein FMNL1. However, no data exists regarding the cellular functions of srGAP2 in cells were FMNL1 is expressed. As FMNL1 has been shown to be expressed in hematopoietic cells and specifically required for macrophages to perform phagocytosis and regulate podosomes (Mersich et al., 2010; Seth et al., 2006), we examined whether srGAP2 played a role in mediating these processes.

These results demonstrate that srGAP2 KO macrophages are deficient at forming podosomes and degrading extracellular matrix (Figure, 4.2, 4.4, 4.6). When srGAP2 deficient macrophages do form podosomes, they predominately form only one podosome, and this podosome is much less able to efficiently degrade an extracellular matrix substrate (Figure 4.6). Additionally, these data show that most WT macrophages have at least one podosome and are capable of forming multiple podosomes, which is
consistent with other data suggesting that podosomes are dynamic structures, with macrophages typically containing many podosomes per cell (Evans and Matsudaira, 2006).

This data above demonstrates that srGAP2 KO macrophages are less able to invade through extracellular matrix (Figure 4.7). Interestingly, these data also suggests that srGAP2 KO macrophages are able to respond to chemo-attractant cues because a large portion of the macrophages sit immediately adjacent to the edge of the matrix but do not invade. This should be investigated further, but initially our data indicates that srGAP2 is important in regulating invasion but could be dispensable for chemo-attractant sensing and subsequent migration.

Several questions remain in regards to our study of srGAP2 and its role in podosomes. Is srGAP2 essential for the formation and/or maintenance of podosomes? Our data suggests that formation is impaired. However, podosomes are quite unique in that formation of podosomes is directly linked to maintenance, as actin turnover is required for efficient podosome formation. If maintaining actin turnover is impaired, initiation of podosome formation is deficient (Chellaiah et al., 2000; Evans et al., 2003; Linder and Aepfelbacher, 2003; Yamaguchi et al., 2005). To determine a more precise role of srGAP2 in podosomes, live imaging of fluorescently tagged srGAP2 will be essential to determining its contribution. Is its role to initiate membrane protrusions, similar to WRP and dendritic filopodia formation? Or is its primary role to regulate Rac
and/or FMNL1 at podosomes? Additionally, as podosomes are also adherent structures, is adherence affected in srGAP2 KO macrophages?

Our work has also demonstrated that srGAP2 is a negative regulator of phagocytosis in macrophages (Figure 4.2). Initially, we expected srGAP2 to regulate formation of filopodia and membrane protrusions that have been seen as essential for recognizing and internalizing external particles (Flannagan et al., 2010). Thus, loss of srGAP2 might delay or impair phagocytosis if its primary role is to facilitate outward protrusion of the phagocytic cup. However, our results demonstrate that srGAP2 may negatively regulate this process. In agreement with our results, work on an ortholog of srGAP2, SRGP-1 in C. elegans, demonstrates that it too is a negative regulator of phagocytosis (Neukomm et al., 2011). Additionally, work on two Rac GAPs, Abr and Bcr, show that a combined loss of both GAPs increases phagocytosis by increasing the amount of active Rac during phagocytosis (Cho et al., 2007). This suggests that perturbing the amount of active Rac in a cell can change phagocytosis rates. One possibility is that instead of deforming the membrane, the IF-BAR domain of srGAP2 may localize srGAP2 within the phagocytic cup where it regulates Rac and FMNL1, rather than to induce phagocytic cup formation. As discussed, prior work on FMNL1 demonstrated that it has a positive effect on phagocytosis, as knockdown of FMNL1 leads to decreased phagocytosis (Seth et al., 2006). It is thought that FMNL1 might play a role in regulation of actin turnover through severing. As our previous work has
demonstrated, srGAP2 can inhibit severing activity of FMNL1. This suggests that srGAP2 may be negatively regulating FMNL1, and if FMNL1 is not being properly turned off, more actin turnover is occurring, allowing for rapid actin remodeling, thus potentiating phagocytosis.

Loss of srGAP2 inhibits matrix degradation by macrophage podosomes. The role of FMNL1 in podosomes is still somewhat unclear, as previous work has not addressed whether FMNL1-associated podosomes are functional and able to degrade extracellular matrix (Mersich et al., 2010). The previous study demonstrates that knock-down of FMNL1 leads to less actin foci but larger actin foci. They suggest that FMNL1 might be playing a role in turning over larger actin podosomes by severing into smaller podosomes, as podosomes have been shown to undergo many fusion and fission events (Evans et al., 2003). We see that srGAP2 increases the number of podosomes associated with degradation. At this time it is unable to correlate our results with these previous finds about FMNL1 as there is no functional data in regards to podosomes in the absence of FMNL1. To address the role of the srGAP2-FMNL1 complex in matrix degradation, it will be essential to assay podosome formation and matrix degradation in the absence of FMNL1.

This study demonstrates a crucial role for srGAP2 in several essential macrophages functions. Loss of srGAP2 increases phagocytosis and decreases invasion. srGAP2 is one of the first mouse models to demonstrate functional macrophage
podosome defects and thus provides an exciting model to study this organelle’s contribution to the immune system.


5 Conclusions and Future Directions

Approximately one percent of the human genome encodes for proteins that regulate or are regulated by Rho-family GTPases. Rho GTPases are molecular switches that regulate many aspects of cell biology, including morphogenesis, polarity, and migration. To perform these functions Rho GTPases signal to activate downstream effectors that remodel the actin cytoskeleton, including a family of proteins called the Formins. Formins are activated by GTP-bound Rho-family proteins, and then they perform a variety of functions on and with actin, including polymerization, bundling, and severing. In cells, these functions must be tightly coordinated and proteins must exist to regulate Formins and Rho GTPases.

One such family of proteins that regulates Rho GTPases is called Rho GTPase Activating Proteins (Rho GAPs), which stimulate the intrinsic rate of hydrolysis by Rho GTPases. This accelerates the transition from active, GTP-bound proteins to inactive, GDP-bound GTPases. This transition is essential not only to turn off specific pathways in a spatial and temporal manner but also to recycle the GTPases, so they can signal again to activate proteins like the Formins. With the human genome encoding for over 70 Rho GAPs and only 22 Rho GTPases, there must be some mechanism for targeting Rho GAPs to specific pools of Rho GTPases.

To investigate this specific question, our lab studies one family of Rho GAPs, the Slit-Robo GAPs (srGAPs). The focus of this work is on an uncharacterized srGAP
protein, srGAP2. To determine what pool of Rho GTPases srGAP2 may be regulating, we performed a yeast two-hybrid screen and identified that srGAP2 binds Formin-like 1. Formin-like 1 is regulated by Rac and polymerizes and severs actin filaments. We identified that srGAP2 can inhibit FMNL1 in two ways: 1) Turning off Rac activating signal; 2) Specifically dampening its actin severing activity.

Additionally, we have made a mouse model of srGAP2. By knocking out the gene for srGAP2, we identified that it is expressed in macrophages and is an important component of a unique organelle called the podosome. Podosomes are actin-rich structures that degrade extracellular matrix, utilized by macrophages to invade in and out of tissues. Once present in tissues, macrophages perform another, more well characterized process called phagocytosis, where they engulf foreign particles. We have identified that srGAP2 is a negative regulator of phagocytosis and loss of srGAP2 increases this process.

This work has identified novel roles for a Rho GTPase regulatory protein: as an inhibitor of Rac and Formin-like 1 and important for phagocytosis and invasion by macrophages. However, several questions remain to be answered.

5.1 srGAP2 Recruitment to Podosomes

Our work demonstrated that srGAP2 is a critical regulator of podosome formation and is essential for matrix degradation by this structure. However, these studies relied on fixed samples. It is unclear if srGAP2 plays a role in podosome
formation or maintenance. It is essential that we utilize live imaging to get a better idea about the role of srGAP2 at podosomes. For example, questions still remain about exactly how podosomes are formed. Are they generated by focused actin polymerization that drives membrane deformation? One hypothesis is that membrane deformation followed by actin polymerization could contribute to the outward protrusions (Linder, 2007).

It is possible that srGAP2 may utilize its IF-BAR domain to induce outward protrusions necessary to induce formation of podosomes, similar to what we see for its homologous family member WRP (Carlson et al., 2011). However, it is also possible that srGAP2 arrives later in the process after podosome formation has begun, potentially to regulate Rac or FMNL1.

To differentiate between these possibilities, we could simultaneously express fluorescently tagged srGAP2 and G-actin (or alternatively an F-actin binding probe such as LifeAct or Utrophin (Rauzi et al., 2010; Riedl et al., 2010)) in macrophages and plate them onto labeled matrices as we have described above. Utilizing live imaging via confocal or TIRF microscopy, we should be able to determine which protein contributes to matrix degradation first: actin or srGAP2. Additionally, using this approach we would be able to address other proteins of interest, including FMNL1 and Rac.

To more directly assay the role of FMNL1, we should develop shRNA to target knock-down of FMNL1 in primary macrophages and determine its affect on matrix
degradation. This will give us a better idea if srGAP2 could be regulating FMNL1 at podosomes. Additionally to determine the contribution of srGAP2 in regulating FMNL1, we could rescue the KO with srGAP2 SH3 mutants described above. If the role of srGAP2 in podosomes is to regulate FMNL1, the SH3 mutant should also have a severe deficit in podosome formation. Alternatively, we could rescue the KO macrophages using srGAP2 with a mutation in the GAP domain, IF-BAR domain or combinations of these mutations. This would help to determine the contributions of these domains to the activities of srGAP2.

A recent study investigating the coordination of Rho, Rac and Cdc42 during cell protrusions utilized a system called “computational multiplexing” (Machacek et al., 2009). This group was able to image two fluorescently labeled GTPases simultaneously and in different combinations, and then using mathematical modeling, determine coordination of activation spatially and temporally. The same approach could be used to determine recruitment of specific components to podosomes.

### 5.2 Does Loss of srGAP2 Affect Adhesion?

Our work has demonstrated that srGAP2 is a critical regulator of podosome formation and is essential for matrix degradation by this structure. However, one aspect we have not addressed is the role of the podosome as an adhesive structure. One common assay to determine adhesion in macrophages is to simply plate onto substrate and counting adherent cells after a period of time (Mersich et al., 2010). Macrophages
with decreased FMNL1 have a moderate but not significant defect in adhesion (Mersich et al., 2010). We should determine if srGAP2 KO macrophages are defective in adhesion.

While it is unclear at this time whether srGAP2 KO macrophages have altered adhesion properties, one thing is clear: these cells have a distinct problem degrading extracellular matrix via podosomes. Macrophages podosomes utilize β1, β2, and β3 integrins to adhere and invade into extracellular matrix (Gimona et al., 2008; Linder and Aepfelbacher, 2003; Linder and Kopp, 2005). We should determine if loss of srGAP2 affects integrin localization via immunostaining. If integrins are mis-localized this could provide a significant problem for podosome stability and affect, ultimately, protease secretion. If integrin localization is normal, then this indicates that another problem is present: potentially mis-regulation of FMNL1 or Rac.

### 5.3 srGAP2 and Actin

One aspect we have not addressed is whether loss of srGAP2 affects formation of actin foci that resemble podosomes. It appears that actin-rich puncta do form (Figure 4.5) but are unable to degrade the matrix. We should quantify the area and numbers of these puncta between wild type and KO macrophages. Are the numbers the same? Would these puncta be suggestive of “would-be” podosomes but they are just unable to induce degradation because they are unstable? Is actin unable to turnover properly because of a mis-regulation of FMNL1, a known actin severing protein (Harris et al., 2004)? To investigate this question, we should express fluorescently labeled G-actin and
perform fluorescence recovery after photobleaching (FRAP). Previous studies, using this method, have demonstrated that actin at podosomes has a turnover rate of approximately 20 seconds (Evans et al., 2003; Evans and Matsudaira, 2006). Utilizing this approach would tell us if loss of srGAP2 affects actin turnover. If there is a larger stable pool without srGAP2, this would indicate that severing rates are lower and there are more stable filaments. This would suggest that cycling FMNL1 activity may be necessary to turnover actin filaments. Alternatively, loss of srGAP2 may decrease the stable pool of actin, which would indicate hyperactivity of FMNL1 and increase severing. Either of these possibilities could explain defects in podosomes. A tight balance between stability and turnover of actin would be expected. Utilizing macrophages lacking FMNL1 would also be extremely informative. We have also recently obtained conditional Rac1 KO mice. Using these in combination with srGAP2 KO and FMNL1 knockdown macrophages would provide a wealth of information.

5.4 Does srGAP2 Play a Role in Regulation of Macrophage Migration?

While our data show that srGAP2 macrophages do not invade matrices as well, we have not addressed whether srGAP2 regulates migration. Prior work on Rac1 and Rac2 in macrophages demonstrates that loss of Rac2 correlates with decreased speed, decreased distance traveled and increased persistence in 2D migration assays (Wheeler et al., 2006). This difference was not evident, however, in 3D migration assays using Transwells. Rac2, however, is not essential for invasion through Transwell invasion
filters. Loss of Rac1 decreases invasion but does not affect migration. These data prove that Rac1 and Rac2 GTPases are important for general macrophage migration and invasion.

These data also suggest that regulation of these GTPases in cells might play an important role in regulating migration. We can utilize identical assays to study whether srGAP2 KO macrophage have altered migratory properties. Utilizing standard 2D migration assays on glass, plastic, and matrix-coated coverslips will provide a wealth of data. Macrophages typically migrate slower on glass than plastic and slower on plastic than on laminin (Wheeler et al., 2006). Substrates with increased rigidity can increase invasion and invadopodia formation while a less rigid substrate typically promotes migration (Alexander et al., 2008; Pelham and Wang, 1997). We should see if srGAP2 has different migratory properties on different substrates. To complement the 2D studies, we will need to determine the difference between WT and KO macrophages using the traditional Transwell Migration and Invasion Assays (Wheeler et al., 2006). Interestingly, Wheeler et al. saw that while Rac2 -/- macrophages were unable to form podosomes, they actually migrated through transwells just fine (Wheeler et al., 2006). This data suggests that migration and invasion may be two separate processes.

5.5 The Role of srGAP2 in Phagocytosis

Our preliminary data suggest that srGAP2 negatively regulates phagocytosis. However, we still have much to learn about what srGAP2 might actually be doing
during this process. To study srGAP2 during phagocytosis, it will be essential to do live imaging. Utilizing reagents we will create to study srGAP2 during podosome formation, we can image srGAP2 during phagocytosis concomitantly with actin, Rac, or FMNL1. Not only will the data be able to tell us when these proteins are recruited to the phagocytic cup and phagosome, but they will also tell us where they localized. Is srGAP2 recruited to the filopodial tips? Is it enriched at the base, like Rac (Hoppe and Swanson, 2004)? One interesting experiment would be to investigate Rac activity states during phagocytosis. This could be done with currently available FRET probes that allow for the determination of Rac activity (Hoppe and Swanson, 2004; Machacek et al., 2009). However, this type of assay can be technically challenging. An easier approach would be to plate WT and KO srGAP2 macrophages and introduce opsonized beads. Over a time course, cells can be lysed and subjected to PAK pulldowns to identify the amount of activated Rac and can be compared to lysates where cells have not been exposed to beads (Cho et al., 2007). We would expect that phagocytic WT cells would have increased active Rac levels as compared to non-phagocytic cells. KO cells should have even more active Rac. Such a finding could help to explain why srGAP2 KO cells exhibit increased phagocytosis.

Additionally, it would be very interesting to do FRAP on actin at the phagocytic cup in WT and KO macrophages, and macrophages where we have knocked down FMNL1 using shRNA. Our expectation would be that actin dynamics would be altered...
during phagocytosis with changes in cellular levels of FMNL1 and srGAP2. Additionally, to better determine the contribution of srGAP2 in regulating FMNL1, we could rescue the KO with srGAP2 SH3 mutants described above.

5.6 Determining the Role of srGAP2 in Infection and Cancer

srGAP2 plays a role in the regulation of phagocytosis and podosome formation in macrophages. Both of these are essential functions for macrophages, as cells that lack podosome components fail to invade in response to adjuvants and could cause a host of immune problems (Cougoule et al., 2010; Linder et al., 1999). However, to our knowledge, no work has been done to look specifically at the role of macrophage podosomes in immune responses (Gimona et al., 2008). We have recently developed a conditional srGAP2 KO mouse, which will allow us to study the loss of srGAP2 in specific cell types. It will be important to use this conditional knockout mouse in examining the role of srGAP2 and macrophage podosomes in the immune response, as it is possible other immune cell types express srGAP2, and complete knockout of this protein could potentially have macrophage-independent effects on immune function. Crossing this mouse to a macrophage specific Cre-driver mouse, such as the murine M lysozyme promoter, would provide specificity to the KO (Clausen et al., 1999; Wang et al., 2008). Alongside these experiments, conditional mice crossed with a CMV-Cre could be used to compare macrophage specific KO effects as compared to total loss of srGAP2 as seen with CMV-Cre.
One classic experiment used to examine macrophage function during an immune response, and specifically to study macrophage invasion, involves inducing peritonitis by injecting an adjuvant into the peritoneal cavity (Cougoule et al., 2010). This allows for recruitment of inflammatory cells, mainly neutrophils and macrophages, through the peritoneal membrane into the peritoneal cavity. At 48 hours, mostly neutrophils are present. After 72 hours, macrophage recruitment peaks. We have performed this experiment several times to isolate macrophages for matrix degradation experiments. However, we have not specifically quantified the recruitment of macrophages. We must do this going forward, to determine whether there is a defect in macrophage recruitment, using either the srGAP2-TmFP mouse described here or our newly generated srGAP2 conditional KO mouse.

Another fascinating area of study with regards to macrophages is their ability to cooperate with cancer cells to increase metastasis and tumor progression (Condeelis and Pollard, 2006; Qian and Pollard, 2010). It is becoming increasingly clear that macrophages are recruited to tumors and that an elevated density of tumor-associated macrophages correlates with poor prognosis (Lin et al., 2002). It is hypothesized that tumors can recruit macrophages to exploit their abilities to remodel the extracellular matrix to allow for intravasation into blood vessels, remodeling of tissues for angiogenesis, and extravasation during metastasis (Condeelis and Pollard, 2006). Cancer cells (in the study highlighted, primary mammary tumor cells were used) secrete Colony
Stimulating Factor-1 (CSF-1) which is a chemoattractant and survival signal for macrophages (Goswami et al., 2005). Macrophages recruited to these tumors express the CSF-1 receptor, while surrounding cells do not (Lin et al., 2002). CSF-1 induces these macrophages to secrete EGF, which attracts cancer cells and thus a paracrine loop of chemotactic cues draws these cells toward blood vessels (Wang et al., 2005). This paracrine loop enhances migration of both cancer cells and the tumor-associated macrophages (Goswami et al., 2005). It is thought that one reason why macrophages and cancer cells potentiate migration of one another is that they can coordinate rapid extracellular matrix degradation through podosomes and invadopodia (Condeelis and Pollard, 2006; Linder, 2007). Several studies have demonstrated that removing macrophages or inhibiting macrophage association with tumors causes a dramatic reduction in metastasis (Condeelis and Pollard, 2006; Lin et al., 2002; Qian and Pollard, 2010).

If metastasis and cancer progression is enhanced by macrophages and their ability to degrade extracellular matrix, loss of srGAP2 may decrease cancer progression. To address this question we could start with a simple experiment: imbedding macrophages with cancer cells, for example MTLn3 mammary carcinoma cells, in a collagen matrix. In this assay, macrophages or cancer cells on their own are unable to induce migration, but when cells are plated together in the matrix, they are able to invade up from the bottom of the plate and into the matrix (Goswami et al., 2005). We
could plate the cancer cells with macrophages expressing or lacking srGAP2 to determine if srGAP2 may play a role in assisting cancer cell invasion. If no difference were seen, this would suggest that srGAP2 is not essential for cancer cell invasion. We would expect, however, that srGAP2 KO macrophages would have a defect in allowing cancer cells to invade because they themselves are unable to invade.

If there is a defect in the ability of srGAP2 KO macrophages to induce cancer migration, it would be intriguing to translate this to a mouse model of metastasis. One classic model of metastasis is the polyoma middle T (PyMT) oncoprotein mouse model of metastatic breast cancer. The PyMT oncoprotein is expressed under the mouse mammary tumor virus promoter and induces transformation in mammary epithelium and metastatic lesions in the lymph nodes and lungs (Fantozzi and Christofori, 2006; Guy et al., 1992). This metastatic phenotype requires a macrophage population that is activated by CSF-1 (Lin et al., 2001). We could cross this mouse with our srGAP2-TmFP KO mouse or macrophage-specific KO and examine whether tumor metastasis is altered. We would expect loss of srGAP2 to decrease metastasis. An alternative mouse model that would also be of interest involves bronchial exposure to Haemophilus influenzae lysate, which results in inflammation in the lung and increased tumorigenesis (Moghaddam et al., 2009). As chronic inflammation can be associated with cancer (Qian and Pollard, 2010), this might be an alternative to the metastasis model described above,
focusing more on the role of macrophages regulating inflammation-induced carcinogenesis.
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