UC Berkeley
UC Berkeley Electronic Theses and Dissertations

Title

Rickettsia Sca2 is a bacterial formin-like mediator of actin-based motility

Permalink

https://escholarship.org/uc/item/6mt5h51w

Author

Haglund, Cathleen Margaret

Publication Date

2011

Peer reviewed|Thesis/dissertation

Rickettsia Sca2 is a bacterial formin-like mediator of actin-based motility

by

Cathleen Margaret Haglund

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Matthew D. Welch, Chair
Professor David Drubin
Professor Daniel A. Portnoy
Professor Kathleen Ryan

Spring 2011

Rickettsia Sca2 is a bacterial formin-like mediator of actin-based motility

© 2011

by

Cathleen Margaret Haglund

ABSTRACT

Rickettsia Sca2 is a bacterial formin-like mediator of actin-based motility

by

Cathleen Margaret Haglund
Doctor of Philosophy in Molecular and Cell Biology
University of California, Berkeley
Professor Matthew D. Welch, Chair

Diverse intracellular pathogens subvert the host actin polymerization machinery to drive movement within and between cells during infection. *Rickettsia* in the spotted fever group (SFG) are Gram-negative, obligate intracellular bacterial pathogens that undergo actin-based motility and assemble distinctive 'comet tails' containing long, unbranched actin filaments. Despite this distinct organization, it was proposed that actin in *Rickettsia* comet tails was nucleated by the host Arp2/3 complex and the bacterial protein RickA, which assemble branched actin networks. To identify additional rickettsial proteins that might function in actin assembly, we searched translated *Rickettsia* genome databases for proteins with WASP homology 2 (WH2) motifs, which are actin-binding peptides found in many cellular proteins. We identified WH2 motifs in *Rickettsia* Sca2 (surface cell antigen 2), a protein of the autotransporter family. Here, we demonstrate that *R. parkeri* Sca2 represents a new class of bacterial actin assembly factor that functionally mimics the eukaryotic formin family of actin nucleators. *R. parkeri* Sca2 nucleates unbranched actin filaments, processively associates with growing barbed ends, requires profilin for efficient elongation, and inhibits the activity of filament capping proteins, all properties shared with formins. Sca2 localizes to the *R. parkeri* surface, is enriched at the actin tail interface, and is sufficient to promote the assembly of long actin filaments in cytoplasmic extract. There is a strong correlation between the domain organization of Sca2 and the actin-based motility phenotype in diverse *Rickettsia* species. These results suggest that Sca2 mimics formins to determine the unique organization of actin filaments in *Rickettsia* tails and drive bacterial motility, independently of host nucleators.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABM: actin-based motility

Arp2/3: actin-related protein 2 and 3

Cc: critical concentration

EHEC: enterohemorrhagic *Escherichia coli*

EPEC: enteropathogenic *Escherichia coli*

F-actin: filamentous (polymeric) actin

FH1: formin homology 1

FH2: formin homology 2

G-actin: globular (monomeric) actin

GST: glutathione S-transferase

IF: intermediate filament

mDia: mammalian diaphanous

NPF: nucleation promoting factor

N-WASP: neuronal Wiskott-Aldrich Syndrome protein

PRD: proline-rich domain

RNAi: RNA interference

Sca2: surface cell antigen 2

SFG: spotted fever group

T3SS: type 3 secretion system

TIRF: total internal reflection fluorescence

WASP: Wiskott-Aldrich Syndrome protein

WH2: WASP homology 2

ACKNOWLEDGMENTS

Graduate school comes with a unique set of challenges when you're older, even more so when you have little prior experience in your field. Thus, I needed the help of a great many people along the way. I am extremely grateful to (in chronological order): My parents, who told me I could do anything and waited four decades to find out what 'anything' would be. My mother, a former biology teacher, drew the first Punnett Square my five-year-old self had seen to explain eye color distribution in our family. It took me thirty years to recognize what an impression that square had made on me. My dad, a seemingly tireless engineer, tackled household projects large and small with equal parts enthusiasm and efficiency (plus a dash of perfectionism) – this dissertation would have been completed sooner and more neatly if he'd been on the job. My sister Kim, who prompted the Punnett Square by wondering where her blue eyes had come from, was one step ahead of me then and continued to be so all along, but she graciously accepted me as an adult long before anyone else did (and probably long before I deserved it). She went back to school in her thirties, proving to me that it was worth the trouble. I hope to repay the favor when we are old and gray by remaining one year less senile than she, so that I can help her record So You Think You Can Dance Season 47.

During the years when I wondered what to do with my life, Bill Hilliard was the ideal boss and helped give me the confidence to change my vocation. Abby Dernberg, Kathleen Ryan, Brandon Brown, and their card-playing buddies inspired me by talking about their science so passionately. UC Berkeley Extension instructor Barbara Bowman flipped a switch in my brain by describing hydrophilic molecules as "sparkly." I couldn't have fulfilled chemistry and biology prerequisites without our amazing and underfunded community college system, in particular Contra Costa College. Microbiology professor Kate Levine is an inspiring whirlwind of bacterial knowledge (some of it terrifying), and I heartily thank her and Caroline Kane for arranging summer internships at Cal for community college students. They placed me in the lab of Kris Niyogi, who was so supportive that I didn't want to leave at the end of the summer. He eventually hired me as a research associate, which was incredibly kind given how little I understood the work in his lab at the time. All the Niyogi lab members were helpful, funny, and smart. Rachel Dent and Marilyn Kobayashi dished out particularly great guidance.

Two years later, I stumbled semi-prepared into the MCB department. Huge thanks to Matt for accepting me first into the program, then into his lab, and for appearing to believe I could finish. I might not have, without his trademark combination of belief, prodding, and insistence on precision in writing and presenting, as well as his example of hard work and professionalism. My early experiments were mostly disasters, but when I finally found something that I felt sure about, Matt took the idea seriously and patiently supported my attempts to produce data. Such data might never have been obtained without the advice of Ken Campellone, who earned a dedicated entry in my protocol notebook (titled "Ken's Little Ways of Doing Everything Perfectly"); Senior Rickettsiologists Robert Jeng and Alisa Serio and "Entry"-level Rickettsiologist Shawna Reed, who collectively eased the slow pain of *Rickettsia* work; lab artist and archivist Taro Ohkawa, who captured the life behind the science; and wise, supportive, and quotable labmates Erin Benanti, Julie Choe, Lee Douglas, Steve Duleh, Peter Hsiue, Elif Firat-Karalar and Haiming Wu, who provided Taro with large quantities of raw material.

My dissertation committee of David Drubin, Dan Portnoy, and Kathleen Ryan were an annual source of good ideas and sage advice. Rebecca Heald and Karsten Weis were paragons of

CHAPTER 1

Using intracellular pathogens to investigate the eukaryotic cytoskeleton

Introduction

Pathogenic microbes offer many advantages for elucidating cytoskeletal function and regulation. They exploit actin, microtubules, septins, and intermediate filaments in diverse ways. They also provide clear functional read-outs, such as infection efficiency or formation of distinct cytoskeletal structures. Finally, microbes often signal in a locally focused or exaggerated fashion, facilitating the dissection of pathways that might be more diffuse or moderate in the host. Pathogens have thus helped us assign function to cytoskeletal proteins, discover new modes of regulation, and unravel temporal and mechanistic interplay between factors involved in filament assembly.

In this chapter, I discuss four infectious processes that have shed light on the host cytoskeleton. The first is pathogen invasion, which exploits cellular uptake pathways that rely on actin, such as phagocytosis and macropinocytosis. Emerging evidence suggests that microtubules and septins also play roles in distinct entry pathways. The second process is establishment of a replication niche, which subverts cytoskeletal functions that normally operate during membrane trafficking and cellular defense. Third, cytoplasmic actin-based motility mimics vesicle rocketing and has also illuminated host cell motility, while actin-based motility at the plasma membrane mimics the pathway responsible for kidney podocyte morphology and function. Finally, pathogen exit and cell-to-cell spread is an emerging field for which the endogenous processes are not clearly defined. I draw examples from three decades of cellular microbiology, focusing on recent developments in the field and on cases where pathogens (mostly bacteria) played particularly noteworthy roles in key discoveries. The pace of new findings suggests that the next three decades will be even more fruitful than the last.

Bacterial invasion of host cells: Many doors, many keys

Entry pathways converge on actin polymerization

Diverse bacteria invade non-phagocytic cells by stimulating endogenous uptake processes, such as phagocytosis and macropinocytosis. Actin polymerization is central to both of these processes, driving plasma membrane extensions that engulf external cargo. In addition to a shared reliance on actin, invading bacteria employ a multitude of upstream signaling molecules, and thus have contributed broadly to our understanding of actin regulation.

Bacterial invasion pathways have historically been classified into two categories, "zipper" and "trigger" (reviewed in Cossart and Sansonetti, 2004). Zipper mechanisms, best studied for invasion of *Listeria monocytogenes* and *Yersinia* spp., occur when specialized bacterial surface proteins bind host cell receptors that signal across the membrane to a phagocytic pathway, which produces limited membrane rearrangement closely apposed to the entering bacterium (Fig. 1.1a). In "trigger" mechanisms, exemplified by *Salmonella enterica* serovar Typhimurium (*Salmonella*) and *Shigella flexneri*, the pathogen injects effector proteins across the host membrane, often via the syringe-like type 3 secretion system (T3SS), inducing a bloom of membrane ruffles that engulf the bacterium and other nearby particles (Fig. 1.1b). Study of *Salmonella* entry established that actin-rich ruffles directly mediate macropinocytosis, a process in which extracellular cargo is taken up non-selectively, and that ruffles produced by endogenous mechanisms, such as by growth factors, have identical uptake behavior (Francis et al., 1993).

Another lesson learned from bacterial entry is the central importance of the Arp2/3 complex in plasma membrane remodeling. The Arp2/3 complex, when activated by nucleation promoting factors (NPFs), nucleates branched actin networks. When this unique actin assembly activity occurs at the plasma membrane, it drives membrane extension, producing structures such as ruffles and phagocytic cups. All phagocytic pathways studied so far utilize the Arp2/3 complex. Even when a second actin nucleator has been implicated as well, as in the case of mDia1 during complement-mediated phagocytosis (Colucci-Guyon et al., 2005), the Arp2/3 complex is also required (May et al., 2000), suggesting that its activity is indispensable for particle engulfment processes. A plethora of injected effectors and host receptors employed by invasive bacteria converge on the Arp2/3 complex through Rho-family GTPases, which recruit and activate NPFs. Although the role of the Arp2/3 complex in phagocytosis has been well studied, recent work continues to impart new information about the pathways that regulate Arp2/3 complex in this process. For instance, when the contributions of the NPFs N-WASP and WAVE2 to *Salmonella* invasion were quantified, genetic deletion of N-WASP was found to double expression of a third Arp2/3 activator, WASH, in uninfected cells (Hanisch et al., 2010). This suggests that WASH can partially compensate for the loss of N-WASP and is one of many examples of cytoskeletal plasticity. In the same study, WASH downregulation decreased *Salmonella* invasion, implicating WASH for the first time in the uptake of large particles.

A more general lesson about actin polymerization is that it requires careful and vigilant modulation to maintain cell function and viability. *Salmonella* illustrates this point nicely, as it injects at least six effectors that control actin rearrangements during entry (reviewed in Haraga et al., 2008; McGhie et al., 2009). Various mechanisms of regulation are employed: two effectors activate Rho GTPases by mimicking host guanine-nucleotide exchange factors (GEFs) (Hardt et al., 1998; Stender et al., 2000); another activates GTPases indirectly, via inositol phosphatase
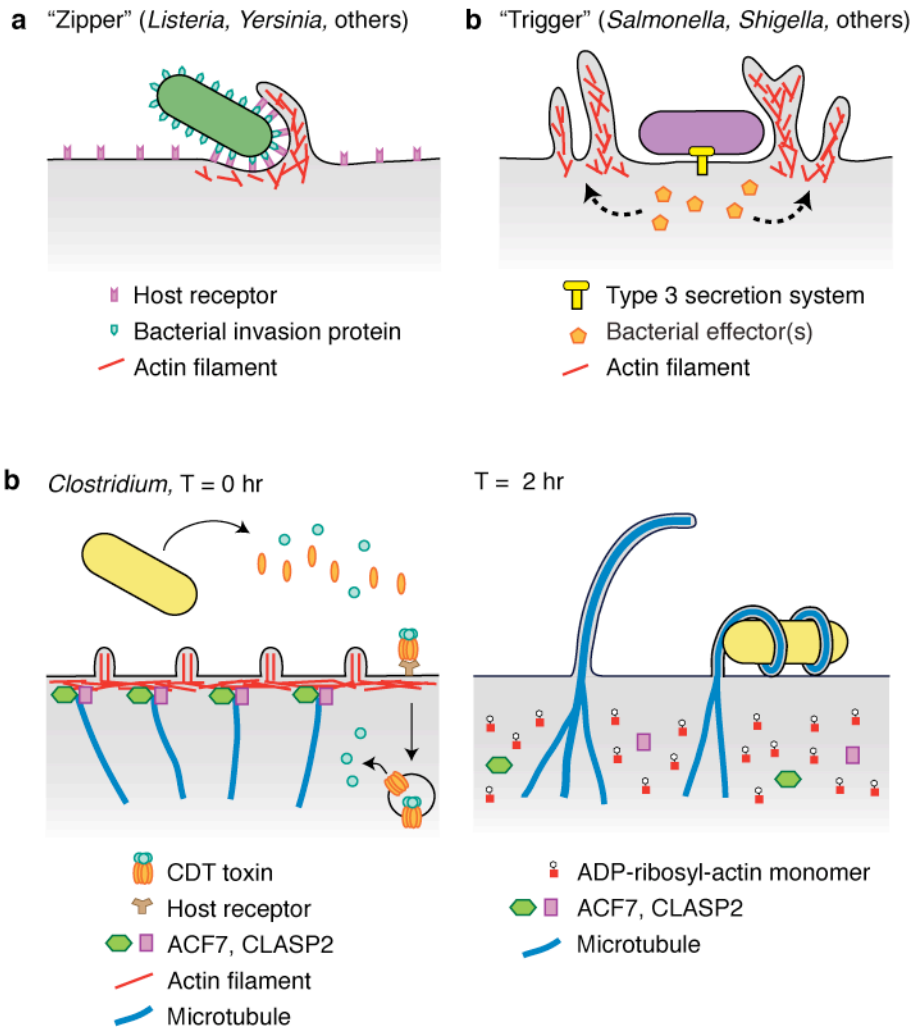
Figure 1.1. Bacteria exploit actin and microtubules to promote invasion and adherence.
(**a**) Zippering bacteria express an invasion protein on their surface, which binds to a host receptor and initiates phagocytosis. (**b**) Triggering bacteria inject protein effector(s) across the host cell membrane, usually via a T3SS, leading to macropinocytosis. (**c**) *Clostridium* CDT, a binary toxin, is endocytosed by intestinal epithelial cells, and the A subunit is released into the cytosol (left panel). CDT toxin ADP-ribosylates actin, promoting actin filament disassembly, effacement of microvilli, and release of cortical proteins that normally capture and stabilize microtubules. Unrestrained microtubule growth produces cellular extensions that wrap around external bacteria.

4

activity (Terebiznik et al., 2002; Zhou et al., 2001); and two effectors directly interact with actin, nucleating and stabilizing filaments near the entry site (Hayward and Koronakis, 1999; Lilic et al., 2003; McGhie et al., 2004; Zhou et al., 1999). After a burst of polymerization, a sixth effector downregulates Rho-family proteins by promoting their GTPase activity, restoring normal actin structure at the cell cortex (Fu and Galan, 1999). The balance between Rho activation and inhibition is regulated both spatially (Patel et al., 2009) and temporally (Kubori and Galan, 2003). Thus, "triggered" entry is not a sudden event like the firing of a gun, but an elaborate, choreographed process. Disrupting this choreography can be detrimental to cell health.

Is actin the only gatekeeper?

Although actin polymerization is critical for uptake processes, recent evidence suggests that other filament networks can also play important roles. The 1990s brought a few reports of microtubule-dependent bacterial invasion; whether actin was also required in these cases was unsettled. Microtubule dependence in certain cell types was even reported for the well-studied *Listeria* (Guzman et al., 1995; Kuhn, 1998) and *Salmonella* (Aiastui et al., 2010). A precise role for microtubules during entry was elusive, in part because the pathway(s) appeared to be cell type and strain specific (Dhakal and Mulvey, 2009; Donnenberg et al., 1990; Oelschlaeger et al., 1993).

In the past few years, progress has been made towards defining a mechanistic basis for microtubule dependence during bacterial entry. Several toxins made by *Clostridium* spp. were found to induce the temporary formation of long, microtubule-filled projections that wrap around bacteria and promote adherence (Schwan et al., 2009) (Fig. 1.1c). Although *Clostridium* do not enter cells, adherence is a prerequisite of invasion, so microtubule-based projections could promote the invasion of other pathogens. Interestingly, the toxins' effect on microtubules is indirect: they ADP-ribosylate actin, leading to actin filament destruction, followed by release from the cell cortex of several proteins that normally capture and stabilize microtubules (CLASP2 and ACF7). For a few hours after toxin application, microtubule-based projections dominate the cell morphology, followed by cell shrinking and rounding. Actin depolymerizing drugs also induce microtubule-based projections, albeit at lower levels. The exaggerated effect produced by *Clostridium* toxins could provide a tool for dissecting the interplay between actin and microtubules at the cell periphery, particularly as it relates to microtubule capture.

There is some evidence that intermediate filaments and septins might also contribute to bacterial entry. The intermediate filament vimentin has been implicated in the uptake of several bacteria, including *Salmonella* (Murli et al., 2001) and *Escherichia coli* K1 (Chi et al., 2010). Depletion of septin-2 reduced invasion efficiency of *Listeria* and *Shigella,* and several septins were observed to form a ring around invading bacteria (Mostowy et al., 2009). For *Listeria*, the effect of septin depletion was specific to one of two receptor-mediated internalization pathways and, as with microtubules, varied with cell type, suggesting that discrete entry pathways are involved.

Given that septin assembly in different cellular contexts can both influence and be influenced by the assembly of actin or microtubules (reviewed in Gilden and Krummel, 2010; Spiliotis, 2010), and that extensive crosstalk occurs between actin, microtubules, and intermediate filaments (Chang and Goldman, 2004; Li and Gundersen, 2008; Rodriguez et al., 2003), it is likely that multiple signals pass between the four filament networks during bacterial entry, complicating the dissection of roles for each filament type. Further complications arise

from findings that blur distinctions between different entry pathways; for instance, zippering bacteria such as *Listeria* exploit the clathrin-mediated endocytic machinery, which is classically associated with smaller cargo (Veiga and Cossart, 2005; Veiga et al., 2007), while *Salmonella* can invade cells independently of ruffle formation (Hanisch et al., 2010; Stender et al., 2000). A systems biology approach, as proposed for viral entry (Damm and Pelkmans, 2006), could help untangle the pathways, and might clarify the variability across cell types. In this approach, RNAi knockdown of numerous host genes is combined with infection by a panel of pathogens to define "functional modules," or host genes that function together in a particular invasion process. A broad panel of bacteria could reveal the total number of separable entry pathways available in the host and define which cytoskeletal factors participate in each.

Barring the door: extracellular pathogens disrupt actin to prevent phagocytosis

In contrast to *Salmonella*, which dampens its effects on the cytoskeleton to maintain host cell viability, extracellular bacteria often treat cells more harshly. Many bacterial toxins covalently modify host cytoskeletal factors such as actin and Rho-family GTPases (Visvikis et al., 2010), thus preventing uptake by phagocytic immune cells. Despite their destructiveness, these toxins' modes of action pertain to endogenous processes. From them, new post-translational modifications and host signaling molecules have been identified. For instance, Rac, a member of the Rho family, was discovered as a substrate of the *Clostridium botulinum* C3 toxin (Didsbury et al., 1989), and the toxin was instrumental in elucidating the roles of Rac and Rho as signaling hubs, with Rac controlling membrane ruffles (Ridley et al., 1992) and Rho controlling stress fibers and focal adhesions (Ridley and Hall, 1992).

More recently, a novel eukaryotic regulatory mechanism, AMPylation, was discovered via the activity of a bacterial toxin. *Vibrio parahaemolyticus* VopS disrupts several Rho-family GTPases by covalent attachment of adenosine 5'-monophosphate (AMP), abolishing downstream actin assembly (Yarbrough et al., 2009). The catalytic domain, called Fic, was also found in eukaryotic proteins (Kinch et al., 2009; Worby et al., 2009). The Fic domain from human HYPE (huntingtin yeast-interacting protein) AMPylates Rho GTPases in vitro (Worby et al., 2009), supporting the notion that AMPylation can serve as an endogenous form of Rho regulation.

Another variation on Rho modification is employed by *Photorhabdus luminescens*, which ADP-ribosylates RhoA, preventing GTP hydrolysis and putting RhoA in a constitutively active state (Lang et al., 2010). This differs from previously-characterized toxins from other pathogens that also ADP-ribosylate Rho proteins, because those toxins target a different residue and inhibit Rho function. A second *Photorhabdus* toxin ADP-ribosylates actin, preventing β-thymosin from sequestering actin monomers and leading to more extensive polymerization (Lang et al., 2010). Again, the toxin targets a different residue and has the opposite effect on actin function compared to previously-studied toxins. Together, these two *Photorhabdus* toxins promote rampant, disruptive actin rearrangements and inhibit phagocytosis. Although the relevance of these forms of regulation to endogenous processes is not clear, it is interesting that phagocytosis can be blocked by both up- and down-regulating actin polymerization. This emphasizes the theme illustrated by *Salmonella* entry: actin polymerization must be carefully controlled to produce useful results, and excessive assembly can bring the network to a halt.

Constructing a niche

Following invasion, many intracellular pathogens remain within the membrane-bound entry compartment, modifying it to suit their needs. This requires subversion of diverse host pathways to acquire resources for growth while manipulating phagosome maturation to prevent destruction within lysosomes. The cytoskeleton is exploited to maintain proper positioning of the pathogen-containing vacuole and to control membrane trafficking and vacuole maturation. Again *Salmonella* provides a useful example, as it expresses multiple effectors with overlapping and antagonistic effects (reviewed in Bakowski et al., 2008; McGhie et al., 2009). In this section, we briefly review how the study of *Salmonella* led to the identification of the kinesin interacting partner SKIP and to potential new roles for cytoskeletal motors in membrane trafficking. We then describe recent insights into IFs and septins, including possible roles in the regulation of autophagy, gleaned from the study of *Chlamydia trachomatis*, *Shigella*, and others.

Salmonella *regulates membrane trafficking via kinesin-1 and SKIP*

Salmonella replicates in a perinuclear compartment called the *Salmonella*-containing vacuole (SCV), from which membrane tubules called Sifs (*Salmonella*-induced filaments) extend. Integrity of the SCV was known to require microtubules, microtubule motors, and numerous bacterial effectors secreted by the T3SS, including SifA. A two-hybrid screen identified the primary interacting partner of SifA, a host protein of previously unknown function now called SKIP (SifA and kinesin-interacting protein) (Boucrot et al., 2005). As the name suggests, SKIP interacts with kinesin-1 in vitro and is required for SCV integrity. However, a role for kinesin in SCV integrity seemed paradoxical: kinesin was not detected on SCVs unless SKIP (or SifA) was depleted. Thus, it was proposed that SKIP negatively regulates the recruitment of kinesin.

Shortly after the identification of SKIP, a different *Salmonella* effector, PipB2, was shown to positively regulate kinesin-1 recruitment to the SCV (Henry et al., 2006). Why *Salmonella* would express both positive and negative regulators of kinesin recruitment was not immediately clear. A functional clue was provided by the discovery that at late time points after infection some SCVs moved toward the cell periphery as a prelude to dissemination, in a manner dependent on microtubules, kinesin-1, and PipB2 (Szeto et al., 2009). At a mechanistic level, subsequent work showed that SKIP binds the late endosomal GTPase Rab9, and that SifA might mimic Rab9 (Jackson et al., 2008). SKIP promotes anterograde movement of late endosomes/lysosomes along microtubules (Dumont et al., 2010; Jackson et al., 2008), possibly through control of membrane tubulation (Ohlson et al., 2008) or scission events (Dumont et al., 2010). SKIP might also contribute to Golgi apparatus positioning, because SKIP overexpression results in peripheral scattering of Golgi markers (Dumont et al., 2010). A model for SCV maintenance was proposed in which inactive kinesin-1 is recruited to SCV membranes by PipB2, followed by formation of a complex containing kinesin, SKIP, and SifA (Fig. 1.2). SKIP could then activate kinesin by an unknown mechanism, possibly through its interaction with the kinesin light chain, promoting membrane scission and release of kinesin-positive vesicles (Dumont et al., 2010). In this model, the lack of kinesin on SCVs is explained by its rapid dispersal on SCV-derived vesicles. The precise biochemical mechanism of SKIP and kinesin function on SCVs remains to be determined. It will be interesting to test this model and to see if an analogous mechanism operates on endogenous SKIP/Rab9-positive compartments.
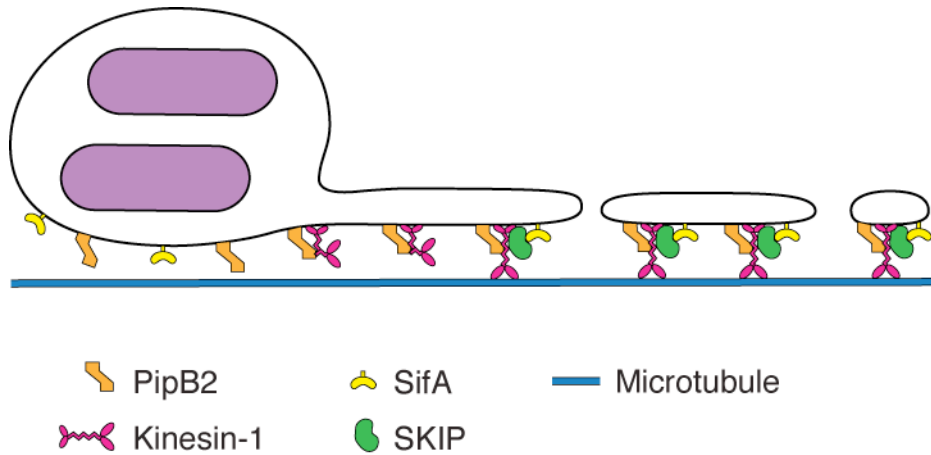
Figure 1.2. Model for kinesin-1 and SKIP activity on SCV membranes. The secreted *Salmonella* effectors PipB2 and SifA recruit kinesin-1 and SKIP, respectively, to SCV membranes. It has been proposed that SKIP might then activate kinsein-1 by binding to the kinesin light chain, and that SKIP:kinesin-1 complexes promote tubulation and/or scission of SCV-derived membrane compartments, which are transported anterogradely along microtubules.

Another potentially interesting parallel between SCVs and endogenous compartments involves the actin-based motor myosin II, which was unexpectedly implicated in SCV positioning and integrity (Wasylnka et al., 2008). Inhibition of myosin II resulted in SCV scattering, similar to the phenotype of SKIP overexpression, implying that myosin activity counteracts the anterograde pull of SKIP/kinesin. In uninfected cells, myosin II, together with Rab6, directly contributes to vesicle fission from the Golgi apparatus (Miserey-Lenkei et al.). By analogy, in *Salmonella*-infected cells, myosin II might promote the release of vesicles from the SCV, perhaps in cooperation with SKIP, kinesin-1 and SifA. The involvement of both myosin II and kinesin-1 in SCV-derived vesicle formation could point to new mechanisms of cooperation between actin- and microtubule-based motors.

Filament "cages": nest or trap?

Intermediate filaments (IFs) and actin also play roles in the establishment of replicative niches. In some cases, IFs, alone or with actin, form stabilizing cages around pathogen-containing vacuoles, apparently protecting them from host recognition. For example, *Chlamydia trachomatis* induces relocalization of the IFs vimentin, cytokeratin-8, and cytokeratin-18, as well as actin filaments to the *Chlamydia* inclusion membrane (Kumar and Valdivia, 2008). IF and actin assembly around the inclusion are interdependent, and disruption of either leads to release of bacteria into the cytoplasm, triggering host defense mechanisms. *Chlamydia* cages bear some resemblance to aggresomes, which are vimentin cages surrounding protein aggregates (Wileman, 2007), but aggresomes do not contain actin, are microtubule-dependent, and facilitate degradation of their contents via proteasomes and autophagy, whereas *Chlamydia* cages do not require microtubules and protect their contents. These cages appear to regulate IF filament assembly in a novel way: the bacterial protease CPAF locally cleaves IF head domains, reducing their cohesiveness but not displacing them from the inclusion, suggesting that controlled proteolysis might allow cage expansion while maintaining structural integrity of the inclusion. The head domain of vimentin is essential for filament assembly in vitro (Herrmann et al., 1996), yet the IFs cleaved by CPAF assemble into higher molecular weight complexes in vitro, and the head domain remains associated with cages. Thus, proteolysis at an unidentified site in the head domain could represent a way to regulate the elasticity of IF networks. The authors proposed that *Chlamydia* cages are built in layers, with an unspecified bacterial T3SS effector recruiting RhoA to initiate actin assembly on the inclusion membrane, followed by the binding of unidentified linker proteins and an outer IF layer, which is progressively cleaved to permit expansion (Kumar and Valdivia, 2008). Many questions remain regarding IF cage structure and CPAF-mediated IF cleavage.

Protective IF cages also coalesce around other intracellular bacteria. *Salmonella* vacuoles recruit the same three IF proteins as *Chlamydia* (vimentin, cytokeratin-8, and cytokeratin-18), and IF disruption results in SCV dispersal (Guignot and Servin, 2008). Vimentin cages form around *Anaplasma phagocytophilum* inclusions, and a bacterial effector that binds vimentin and indirectly promotes survival was identified (Sukumaran et al., 2011), supporting the hypothesis that caging is a protective, pathogen-controlled "nesting" process. Characterizing microbial effectors that mediate cage-building could provide additional information about IF regulation.

In contrast, a distinct type of cage acts as a host-mediated trap that promotes destruction of the pathogen by autophagy. A proportion of cytosolic *Shigella* become wrapped in septin filaments, in a myosin II-dependent manner, concurrently with acquisition of markers of

autophagy (Mostowy et al., 2010). Although initial cage assembly requires actin polymerization, an inverse correlation exists between the presence of a cage surrounding a bacterium and productive actin-based motility, and also between myosin activity and motility, indicating that a delicate balance of cytoskeletal forces determines the fate of each bacterium. Because septin cages initially require, but then antagonize actin polymerization, the authors looked for septins around other bacteria that undergo actin-based motility. Cages were detected around *Mycobacterium marinum* but not *Listeria* or *Rickettsia conorii*, suggesting that caging is influenced by bacterial factors, which could provide routes to understand its regulation. In any case, the connection between septin cages and autophagy points to a new cellular function for septins and merits further study. Given that a connection also exists between aggresome vimentin cages and autophagy, it is tempting to speculate that certain filament assemblies, such as septins or vimentin, simultaneously immobilize cytoplasmic contents and mark them for the autophagy machinery. If this is the case, then pathogens would be expected to evolve countermeasures to modify filament traps to avoid autophagy. For example, if vimentin acts as an autophagy signal, perhaps recruitment of additional IFs (such as cytokeratins) might serve to mask this signal. Intracellular pathogens, by serving as both targets and manipulators of the autophagy pathway, are invaluable tools for investigating its regulation.

Tiny rocket scientists: Diverse pathogens "discovered" actin-based motility

Some bacterial species escape from the phagocytic vacuole and replicate freely in the host cytoplasm. A subset of these pathogens express factors that trigger actin polymerization against their surface, producing mechanical force that propels them through the cell and facilitates spread to neighboring cells. This form of motility evolved independently in *Listeria, Shigella, Rickettsia*, and *Burkholderia*, and each of these genera use a distinct mechanism triggered by a single bacterial protein, demonstrating that, in contrast to vacuole positioning, actin-based motility (ABM) is relatively simple to achieve. Thus, it is not surprising that some viruses – vaccinia and other poxviruses (Cudmore et al., 1995; Dodding and Way, 2009), and a baculovirus (Ohkawa et al., 2010) – have also evolved ABM, although poxviruses trigger it from outside of the cell. The study of pathogen ABM, particularly of *Listeria*, revolutionized our understanding of cellular actin-based propulsion, such as vesicle rocketing and lamellipodia-driven protrusion during cell migration. First, pathogen ABM enabled the identification of key players in these processes, and later it provided a tractable system to elucidate the activities of proteins involved in nucleation, crosslinking, and turnover of actin networks, as well as upstream signaling. We review discoveries that established the ABM field, and then highlight recent studies that shed new light on actin dynamics.

Motility through the cytoplasm

By the 1990s, it was well-established that actin polymerization was involved in the motility of eukaryotic cells, and it was inferred from the study of *Listeria* actin "comet tails" (Tilney and Portnoy, 1989) that the motive force for lamellipodia protrusion during cell migration is derived from polymerization itself, rather than myosin activity along a cytoskeletal track (Theriot et al., 1992). However, the cellular mechanisms of actin filament nucleation, organization, and disassembly in lamellipodia were unknown. The structure of comet tails produced by *Listeria* ActA or *Shigella* IcsA were highly similar to each other, suggesting that pathways controlling
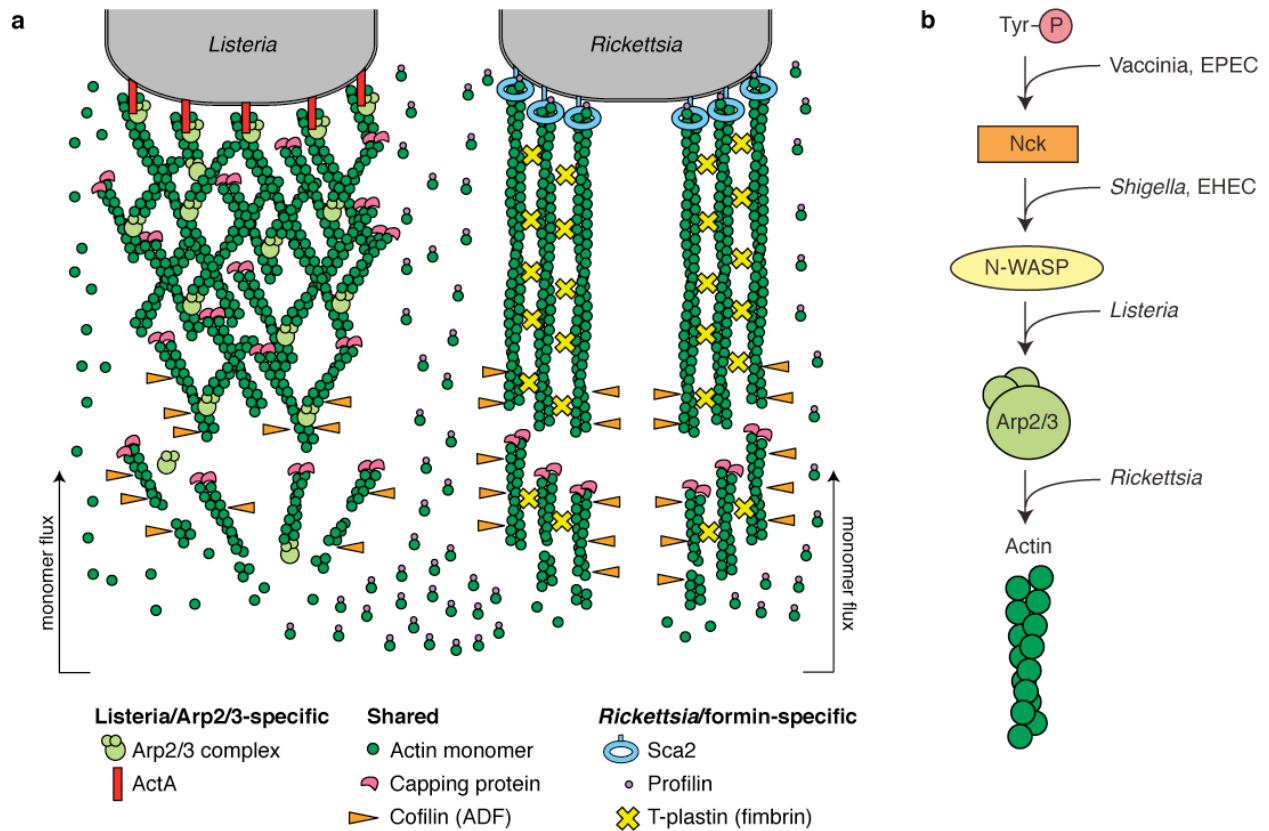
Figure 1.3. Pathogens employ distinct actin-based motility mechanisms. (a) The actin-based motility of *Listeria* and *Rickettsia* have distinct host protein requirements. *Listeria* expresses ActA on its surface, which activates the host Arp2/3 complex, producing branched actin filaments. Actin monomers or profilin:actin complexes can polymerize onto filament ends in *Listeria* tails. *Rickettsia* expresses the formin-like protein Sca2 on its surface, which nucleates unbranched actin filaments and requires profilin for filament elongation. T-plastin is also important for *Rickettsia* tail formation. Both systems require capping protein and cofilin. **(b)** Pathogens intercept actin assembly pathways at different levels. Vaccinia virus and EPEC mimic host phosphotyrosine motifs to recruit the adaptor protein Nck. *Shigella* and EHEC recruit and activate N-WASP. *Listeria* mimics N-WASP to activate the host Arp2/3 complex. *Rickettsia* bypasses host nucleators to interact directly with actin.

actin tail morphology and stability were controlled by the host. It was clear that identifying host binding partners of either ActA or IcsA would advance our understanding of cellular actin dynamics.

The first binding partner identified was the Arp2/3 complex, a highly conserved seven-subunit complex that was found to be sufficient for actin assembly at the *Listeria* surface (Welch et al., 1997). At the biochemical level, the Arp2/3 complex weakly promoted actin nucleation on its own and was strongly activated by the N-terminal domain of ActA (Welch et al., 1998). *Shigella* also exploits Arp2/3 complex, but via a distinct mechanism: *Shigella* IcsA binds and activates host N-WASP to recruit Arp2/3 and actin (Egile et al., 1999). The importance of Arp2/3 in cellular processes such as lamellipodia protrusion and phagocytosis was quickly recognized, and endogenous proteins such as WASP/N-WASP and WAVE were shown to activate Arp2/3 complex in the same manner as ActA (reviewed in Goley and Welch, 2006). Collectively, these Arp2/3 activators became known as nucleation-promoting factors (NPFs). Additional NPFs, such as WHAMM, WASH, and JMY, continue to be identified and characterized, uncovering new roles for the Arp2/3 complex in processes such as ER-to-Golgi transport and endosome trafficking (Rottner et al., 2010).

Recapitulating Arp2/3 complex-mediated motility in a defined system of purified components was achieved by recognizing that nucleation is not the only activity required for productive ABM. Sustained movement was achieved using the following components: *Listeria*, or *Escherichia coli* expressing IcsA and coated with N-WASP; Arp2/3 complex to nucleate filaments; actin depolymerizing factor (ADF, also known as cofilin) to accelerate depolymerization to maintain the actin monomer pool; and capping protein to prevent non-productive growth of filaments away from the bacterial surface (Loisel et al., 1999) (Fig. 1.3a). Profilin, which binds actin monomers and enhances depolymerization from pointed ends, increased the rate of movement but was not strictly required. This landmark study laid the foundation for elucidating the chemical and physical bases of force production by actin assembly, which directly informs the cellular process of vesicle rocketing (Marchand et al., 1995; Merrifield et al., 1999; Taunton et al., 2000). Moreover, the Arp2/3 complex nucleation machinery and the signaling molecules involved in ABM are substantially similar to those driving the protrusion of cellular lamellipodia and pseudopodia, although these structures differ from rocketing particles in size and shape (Borisy and Svitkina, 2000; Bugyi et al., 2008; Pollard and Borisy, 2003). Reconstitution of motility also provides a tractable system to dissect the activities of other proteins that regulate actin dynamics. To list just a few examples, it has been used to demonstrate filament capping by twinfilin (Helfer et al., 2006), severing by villin (Revenu et al., 2007), and enhancement of the N-WASP/Arp2/3 interaction by the adaptor protein Grb2 (Carlier et al., 2000).

There is still more to learn from pathogens moving through cytoplasm. Twenty-two years after Tilney's micrographs of *Listeria* ABM, the biophysical mechanisms of its propulsion are still being debated and tested (reviewed in Dickinson, 2009; Mogilner, 2006). Furthermore, alternative mechanisms of ABM have recently been discovered. *Rickettsia* is the first pathogen found to bypass the Arp2/3 complex for ABM (Serio et al., 2010), instead encoding its own formin-like nucleator (Haglund et al., 2010). This type of motility requires a different set of host factors compared to *Listeria* and *Shigella* (Fig. 1.3a). Specifically, profilin and the actin bundling protein T-plastin (fimbrin) are critical for *Rickettsia* ABM (Serio et al., 2010). *Rickettsia* may subvert an endogenous motility pathway driven by formins, as host formins mediate movements

such as the translocation of oocyte chromosomes towards the cortex during meiosis I (Li et al., 2008), and might also play a role in ER positioning in somatic cells (Chhabra et al., 2009). Whether *Rickettsia* ABM will prove useful in understanding the movements of these or other cellular cargoes remains to be determined.

Motility across the membrane

A distinct "surfing" form of ABM is employed by extracellular vaccinia virus and pathogenic strains of *E. coli*, which intercept receptor tyrosine-kinase signaling pathways, resulting in the formation of a moving, actin-rich pedestal beneath the pathogen. Each of these divergent microbes encodes a transmembrane protein that becomes phosphorylated by Src- and Abl-family kinases, leading to recruitment of the adaptor proteins Nck1 and Nck2, which activate N-WASP to stimulate Arp2/3-mediated actin polymerization (Campellone, 2010; Frischknecht et al., 1999) (Fig. 1.3b). Studies of vaccinia and enteropathogenic *E. coli* (EPEC) demonstrated the physiological relevance of the phosphotyrosine/Nck/N-WASP pathway for signaling to actin assembly. Following its elucidation, this pathway was found to be essential in kidney podocytes, signaling through the host receptor nephrin to produce the actin-rich cellular extensions that are critical for kidney filtration function (Jones et al., 2006). In fact, the phoshotyrosine motifs from EPEC can functionally replace those of nephrin (Blasutig et al., 2008). Stimulated T cell antigen receptors (TCRs) also induce actin polymerization by recruiting Nck, although the pathway employs WASP instead of N-WASP (Barda-Saad et al., 2005). Nck also signals to actin downstream of other receptor tyrosine kinases, including neuronal axon guidance receptors such as Ephrin A4 (Fawcett et al., 2007) and growth factor receptors such as PDGF-R (Rivera et al., 2006; Ruusala et al., 2008), although the downstream pathways and potential roles of Nck in axon guidance or growth factor signaling are not clearly defined. Nonetheless, pathogen signaling through Nck/N-WASP is relevant to several endogenous processes.

One recent advance derived from the study of these pathogens is the dynamic interplay between Arp2/3, NPFs, and their regulatory partners. A recent report used vaccinia virus to examine the recruitment and turnover of Nck, N-WASP, WASP interacting protein (WIP), and the adaptor protein Grb2 during actin assembly (Weisswange et al., 2009). Surprisingly, although Nck and WIP are thought to recruit N-WASP, the turnover rate for N-WASP was much slower than for Nck and WIP, implying that other interactions stabilize N-WASP in vaccinia tails. N-WASP stabilization activity was attributed to Grb2 and, more importantly, to the Arp2/3 complex. In fact, N-WASP did not turn over when its ability to stimulate Arp2/3-mediated nucleation was disrupted, suggesting that interaction with the Arp2/3 complex is required to dissociate N-WASP from its binding partners on the surface of the motile particle. Presumably, this requirement applies to N-WASP-mediated nucleation on rocketing vesicles as well, and possibly to Arp2/3 activation by other NPFs, although this remains to be tested. These molecular interactions have implications at the level of virus motility. The rate of N-WASP exchange correlated positively with the rate of virus motility, but inversely with the number of tails, illustrating the need to balance speed with stability to achieve productive motility. The vaccinia system is well-suited to future studies of the dynamic interactions within actin nucleation complexes.

Recent work with EPEC has shed light on membrane phosphoinosotide signals regulating actin assembly. The EPEC protein that recruits Nck, called Tir, also binds host phosphoinositide 3-kinase (PI3K; (Sason et al., 2009; Selbach et al., 2009) and the inositol-5-phosphatase SHIP2

(Smith et al., 2010). The combined activities of these two enzymes can convert $PI(4,5)P_2$ to $PI(3,4)P_2$, the predominant membrane phosophoinositide in stable wild-type pedestals. When SHIP2 recruitment is prevented, $PI(3,4,5)P_3$ accumulates instead, and bacteria are associated with multiple, aberrantly long pedestals (Smith et al., 2010), suggesting that $PI(3,4)P_2$ is required to downregulate signaling after an initial burst of $PI(3,4,5)P_3$-enhanced actin polymerization. The lipid requirements for EPEC pedestal formation might have parallels to endogenous processes. The motif in Tir that recruits SHIP2 is homologous to host immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which also recruit SHIP2-like phosphatases to downregulate signaling from TCRs and other receptors. Conversely, PI3K is recruited during T cell activation (Smith-Garvin et al., 2009) and also during nephrin-mediated actin reorganization (Huber et al., 2003; Zhu et al., 2008). Because EPEC pedestals provide a clear read-out of the extent of actin assembly, they could be useful for investigating the mechanisms by which $PI(3,4)P_2$ downregulates actin assembly.

Enterohemorrhagic *E. coli* (EHEC), while closely related to EPEC, has evolved a distinct mechanism of pedestal formation that has shed light on N-WASP regulation. Instead of recruiting Nck, EHEC Tir recruits the bacterial effector $EspF_U$, which contains 2-6 repeated sequences that directly bind to and activate N-WASP. Dissection of $EspF_U$ demonstrated the critical role of multivalency in N-WASP activation, as the repeated $EspF_U$ peptides are efficient activators only when they can recruit multiple copies of N-WASP (Sallee et al., 2008). In contrast, isolated $EspF_U$ repeats can bind N-WASP, but do not promote robust actin polymerization (Campellone et al., 2008; Sallee et al., 2008). The importance of oligomerization as a universal mode of NPF regulation is supported by in vitro work showing that dimerized NPF activation domains have ~100x greater affinity for (Padrick et al., 2008) and activity towards the Arp2/3 complex (Higgs and Pollard, 2000; Padrick et al., 2008) compared to monomeric NPFs. Oligomerization could also explain results in uninfected cells in which artificial clustering of WASP or an upstream binding partner at the plasma membrane stimulated downstream actin polymerization (Castellano et al., 1999; Rivera et al., 2004).

As with cytoplasmic ABM, more remains to be learned from pathogen motility on the plasma membrane. For instance, even the relatively well-studied molecule EPEC Tir contains peptides whose effects on actin pedestals are not understood (reviewed in Campellone, 2010). Intriguingly, both EPEC and host nephrin employ secondary mechanisms of actin assembly in addition to the primary phosphotyrosine- and Nck-dependent pathway, leaving the possibility that additional parallels exist between the two systems. Finally, EHEC $EspF_U$ can be used to explore how cells generate plasma membrane protrusions independent of tyrosine kinase signaling. Continued study of pathogens that promote ABM across membranes will shed light on signal transduction pathways and actin assembly mechanisms at the cell boundary.

New directions: Pathogen exit and dissemination

Pathogens can quickly consume a host cell's resources and must spread to new cells to continue their life cycle. Although spread can follow host cell lysis, it is often advantageous for a microorganism to exit cells in a controlled, non-lytic manner. Far less is known about molecular mechanisms of dissemination compared to entry or intracellular movement, but several exit modes – protrusion-mediated spread, extrusion of a vacuole, and non-lytic ejection – have been shown to utilize the actin cytoskeleton (Fig. 1.4). It is likely that normal host cell processes are
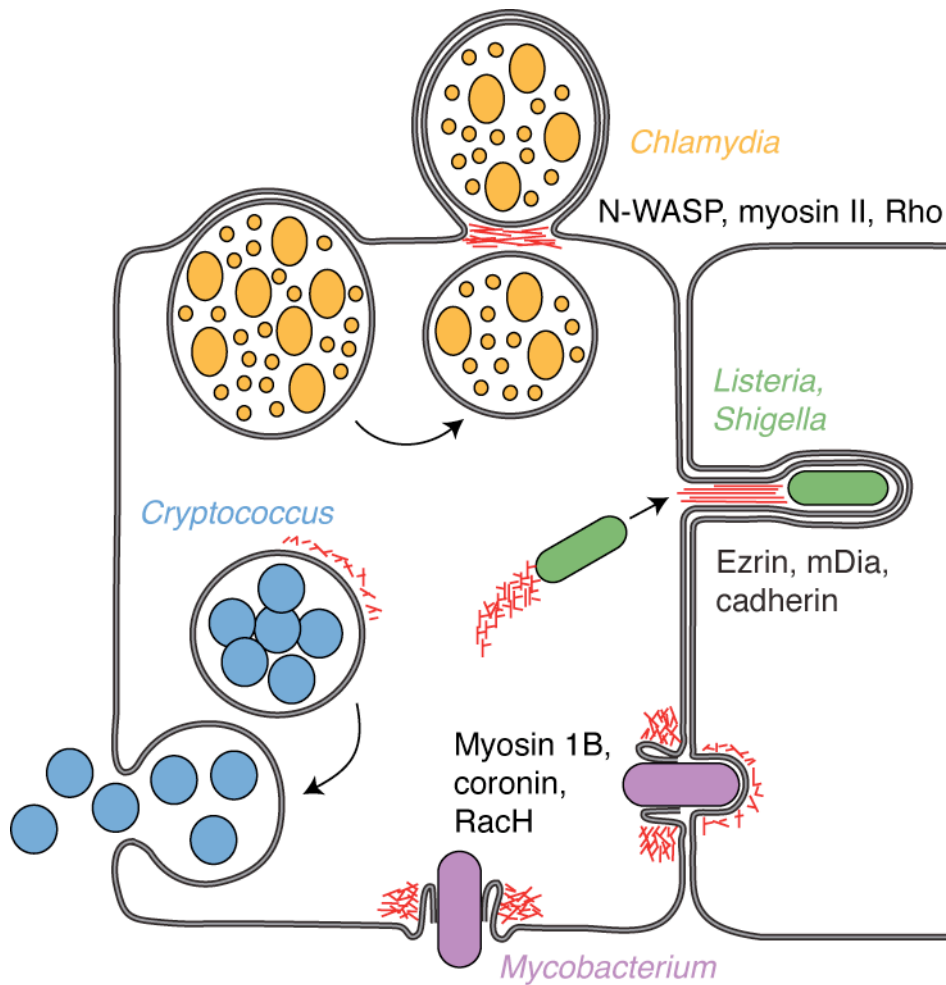
Figure 1.4. Exit strategies of diverse intracellular pathogens use actin. *Chlamydia* (orange) vacuoles are extruded through a cortical constriction, and the plasma membrane seals around the constriction point in an actin-dependent manner, releasing a double-membrane-bound bacterial compartment. *Listeria* (green) and *Shigella*, propelled by actin-based motility, enter plasma membrane protrusions and are taken up by neighboring cells. *Mycobacterium* (purple) exits cells through a plasma membrane break surrounded by a barrel-shaped ejectosome rich in actin, myosin 1B, and coronin. *Cryptococcus* (blue) phagosomes fuse with the plasma membrane, and intermittent actin polymerization around the phagosome inhibits this fusion.

exploited during exit, as during entry and intracellular motility, but these processes are poorly defined. This makes pathogen exit an exciting area for future research.

Bacteria that undergo ABM, such as *Listeria* and *Shigella*, subsequently enter long, plasma-membrane-bound protrusions from infected cells into adjacent cells (Kadurugamuwa et al., 1991; Tilney and Portnoy, 1989), and, about 40 minutes later, escape into the neighboring cell's cytoplasm (Monack and Theriot, 2001; Robbins et al., 1999). Within protrusions, actin tail morphology changes: filaments become longer and more densely bundled (Gouin et al., 1999; Sechi et al., 1997) and more stable (Robbins et al., 1999). The first host factors implicated in protrusion-mediated spread were adherens junction proteins. Vinculin localized to *Shigella* protrusions (Kadurugamuwa et al., 1991), and cadherins contributed to protrusion formation in a manner beyond their role in cell-cell adhesion (Sansonetti et al., 1994). Because cadherin cytoplasmic domains link to actin filaments via vinculin and other junction components, it was speculated that junction proteins might bind actin comet tails, inducing changes to actin filament organization that promote the formation and rigidity of protrusions. Analogously, disruption of ezrin, an actin-membrane linker that localizes to *Listeria* protrusions but not cytoplasmic tails, results in short, crumpled protrusions (Pust et al., 2005). In contrast, the junction protein Tuba, which has N-WASP binding, scaffolding, and GEF activity, negatively regulates protrusion formation (Rajabian et al., 2009), apparently by promoting cortical tension in epithelial cell layers (Otani et al., 2006; Rajabian et al., 2009). *Listeria* has evolved an effector, InlC, that interferes with Tuba:N-WASP binding, relaxing cortical tension and promoting protrusions. Thus, protrusion formation appears to require an initial relaxation of cortical rigidity, followed by promotion of rigidity, presumably by a different set of factors, around the bacterial actin tail. *Listeria* protrusions could serve as a useful, easily-studied structure to characterize host proteins involved in the maintenance of cortical tension.

Recently, the formin-family actin nucleators Dia1 and Dia2 were found to support spreading of *Shigella* (Heindl et al., 2010). As with ezrin, Dia1 and Dia2 localize to actin in protrusions but not to cytoplasmic tails, and disruption of Dia function reduces the frequency and length of protrusions. The involvement of formins, which generate long, unbranched actin filaments, is consistent with the parallel filament bundles found in protrusions. Together, the effects of formins, ezrin, vinculin and cadherin on protrusions but not cytoplasmic tails suggest that a distinct set of actin regulatory factors interacts with motile bacteria after they contact the plasma membrane. More work is required to determine which cellular factors help build protrusions, the order in which they act, and how the transition is regulated. Moreover, how these cytoskeletal alterations promote uptake by neighboring cells is unclear. In uninfected cells, uptake of vesicles derived from neighboring cells has been observed and was named paracytophagy (Robbins et al., 1999). Paracytophagy's role in cells has not been described, but it is possible that study of protrusion-mediated spread could provide clues to the conditions under which it occurs.

Another actin-dependent mode of exit is the extrusion of *Chlamydia*-filled inclusions into the extracellular space (Hybiske and Stephens, 2007). Compartments containing a few to hundreds of *Chlamydia*, surrounded by both inclusion and host plasma membranes, were observed to balloon out from a constriction point in the host cell over the course of several hours, eventually being released into the media. The process requires actin, N-WASP, and myosin II, and the final pinching-off step requires the GTPase Rho. *Chlamydia* have a second, lytic exit strategy, and usually both mechanisms are employed with equal frequency. Curiously, the

authors found that jasplakinolide, which prevents actin depolymerization but not polymerization, abrogated the lytic strategy and quickly induced extrusions, even early in infection when *Chlamydia* do not normally exit. This suggests that robust actin polymerization alone is sufficient to induce extrusion, which implies that it is a host-driven process. However, the endogenous function of extrusion, if any, remains a mystery.

A third actin-dependent mode of exit was recently described for *Mycobacterium marinum* and *M. tuberculosis* (Hagedorn et al., 2009). These bacteria, which spend part of their life cycle free in the host cytoplasm, were observed exiting host amoebae through a barrel-shaped structure rich in actin, myosin 1B, and coronin, which the authors called an ejectosome. The ejection process occurred without long membrane extensions, did not require a comet tail or a recipient cell, and took only a few minutes to complete, distinguishing it from protrusion-mediated spread and extrusion. Ejection resulted in direct breakage of the plasma membrane and exit into either the media or an adjacent cell, which formed a phagocytic-cup-like structure around the invading bacterium. In spite of membrane rupture, leakage of cellular contents did not occur, apparently due to a tight septum formed by the actin ring. The authors proposed that ejectosomes might have originated as a mechanism for plasma membrane resealing. Actin polymerization and actomyosin contraction occur around plasma membrane wounds (Mandato and Bement, 2001), but this process employs myosin 2 (Mandato and Bement, 2001; Togo and Steinhardt, 2004), whereas myosin 2 was not detected at ejectosomes. Thus, the connection between ejectosomes and plasma membrane repair pathways requires further investigation.

A membrane-sealing function might also explain the cycles of transient actin polymerization observed around phagosomes containing the fungal pathogen *Cryptococcus neoformans* (Johnston and May, 2010) or bacteria such as *Listeria* (Yam and Theriot, 2004). In the case of *Cryptococcus,* actin "flashes" follow phagosome permeabilization events, in an Arp2/3-complex- and WASP/N-WASP-dependent manner. *Cryptococcus* exits cells by phagosomal fusion with the plasma membrane, and fusion is usually preceded by phagosome permeabilization. In contrast to the above exit strategies, fungal exit is enhanced when actin polymerization is disrupted, implying that host cells use actin polymerization to limit dissemination. Flashes were also observed on phagosomes containing transferrin-coated beads, on mechanically-induced plasma membrane wounds, and at membrane invaginations around particles too large to phagocytose (Yam and Theriot, 2004), demonstrating that flashing is an endogenous process, induced by membrane breakage and possibly by the presence of large internalized particles. Although dynamic actin accumulates around plasma membrane wounds, the role of actin polymerization on phagosomes is less clear. Actin might contribute to phagosomal membrane repair, or could form a barrier to limit mixing of vesicle contents with cytosol while other repair mechanisms occur. In either case, actin polymerization appears to help maintain the integrity of phagosomes.

Collectively, these reports will certainly lead to further discoveries about the role of actin in ushering pathogens or other particles out of cells, including insights into membrane resealing. As mentioned above, the endogenous pathways exploited during pathogen exit are not defined, and it is currently unknown if other cytoskeletal structures are involved in these processes.

Future Perspectives

Given the numerous ways in which pathogens have contributed to our understanding of the cytoskeleton, it is obvious that future study of pathogen-cytoskeleton interactions will uncover

important new insights. In particular, pathogens might reveal key clues about the role(s) of actin in the nucleus, an area that is just beginning to be explored (Skarp and Vartiainen, 2010). For instance, baculovirus replication requires nuclear translocation and polymerization of actin (Goley et al., 2006). Furthermore, *Anaplasma phagocytophilum* induces phosphorylation of actin in its host, leading to increased nuclear G-actin and phospho-actin-dependent upregulation of a host gene required for bacterial survival (Sultana et al., 2010). These microbes might serve as useful probes for the study of nuclear actin. Pathogens also have additional tricks up their sleeves that were not discussed here, such as destabilization (Coureuil et al., 2009) or reinforcement (Kim et al., 2009) of intercellular junctions, as well as promotion of host cell motility (Worley et al., 2006), suggesting insights into these processes will be forthcoming. Advancements will also come from technological improvements, for example in imaging methods, as well as new approaches, such as systems-level analyses and mathematical modeling. In addition to revealing fundamental cellular mechanisms, future studies of the host-pathogen relationship will enhance our understanding of pathogenesis and disease, and may lead to improved diagnostics and treatments for microbial infections.

References

Aiastui, A., M.G. Pucciarelli, and F. Garcia-del Portillo. 2010. *Salmonella enterica* serovar typhimurium invades fibroblasts by multiple routes differing from the entry into epithelial cells. *Infect Immun*. 78:2700-13.

Bakowski, M.A., V. Braun, and J.H. Brumell. 2008. *Salmonella*-containing vacuoles: directing traffic and nesting to grow. *Traffic*. 9:2022-31.

Barda-Saad, M., A. Braiman, R. Titerence, S.C. Bunnell, V.A. Barr, and L.E. Samelson. 2005. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat Immunol*. 6:80-9.

Blasutig, I.M., L.A. New, A. Thanabalasuriar, T.K. Dayarathna, M. Goudreault, S.E. Quaggin, S.S. Li, S. Gruenheid, N. Jones, and T. Pawson. 2008. Phosphorylated YDXV motifs and Nck SH2/SH3 adaptors act cooperatively to induce actin reorganization. *Mol Cell Biol*. 28:2035-46.

Borisy, G.G., and T.M. Svitkina. 2000. Actin machinery: pushing the envelope. *Curr Opin Cell Biol*. 12:104-12.

Boucrot, E., T. Henry, J.P. Borg, J.P. Gorvel, and S. Meresse. 2005. The intracellular fate of *Salmonella* depends on the recruitment of kinesin. *Science*. 308:1174-8.

Bugyi, B., C. Le Clainche, G. Romet-Lemonne, and M.F. Carlier. 2008. How do in vitro reconstituted actin-based motility assays provide insight into in vivo behavior? *FEBS Lett*. 582:2086-92.

Campellone, K.G. 2010. Cytoskeleton-modulating effectors of enteropathogenic and enterohaemorrhagic Escherichia coli: Tir, EspFU and actin pedestal assembly. *FEBS J*. 277:2390-402.

Campellone, K.G., H.C. Cheng, D. Robbins, A.D. Siripala, E.J. McGhie, R.D. Hayward, M.D. Welch, M.K. Rosen, V. Koronakis, and J.M. Leong. 2008. Repetitive N-WASP-binding elements of the enterohemorrhagic *Escherichia coli* effector EspF(U) synergistically activate actin assembly. *PLoS Pathog*. 4:e1000191.

Carlier, M.F., P. Nioche, I. Broutin-L'Hermite, R. Boujemaa, C. Le Clainche, C. Egile, C. Garbay, A. Ducruix, P. Sansonetti, and D. Pantaloni. 2000. GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. *J Biol Chem*. 275:21946-52.

Castellano, F., P. Montcourrier, J.C. Guillemot, E. Gouin, L. Machesky, P. Cossart, and P. Chavrier. 1999. Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr Biol*. 9:351-60.

Chhabra, E.S., V. Ramabhadran, S.A. Gerber, and H.N. Higgs. 2009. INF2 is an endoplasmic reticulum-associated formin protein. *J Cell Sci*. 122:1430-40.

Chang, L., and R.D. Goldman. 2004. Intermediate filaments mediate cytoskeletal crosstalk. *Nat Rev Mol Cell Biol*. 5:601-13.

Chi, F., T.D. Jong, L. Wang, Y. Ouyang, C. Wu, W. Li, and S.H. Huang. 2010. Vimentin-mediated signalling is required for IbeA+ *E. coli* K1 invasion of human brain microvascular endothelial cells. *Biochem J*. 427:79-90.

Colucci-Guyon, E., F. Niedergang, B.J. Wallar, J. Peng, A.S. Alberts, and P. Chavrier. 2005. A role for mammalian diaphanous-related formins in complement receptor (CR3)-mediated phagocytosis in macrophages. *Curr Biol*. 15:2007-12.

Cossart, P., and P.J. Sansonetti. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science*. 304:242-8.

Coureuil, M., G. Mikaty, F. Miller, H. Lecuyer, C. Bernard, S. Bourdoulous, G. Dumenil, R.M. Mege, B.B. Weksler, I.A. Romero, P.O. Couraud, and X. Nassif. 2009. Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science*. 325:83-7.

Cudmore, S., P. Cossart, G. Griffiths, and M. Way. 1995. Actin-based motility of vaccinia virus. *Nature*. 378:636-8.

Damm, E.M., and L. Pelkmans. 2006. Systems biology of virus entry in mammalian cells. *Cell Microbiol*. 8:1219-27.

Dhakal, B.K., and M.A. Mulvey. 2009. Uropathogenic *Escherichia coli* invades host cells via an HDAC6-modulated microtubule-dependent pathway. *J Biol Chem*. 284:446-54.

Dickinson, R.B. 2009. Models for actin polymerization motors. *J Math Biol*. 58:81-103.

Didsbury, J., R.F. Weber, G.M. Bokoch, T. Evans, and R. Snyderman. 1989. rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J Biol Chem*. 264:16378-82.

Dodding, M.P., and M. Way. 2009. Nck- and N-WASP-dependent actin-based motility is conserved in divergent vertebrate poxviruses. *Cell Host Microbe*. 6:536-50.

Donnenberg, M.S., A. Donohue-Rolfe, and G.T. Keusch. 1990. A comparison of HEp-2 cell invasion by enteropathogenic and enteroinvasive *Escherichia coli*. *FEMS Microbiol Lett*. 57:83-6.

Dumont, A., E. Boucrot, S. Drevensek, V. Daire, J.P. Gorvel, C. Pous, D.W. Holden, and S. Meresse. 2010. SKIP, the host target of the Salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges. *Traffic*. 11:899-911.

Egile, C., T.P. Loisel, V. Laurent, R. Li, D. Pantaloni, P.J. Sansonetti, and M.F. Carlier. 1999. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J Cell Biol*. 146:1319-32.

Fawcett, J.P., J. Georgiou, J. Ruston, F. Bladt, A. Sherman, N. Warner, B.J. Saab, R. Scott, J.C. Roder, and T. Pawson. 2007. Nck adaptor proteins control the organization of neuronal circuits important for walking. *Proc Natl Acad Sci U S A*. 104:20973-8.

Francis, C.L., T.A. Ryan, B.D. Jones, S.J. Smith, and S. Falkow. 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature*. 364:639-42.

Frischknecht, F., V. Moreau, S. Röttger, S. Gonfloni, I. Reckmann, G. Superti-Furga, and M. Way. 1999. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature*. 401:926-9.

Fu, Y., and J.E. Galan. 1999. A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature*. 401:293-7.

Gilden, J., and M.F. Krummel. 2010. Control of cortical rigidity by the cytoskeleton: emerging roles for septins. *Cytoskeleton (Hoboken)*. 67:477-86.

Goley, E.D., T. Ohkawa, J. Mancuso, J.B. Woodruff, J.A. D'Alessio, W.Z. Cande, L.E. Volkman, and M.D. Welch. 2006. Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein. *Science*. 314:464-7.

Goley, E.D., and M.D. Welch. 2006. The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol*. 7:713-26.

Gouin, E., H. Gantelet, C. Egile, I. Lasa, H. Ohayon, V. Villiers, P. Gounon, P.J. Sansonetti, and P. Cossart. 1999. A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia conorii*. *J Cell Sci*. 112:1697-708.

Guignot, J., and A.L. Servin. 2008. Maintenance of the *Salmonella*-containing vacuole in the juxtanuclear area: a role for intermediate filaments. *Microb Pathog*. 45:415-22.

Guzman, C.A., M. Rohde, T. Chakraborty, E. Domann, M. Hudel, J. Wehland, and K.N. Timmis. 1995. Interaction of *Listeria monocytogenes* with mouse dendritic cells. *Infect Immun*. 63:3665-73.

Hagedorn, M., K.H. Rohde, D.G. Russell, and T. Soldati. 2009. Infection by tubercular mycobacteria is spread by nonlytic ejection from their amoeba hosts. *Science*. 323:1729-33.

Haglund, C.M., J.E. Choe, C.T. Skau, D.R. Kovar, and M.D. Welch. 2010. *Rickettsia* Sca2 is a bacterial formin-like mediator of actin-based motility. *Nat Cell Biol*. 12:1057-63.

Hanisch, J., J. Ehinger, M. Ladwein, M. Rohde, E. Derivery, T. Bosse, A. Steffen, D. Bumann, B. Misselwitz, W.D. Hardt, A. Gautreau, T.E. Stradal, and K. Rottner. 2010. Molecular dissection of *Salmonella*-induced membrane ruffling versus invasion. *Cell Microbiol*. 12:84-98.

Haraga, A., M.B. Ohlson, and S.I. Miller. 2008. Salmonellae interplay with host cells. *Nat Rev Microbiol*. 6:53-66.

Hardt, W.D., L.M. Chen, K.E. Schuebel, X.R. Bustelo, and J.E. Galan. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*. 93:815-26.

Hayward, R.D., and V. Koronakis. 1999. Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. *EMBO J*. 18:4926-34.

Heindl, J.E., I. Saran, C.R. Yi, C.F. Lesser, and M.B. Goldberg. 2010. Requirement for formin-induced actin polymerization during spread of *Shigella flexneri*. *Infect Immun*. 78:193-203.

Helfer, E., E.M. Nevalainen, P. Naumanen, S. Romero, D. Didry, D. Pantaloni, P. Lappalainen, and M.F. Carlier. 2006. Mammalian twinfilin sequesters ADP-G-actin and caps filament barbed ends: implications in motility. *EMBO J*. 25:1184-95.

Henry, T., C. Couillault, P. Rockenfeller, E. Boucrot, A. Dumont, N. Schroeder, A. Hermant, L.A. Knodler, P. Lecine, O. Steele-Mortimer, J.P. Borg, J.P. Gorvel, and S. Meresse. 2006. The *Salmonella* effector protein PipB2 is a linker for kinesin-1. *Proc Natl Acad Sci U S A*. 103:13497-502.

Herrmann, H., M. Haner, M. Brettel, S.A. Muller, K.N. Goldie, B. Fedtke, A. Lustig, W.W. Franke, and U. Aebi. 1996. Structure and assembly properties of the intermediate filament protein vimentin: the role of its head, rod and tail domains. *J Mol Biol*. 264:933-53.

Higgs, H.N., and T.D. Pollard. 2000. Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol*. 150:1311-20.

Huber, T.B., B. Hartleben, J. Kim, M. Schmidts, B. Schermer, A. Keil, L. Egger, R.L. Lecha, C. Borner, H. Pavenstadt, A.S. Shaw, G. Walz, and T. Benzing. 2003. Nephrin and CD2AP

associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol*. 23:4917-28.

Hybiske, K., and R.S. Stephens. 2007. Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc Natl Acad Sci U S A*. 104:11430-5.

Jackson, L.K., P. Nawabi, C. Hentea, E.A. Roark, and K. Haldar. 2008. The *Salmonella* virulence protein SifA is a G protein antagonist. *Proc Natl Acad Sci U S A*. 105:14141-6.

Johnston, S.A., and R.C. May. 2010. The human fungal pathogen *Cryptococcus neoformans* escapes macrophages by a phagosome emptying mechanism that is inhibited by Arp2/3 complex-mediated actin polymerisation. *PLoS Pathog*. 6.

Jones, N., I.M. Blasutig, V. Eremina, J.M. Ruston, F. Bladt, H. Li, H. Huang, L. Larose, S.S. Li, T. Takano, S.E. Quaggin, and T. Pawson. 2006. Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature*. 440:818-23.

Kadurugamuwa, J.L., M. Rohde, J. Wehland, and K.N. Timmis. 1991. Intercellular spread of *Shigella flexneri* through a monolayer mediated by membranous protrusions and associated with reorganization of the cytoskeletal protein vinculin. *Infect Immun*. 59:3463-71.

Kim, M., M. Ogawa, Y. Fujita, Y. Yoshikawa, T. Nagai, T. Koyama, S. Nagai, A. Lange, R. Fassler, and C. Sasakawa. 2009. Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment. *Nature*. 459:578-82.

Kinch, L.N., M.L. Yarbrough, K. Orth, and N.V. Grishin. 2009. Fido, a novel AMPylation domain common to fic, doc, and AvrB. *PLoS One*. 4:e5818.

Kubori, T., and J.E. Galan. 2003. Temporal regulation of *Salmonella* virulence effector function by proteasome-dependent protein degradation. *Cell*. 115:333-42.

Kuhn, M. 1998. The microtubule depolymerizing drugs nocodazole and colchicine inhibit the uptake of *Listeria monocytogenes* by P388D1 macrophages. *FEMS Microbiol Lett*. 160:87-90.

Kumar, Y., and R.H. Valdivia. 2008. Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. *Cell Host Microbe*. 4:159-69.

Lang, A.E., G. Schmidt, A. Schlosser, T.D. Hey, I.M. Larrinua, J.J. Sheets, H.G. Mannherz, and K. Aktories. 2010. *Photorhabdus luminescens* toxins ADP-ribosylate actin and RhoA to force actin clustering. *Science*. 327:1139-42.

Li, H., F. Guo, B. Rubinstein, and R. Li. 2008. Actin-driven chromosomal motility leads to symmetry breaking in mammalian meiotic oocytes. *Nat Cell Biol*. 10:1301-8.

Li, R., and G.G. Gundersen. 2008. Beyond polymer polarity: how the cytoskeleton builds a polarized cell. *Nat Rev Mol Cell Biol*. 9:860-73.

Lilic, M., V.E. Galkin, A. Orlova, M.S. VanLoock, E.H. Egelman, and C.E. Stebbins. 2003. *Salmonella* SipA polymerizes actin by stapling filaments with nonglobular protein arms. *Science*. 301:1918-21.

Loisel, T.P., R. Boujemaa, D. Pantaloni, and M.F. Carlier. 1999. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature*. 401:613-6.

Mandato, C.A., and W.M. Bement. 2001. Contraction and polymerization cooperate to assemble and close actomyosin rings around Xenopus oocyte wounds. *J Cell Biol*. 154:785-97.

Marchand, J.B., P. Moreau, A. Paoletti, P. Cossart, M.F. Carlier, and D. Pantaloni. 1995. Actin-based movement of *Listeria monocytogenes*: actin assembly results from the local

maintenance of uncapped filament barbed ends at the bacterium surface. *J Cell Biol*. 130:331-43.

May, R.C., E. Caron, A. Hall, and L.M. Machesky. 2000. Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. *Nat Cell Biol*. 2:246-8.

McGhie, E.J., L.C. Brawn, P.J. Hume, D. Humphreys, and V. Koronakis. 2009. *Salmonella* takes control: effector-driven manipulation of the host. *Curr Opin Microbiol*. 12:117-24.

McGhie, E.J., R.D. Hayward, and V. Koronakis. 2004. Control of actin turnover by a *Salmonella* invasion protein. *Mol Cell*. 13:497-510.

Merrifield, C.J., S.E. Moss, C. Ballestrem, B.A. Imhof, G. Giese, I. Wunderlich, and W. Almers. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. *Nat Cell Biol*. 1:72-4.

Miserey-Lenkei, S., G. Chalancon, S. Bardin, E. Formstecher, B. Goud, and A. Echard. 2010. Rab and actomyosin-dependent fission of transport vesicles at the Golgi complex. *Nat Cell Biol*. 12:645-54.

Mogilner, A. 2006. On the edge: modeling protrusion. *Curr Opin Cell Biol*. 18:32-9.

Monack, D.M., and J.A. Theriot. 2001. Actin-based motility is sufficient for bacterial membrane protrusion formation and host cell uptake. *Cell Microbiol*. 3:633-47.

Mostowy, S., M. Bonazzi, M.A. Hamon, T.N. Tham, A. Mallet, M. Lelek, E. Gouin, C. Demangel, R. Brosch, C. Zimmer, A. Sartori, M. Kinoshita, M. Lecuit, and P. Cossart. 2010. Entrapment of intracytosolic bacteria by septin cage-like structures. *Cell Host Microbe*. 8:433-44.

Mostowy, S., T. Nam Tham, A. Danckaert, S. Guadagnini, S. Boisson-Dupuis, J. Pizarro-Cerda, and P. Cossart. 2009. Septins regulate bacterial entry into host cells. *PLoS One*. 4:e4196.

Murli, S., R.O. Watson, and J.E. Galan. 2001. Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of *Salmonella* with host cells. *Cell Microbiol*. 3:795-810.

Oelschlaeger, T.A., P. Guerry, and D.J. Kopecko. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci U S A*. 90:6884-8.

Ohkawa, T., L.E. Volkman, and M.D. Welch. 2010. Actin-based motility drives baculovirus transit to the nucleus and cell surface. *J Cell Biol*. 190:187-95.

Ohlson, M.B., Z. Huang, N.M. Alto, M.P. Blanc, J.E. Dixon, J. Chai, and S.I. Miller. 2008. Structure and function of *Salmonella* SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation. *Cell Host Microbe*. 4:434-46.

Otani, T., T. Ichii, S. Aono, and M. Takeichi. 2006. Cdc42 GEF Tuba regulates the junctional configuration of simple epithelial cells. *J Cell Biol*. 175:135-46.

Padrick, S.B., H.C. Cheng, A.M. Ismail, S.C. Panchal, L.K. Doolittle, S. Kim, B.M. Skehan, J. Umetani, C.A. Brautigam, J.M. Leong, and M.K. Rosen. 2008. Hierarchical regulation of WASP/WAVE proteins. *Mol Cell*. 32:426-38.

Patel, J.C., K. Hueffer, T.T. Lam, and J.E. Galan. 2009. Diversification of a *Salmonella* virulence protein function by ubiquitin-dependent differential localization. *Cell*. 137:283-94.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-65.

Pust, S., H. Morrison, J. Wehland, A.S. Sechi, and P. Herrlich. 2005. *Listeria monocytogenes* exploits ERM protein functions to efficiently spread from cell to cell. *EMBO J*. 24:1287-300.

Rajabian, T., B. Gavicherla, M. Heisig, S. Muller-Altrock, W. Goebel, S.D. Gray-Owen, and K. Ireton. 2009. The bacterial virulence factor InlC perturbs apical cell junctions and promotes cell-to-cell spread of *Listeria*. *Nat Cell Biol*. 11:1212-8.

Revenu, C., M. Courtois, A. Michelot, C. Sykes, D. Louvard, and S. Robine. 2007. Villin severing activity enhances actin-based motility in vivo. *Mol Biol Cell*. 18:827-38.

Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 70:389-99.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401-10.

Rivera, G.M., S. Antoku, S. Gelkop, N.Y. Shin, S.K. Hanks, T. Pawson, and B.J. Mayer. 2006. Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. *Proc Natl Acad Sci U S A*. 103:9536-41.

Rivera, G.M., C.A. Briceno, F. Takeshima, S.B. Snapper, and B.J. Mayer. 2004. Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. *Curr Biol*. 14:11-22.

Robbins, J.R., A.I. Barth, H. Marquis, E.L. de Hostos, W.J. Nelson, and J.A. Theriot. 1999. *Listeria monocytogenes* exploits normal host cell processes to spread from cell to cell. *J Cell Biol*. 146:1333-50.

Rodriguez, O.C., A.W. Schaefer, C.A. Mandato, P. Forscher, W.M. Bement, and C.M. Waterman-Storer. 2003. Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat Cell Biol*. 5:599-609.

Rottner, K., J. Hanisch, and K.G. Campellone. 2010. WASH, WHAMM and JMY: regulation of Arp2/3 complex and beyond. *Trends Cell Biol*. 20:650-61.

Ruusala, A., T. Pawson, C.H. Heldin, and P. Aspenstrom. 2008. Nck adapters are involved in the formation of dorsal ruffles, cell migration, and Rho signaling downstream of the platelet-derived growth factor beta receptor. *J Biol Chem*. 283:30034-44.

Sallee, N.A., G.M. Rivera, J.E. Dueber, D. Vasilescu, R.D. Mullins, B.J. Mayer, and W.A. Lim. 2008. The pathogen protein EspF(U) hijacks actin polymerization using mimicry and multivalency. *Nature*. 454:1005-8.

Sansonetti, P.J., J. Mounier, M.C. Prevost, and R.M. Mege. 1994. Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. *Cell*. 76:829-39.

Sason, H., M. Milgrom, A.M. Weiss, N. Melamed-Book, T. Balla, S. Grinstein, S. Backert, I. Rosenshine, and B. Aroeti. 2009. Enteropathogenic *Escherichia coli* subverts phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate upon epithelial cell infection. *Mol Biol Cell*. 20:544-55.

Schwan, C., B. Stecher, T. Tzivelekidis, M. van Ham, M. Rohde, W.D. Hardt, J. Wehland, and K. Aktories. 2009. *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog*. 5:e1000626.

Sechi, A.S., J. Wehland, and J.V. Small. 1997. The isolated comet tail pseudopodium of *Listeria monocytogenes*: a tail of two actin filament populations, long and axial and short and random. *J Cell Biol*. 137:155-67.

Selbach, M., F.E. Paul, S. Brandt, P. Guye, O. Daumke, S. Backert, C. Dehio, and M. Mann. 2009. Host cell interactome of tyrosine-phosphorylated bacterial proteins. *Cell Host Microbe*. 5:397-403.

Serio, A.W., R.L. Jeng, C.M. Haglund, S.C. Reed, and M.D. Welch. 2009. Defining a core set of actin cytoskeletal proteins critical for actin-based motility of *Rickettsia*. *Cell Host Microbe*. 7:388-98.

Skarp, K.P., and M.K. Vartiainen. 2010. Actin on DNA-an ancient and dynamic relationship. *Cytoskeleton (Hoboken)*. 67:487-95.

Smith, K., D. Humphreys, P.J. Hume, and V. Koronakis. 2010. Enteropathogenic *Escherichia coli* recruits the cellular inositol phosphatase SHIP2 to regulate actin-pedestal formation. *Cell Host Microbe*. 7:13-24.

Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan. 2009. T cell activation. *Annu Rev Immunol*. 27:591-619.

Spiliotis, E.T. 2010. Regulation of microtubule organization and functions by septin GTPases. *Cytoskeleton (Hoboken)*. 67:339-45.

Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W.D. Hardt. 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. *Mol Microbiol*. 36:1206-21.

Sukumaran, B., J.E. Mastronunzio, S. Narasimhan, S. Fankhauser, P.D. Uchil, R. Levy, M. Graham, T.M. Colpitts, C.F. Lesser, and E. Fikrig. 2011. *Anaplasma phagocytophilum* AptA modulates Erk1/2 signalling. *Cell Microbiol*. 13:47-61.

Sultana, H., G. Neelakanta, F.S. Kantor, S.E. Malawista, D. Fish, R.R. Montgomery, and E. Fikrig. 2010. *Anaplasma phagocytophilum* induces actin phosphorylation to selectively regulate gene transcription in Ixodes scapularis ticks. *J Exp Med*. 207:1727-43.

Szeto, J., A. Namolovan, S.E. Osborne, B.K. Coombes, and J.H. Brumell. 2009. *Salmonella*-containing vacuoles display centrifugal movement associated with cell-to-cell transfer in epithelial cells. *Infect Immun*. 77:996-1007.

Taunton, J., B.A. Rowning, M.L. Coughlin, M. Wu, R.T. Moon, T.J. Mitchison, and C.A. Larabell. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. *J Cell Biol*. 148:519-30.

Terebiznik, M.R., O.V. Vieira, S.L. Marcus, A. Slade, C.M. Yip, W.S. Trimble, T. Meyer, B.B. Finlay, and S. Grinstein. 2002. Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by *Salmonella*. *Nat Cell Biol*. 4:766-73.

Theriot, J.A., T.J. Mitchison, L.G. Tilney, and D.A. Portnoy. 1992. The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. *Nature*. 357:257-60.

Tilney, L.G., and D.A. Portnoy. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J Cell Biol*. 109:1597-608.

Togo, T., and R.A. Steinhardt. 2004. Nonmuscle myosin IIA and IIB have distinct functions in the exocytosis-dependent process of cell membrane repair. *Mol Biol Cell*. 15:688-95.

Veiga, E., and P. Cossart. 2005. *Listeria* hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat Cell Biol*. 7:894-900.

Veiga, E., J.A. Guttman, M. Bonazzi, E. Boucrot, A. Toledo-Arana, A.E. Lin, J. Enninga, J. Pizarro-Cerda, B.B. Finlay, T. Kirchhausen, and P. Cossart. 2007. Invasive and adherent bacterial pathogens co-opt host clathrin for infection. *Cell Host Microbe*. 2:340-51.

Visvikis, O., M.P. Maddugoda, and E. Lemichez. 2010. Direct modifications of Rho proteins: deconstructing GTPase regulation. *Biol Cell*. 102:377-89.

Wasylnka, J.A., M.A. Bakowski, J. Szeto, M.B. Ohlson, W.S. Trimble, S.I. Miller, and J.H. Brumell. 2008. Role for myosin II in regulating positioning of *Salmonella*-containing vacuoles and intracellular replication. *Infect Immun*. 76:2722-35.

Weisswange, I., T.P. Newsome, S. Schleich, and M. Way. 2009. The rate of N-WASP exchange limits the extent of ARP2/3-complex-dependent actin-based motility. *Nature*. 458:87-91.

Welch, M.D., A. Iwamatsu, and T.J. Mitchison. 1997. Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature*. 385:265-9.

Welch, M.D., J. Rosenblatt, J. Skoble, D.A. Portnoy, and T.J. Mitchison. 1998. Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science*. 281:105-8.

Wileman, T. 2007. Aggresomes and pericentriolar sites of virus assembly: cellular defense or viral design? *Annu Rev Microbiol*. 61:149-67.

Worby, C.A., S. Mattoo, R.P. Kruger, L.B. Corbeil, A. Koller, J.C. Mendez, B. Zekarias, C. Lazar, and J.E. Dixon. 2009. The fic domain: regulation of cell signaling by adenylylation. *Mol Cell*. 34:93-103.

Worley, M.J., G.S. Nieman, K. Geddes, and F. Heffron. 2006. *Salmonella typhimurium* disseminates within its host by manipulating the motility of infected cells. *Proc Natl Acad Sci U S A*. 103:17915-20.

Yam, P.T., and J.A. Theriot. 2004. Repeated cycles of rapid actin assembly and disassembly on epithelial cell phagosomes. *Mol Biol Cell*. 15:5647-58.

Yarbrough, M.L., Y. Li, L.N. Kinch, N.V. Grishin, H.L. Ball, and K. Orth. 2009. AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. *Science*. 323:269-72.

Zhou, D., L.M. Chen, L. Hernandez, S.B. Shears, and J.E. Galan. 2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol Microbiol*. 39:248-59.

Zhou, D., M.S. Mooseker, and J.E. Galan. 1999. Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. *Science*. 283:2092-5.

Zhu, J., N. Sun, L. Aoudjit, H. Li, H. Kawachi, S. Lemay, and T. Takano. 2008. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. *Kidney Int*. 73:556-66.

CHAPTER 2

Rickettsia parkeri Sca2 is a formin-like actin nucleation and elongation factor

Introduction

Diverse intracellular pathogens subvert the host actin polymerization machinery to drive movement within and between cells during infection. *Rickettsia* in the spotted fever group (SFG) are Gram-negative, obligate intracellular bacterial pathogens that undergo actin-based motility and assemble distinctive comet tails consisting of long, unbranched actin filaments (Gouin et al., 1999; Van Kirk et al., 2000). Despite this distinct organization, it was proposed that actin in *Rickettsia* comet tails was nucleated by the host Arp2/3 complex and the bacterial protein RickA, which assemble branched actin networks (Gouin et al., 1999; Jeng et al., 2004). However, a second bacterial gene, *sca2*, was recently implicated in actin tail formation by *R. rickettsii* (Kleba et al., 2010). In this chapter, we demonstrate that Sca2 represents a new class of bacterial actin assembly factor that functionally mimics eukaryotic formin proteins. Sca2 nucleates unbranched actin filaments, processively associates with growing barbed ends, requires profilin for efficient elongation, and inhibits the activity of capping protein, all properties shared with formins. Sca2 localizes to the *Rickettsia* surface and is sufficient to promote the assembly of actin filaments in cytoplasmic extract. These results suggest that Sca2 mimics formins to determine the unique organization of actin filaments in *Rickettsia* tails and drive bacterial motility, independently of host nucleators.

The geometry of actin filaments in eukaryotic cells is specified by nucleation and elongation factors. These include the Arp2/3 complex together with its nucleation-promoting factors (NPFs) that assemble branched networks, and formins and tandem-monomer-binding proteins that assemble unbranched filaments (Campellone and Welch, 2010). The Arp2/3 complex is essential for the motility of diverse microbial pathogens, including *Listeria monocytogenes*, *Shigella flexneri*, and vaccinia virus, which each express a factor that mimics or recruits host NPFs (Gouin et al., 2005). These pathogens have been useful tools for investigating the assembly of branched actin arrays, such as those in cellular lamellipodia. Although many *Rickettsia* species express the NPF RickA (Gouin et al., 2004; Jeng et al., 2004), the failure to observe Arp2/3 complex subunits in *Rickettsia* comet tails (Gouin et al., 1999; Harlander et al., 2003; Heinzen, 2003; Serio et al., 2010), the ability of *Rickettsia* to undergo motility in cells in which Arp2/3 is inhibited (Harlander et al., 2003; Heinzen, 2003; Serio et al., 2010), and the unbranched organization of *Rickettsia* tails point to an Arp2/3-independent polymerization mechanism. Other bacterial pathogens express tandem-monomer-binding nucleators, including VopF from *Vibrio cholerae* (Tam et al., 2007), VopL from *Vibrio parahaemolyticus* (Liverman et al., 2007), and TARP from *Chlamydia trachomatis* (Jewett et al., 2006; Jewett et al., 2010). However, these secreted effectors are not implicated in actin-based motility. Because SFG *Rickettsia* assemble distinctive parallel actin filament arrays to drive motility, they could be a model for investigating the forces that such arrays impart on intracellular cargo, or for studying actin assembly pathways in cellular structures such as filopodia and microvilli.

Results and Discussion

To identify bacterial factors that contribute to actin nucleation, we searched translated *Rickettsia* genome databases for proteins with WASP homology 2 (WH2) motifs, which are actin-binding peptides in NPFs, some formins, and tandem-monomer-binding nucleators (Campellone and Welch, 2010). We identified WH2 motifs in *Rickettsia* Sca2 (surface cell antigen 2), a protein that was recently implicated in *R. conorii* invasion of mammalian cells (Cardwell and Martinez, 2009) and, importantly, in *R. rickettsii* actin tail formation and virulence (Kleba et al., 2010). Sca2 has a conserved autotransporter domain, predicted to anchor it in the outer membrane, and a large passenger domain, predicted to be exposed on the bacterial surface (Ngwamidiba et al., 2005) (Fig. 2.1a). In *R. parkeri*, a representative SFG species, Sca2 contains a central cluster of three putative WH2 motifs, reminiscent of tandem-monomer-binding nucleators (Fig. 2.1a, b; these putative WH2 motifs differ from those proposed by Kleba et al. (Kleba et al., 2010). This WH2 cluster is flanked by two proline-rich domains (PRDs) that are predicted to interact with the actin monomer-binding protein profilin (Holt and Koffer, 2001), similar to formin homology 1 (FH1) domains of formins (Fig. 2.1c). Thus, Sca2 shares sequence motifs with both formins and tandem-monomer-binding nucleators.

We therefore investigated whether purified recombinant *R. parkeri* Sca2 passenger domain (GST-Sca2) affected the assembly kinetics of pyrene-labeled actin in vitro. In this assay, Sca2 passenger domain exhibited dose-dependent nucleation activity (Fig. 2.2a, b), eliminating the lag phase of polymerization (Fig. 2.2a, left panel). Similar kinetics were observed using Sca2 lacking the GST tag (not shown). The intrinsic nucleating activity of Sca2 distinguishes it from RickA, which requires host Arp2/3 complex to nucleate actin (Gouin et al., 2004; Jeng et al., 2004). The combination of purified Sca2 and RickA assembled actin with the same kinetics as Sca2 alone (Fig. 2.2c), indicating that RickA does not affect nucleation by Sca2.

To map the domains in Sca2 responsible for nucleation, we tested the activity of truncated Sca2 derivatives (Fig. 2.1a). A fragment containing the N-terminus, WH2 cluster, and PRDs (GST-Sca2-1106) nucleated actin assembly (Fig. 2.2d). However, it was less potent than GST-Sca2 and lost activity at salt concentrations above 50 mM, suggesting that the missing repetitive sequences are necessary for optimal activity. The Sca2 N-terminal domain (GST-Sca2-670) also displayed weak nucleation activity, with slightly different assembly kinetics compared to GST-Sca2-N1106 (Fig. 2.2e). In contrast, a derivative containing only the putative WH2 cluster and PRDs (GST-Sca2-646-1106) caused dose-dependent inhibition of polymerization (Fig. 2.2f). Potent inhibition required concentrations approaching that of actin, suggesting that it bound and sequestered actin monomers. The failure of the WH2 cluster to nucleate actin implies that Sca2 does not belong to the tandem-monomer-binding class of nucleators, distinguishing it from VopF, VopL, and TARP. Collectively, these results indicate that efficient actin nucleation requires the N-terminal domain, WH2 cluster, and PRDs.

Two features of the bulk polymerization kinetics in the presence of GST-Sca2 and GST-Sca2-1106 suggested that Sca2 might affect actin assembly at fast-growing barbed ends, a property of formins. First, reactions containing low concentrations of Sca2 reached steady state more slowly than reactions lacking Sca2. Second, all concentrations of Sca2 lowered the steady-state level of actin polymer, suggesting that Sca2 raises the critical concentration for actin assembly (Cc). We measured the effect of Sca2 on the Cc by polymerizing actin overnight in the presence of a range of GST-Sca2 concentrations (Fig. 2.3a). Saturating concentrations of GST-Sca2 raised the Cc of actin to 0.7 μM, approximately that of pointed ends, suggesting that Sca2
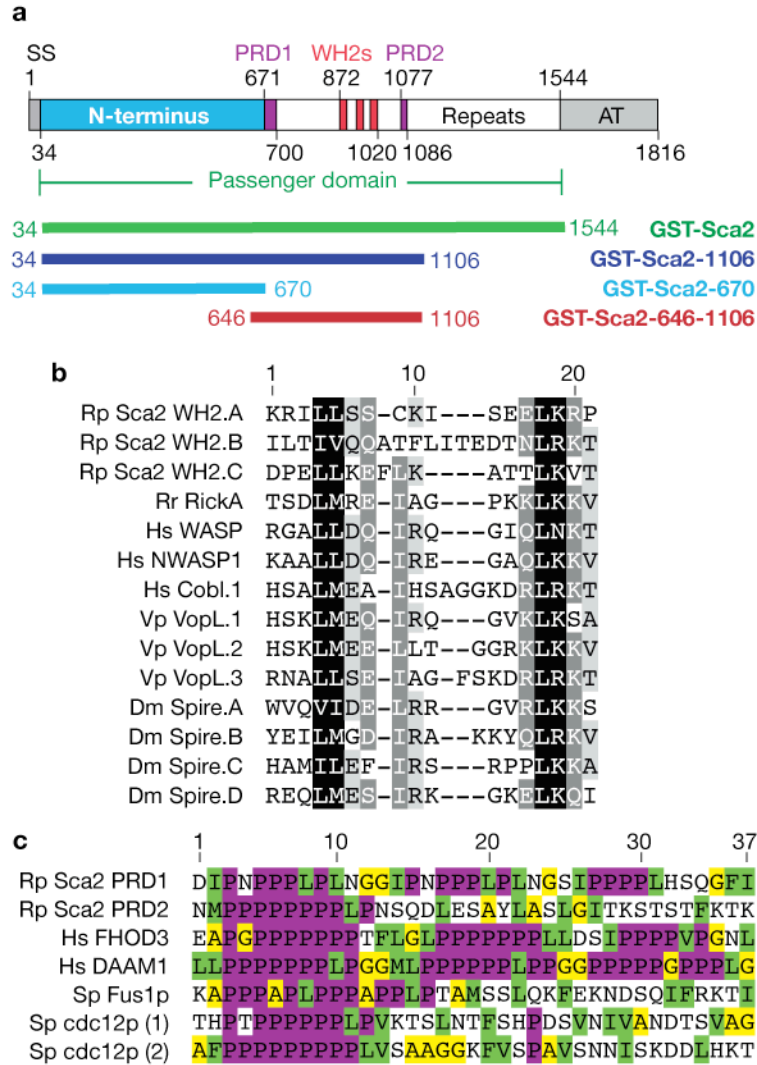
Figure 2.1. Sca2 shares sequence motifs with actin assembly factors. (**a**) Domain organization of *R. parkeri* Sca2 and truncation derivatives. SS = signal sequence, PRD = proline-rich domain, WH2 = WASP homology 2, AT = autotransporter. (**b**) Alignment of putative WH2 motifs in *R. parkeri* Sca2 with WH2 domains in actin nucleating proteins from various species (Hs = *Homo sapiens*, Vp = *Vibrio parahaemolyticus*, Dm = *Drosophila melanogaster*). Similarity shading is based on the BLOSUM45 matrix. (**c**) Alignment of the proline-rich domains (PRDs) of *R. parkeri* Sca2 with FH1 domains in representative formins (Sp = *Schizosaccharomyces pombe*). Proline residues are shaded in purple, hydrophobic residues in green, and alanine and glycine residues in yellow.
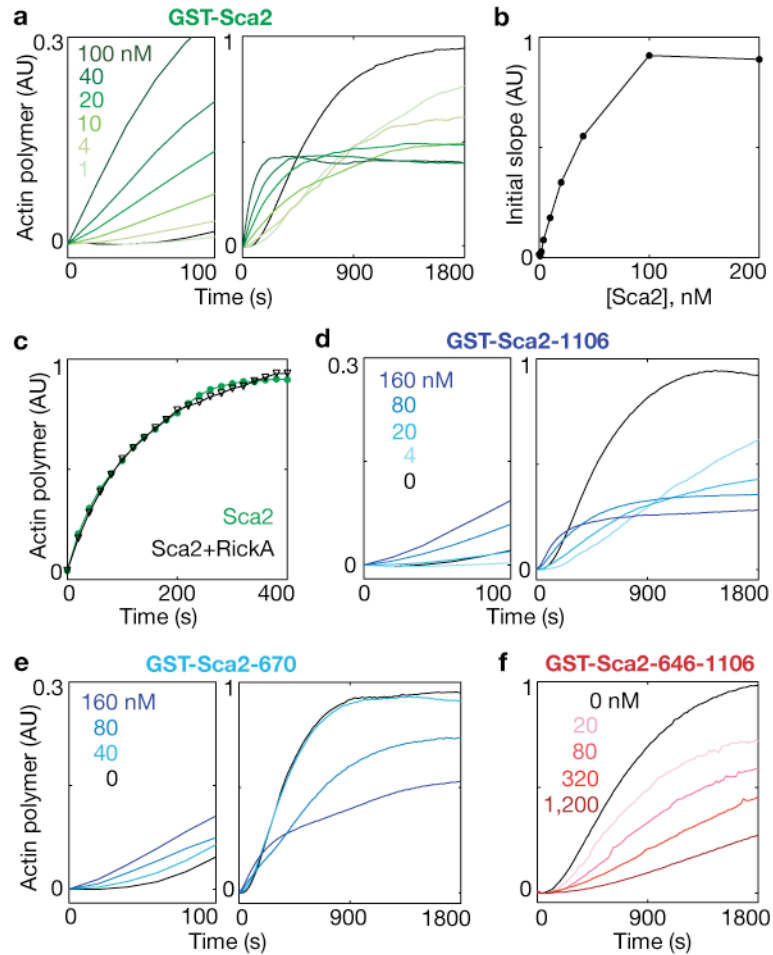
Figure 2.2. Sca2 nucleates actin filaments. (**a**) Polymerization of pyrene-actin (1 μM, 10% pyrene labeled, same for all panels) over time with GST alone (160 nM, black) or increasing concentrations of GST-Sca2 (green). Left panel: magnified view of the first 100 s. (**b**) Dependence of the initial polymerization rate on Sca2 concentration. (**c**) Polymerization of pyrene-actin over time with GST-Sca2 alone (40 nM, green) or Sca2 and GST-RickA (40 nM and 200 nM, black). (**d**) Polymerization of pyrene-actin over time with increasing concentrations of GST-Sca2-1106 (blue). Left panel: magnified view of the first 100 s. (**e**) Polymerization of pyrene-actin over time with increasing concentrations of GST-Sca2-670 (blue). Left panel: magnified view of the first 100 s. (**f**) Polymerization of pyrene-actin over time with increasing concentrations of GST-Sca2-646-1106 (red).

Figure 2.3. Sca2 is a profilin-dependent actin filament elongation factor that protects barbed ends from capping protein. (**a**) Actin polymer formed after overnight polymerization of pyrene-actin (1 μM, 10% pyrene labeled) in the presence of a range of GST-Sca2 concentrations. (**b**) Elongation of preformed actin filaments (unlabeled) in t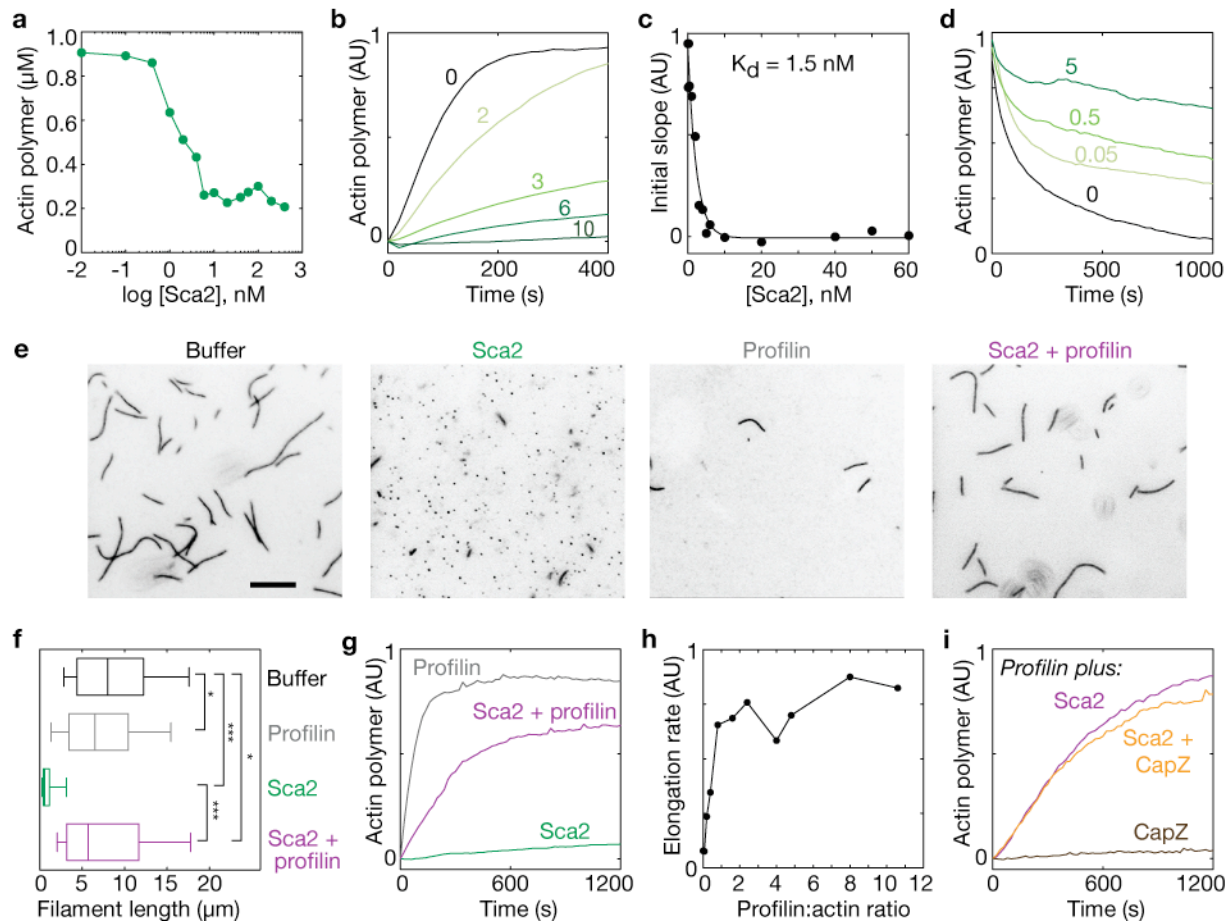he presence of 0.4 μM actin monomers (10% pyrene labeled) and the indicated amounts of GST-Sca2 (nM). (**c**) Dependence of the initial elongation rate on the concentration of GST-Sca2. (**d**) Disassembly of preformed actin filaments (1 μM, 50% pyrene labeled) following dilution into polymerization buffer containing the indicated concentrations of GST-Sca2 (nM). (**e**) Actin (1 μM, 10% pyrene labeled) was polymerized for 10 min in the presence of buffer, GST-Sca2 (10 nM), profilin (4 μM), or profilin and GST-Sca2 together. Rhodamine-phalloidin (1 μM) was added, and diluted samples were observed by epifluorescence microscopy. Scale bar 10 μm. (**f**) Filament length distributions of the reactions pictured in **e**. The boxes cover percentiles 25-75 with lines marking the medians. The whiskers mark percentiles 10 and 90. The p-values were determined using the Kruskal-Wallis test: * = p < 0.05, *** = p < 0.001. (**g**) Elongation of filament seeds after the addition of pyrene-actin monomers (0.5 μM, 10% pyrene labeled) in the presence of profilin (0.5 μM), GST-Sca2 (6 nM), or GST-Sca2 and profilin together. (**h**) Dependence of the initial elongation rate on the concentration of profilin, in the presence of 20 nM GST-Sca2. (**i**) Elongation of filament seeds in the presence of CapZ (10 nM) or CapZ and GST-Sca2 (12 nM). All reactions included profilin (1 μM).

significantly inhibits barbed-end dynamics. To test this, we monitored pyrene-actin assembly from filament seeds at a monomer concentration below the Cc for pointed ends. Under these conditions, low nanomolar amounts of Sca2 inhibited barbed-end elongation in a dose-dependent manner (Fig. 2.3b, c). Sca2 also slowed the depolymerization of preformed actin filaments (Fig. 2.3d). The K_d of Sca2 for barbed ends, derived from its inhibition of barbed-end assembly, was 1.5 nM. Thus, Sca2 binds barbed ends with high affinity, a conserved property of formins (Chesarone et al., 2010).

Sca2 behaves like the *Schizosaccharomyces pombe* formin Cdc12p (Kovar et al., 2003; Skau et al., 2009), the *Drosophila melanogaster* formin DAAM (Barko et al., 2010), and the mammalian formin mDia2 (Kovar et al., 2006), which dramatically slow barbed-end elongation in the absence of profilin. Because profilin enables elongation by these formins (Kovar et al., 2006; Romero et al., 2004) and Sca2 contains predicted profilin binding sites in its PRDs, we tested whether human platelet profilin could accelerate barbed-end elongation in the presence of Sca2. We first measured the lengths of filaments nucleated by GST-Sca2 prior to the reaction reaching steady state (Fig. 2.3e, f). Control reactions containing actin alone produced filaments with a median length of 8.0 μm. In contrast, filaments nucleated by GST-Sca2 had a median length of only 0.6 μm (p<0.001, Kruskal-Wallis test). When profilin and Sca2 were both included, the length of filaments was increased to a median of 5.7 μm. The filaments polymerized by Sca2 and profilin were unbranched, like filaments in *Rickettsia* comet tails. To confirm that profilin stimulated elongation at barbed ends, we monitored pyrene-actin assembly from filament seeds at a monomer concentration below the Cc for pointed ends (Fig. 2.3g). Inclusion of profilin with GST-Sca2 significantly accelerated elongation, although the rate was slower than with actin and profilin alone, suggesting that Sca2 remains bound to polymerizing barbed ends in the presence of profilin. The effect of profilin was dose-dependent and saturated at a 1:1 profilin:actin ratio (Fig. 2.3h), consistent with the idea that Sca2 assembles profilin-actin complexes more efficiently than actin alone. Thus, Sca2 behaves like formins as a profilin-dependent barbed-end elongation factor.

By associating with barbed ends, formins can compete with capping proteins and prevent termination of elongation (Zigmond et al., 2003). We tested whether Sca2 could compete with the capping protein CapZ using the pyrene-actin elongation assay (Fig. 2.3i). In control reactions containing CapZ and profilin, CapZ fully capped barbed ends. However, Sca2 and profilin competed with CapZ, permitting elongation at a rate equivalent to that of Sca2 and profilin alone. This further supports the hypothesis that Sca2 remains bound to barbed ends as they polymerize.

To directly test whether Sca2 processively associates with barbed ends, we used TIRF microscopy to observe the effect of Sca2 on the polymerization of fluorescently labeled actin filaments anchored to coverslips by inactivated myosin. In a control reaction containing actin alone, filaments grew at 11.9 ± 0.3 subunits/sec (mean ± SD, n = 8) (Fig. 2.4a-c, Video 1). In the presence of subnanomolar concentrations of GST-Sca2, we observed two filament populations: free filaments that grew at a rate similar to controls (14.4 ± 0.9 subunits/sec, n = 8), and Sca2-bound filaments that grew extremely slowly (0.6 ± 0.2 subunits/sec) (Fig. 2.4d-f, Video 2). The proportion of slow-growing filaments depended on the dose of Sca2. Sca2 slowed elongation more dramatically than did the mammalian formin mDia2 (Fig. 2.5, Videos 3, 4), to a rate comparable that observed with Cdc12p (Kovar et al., 2003; Skau et al., 2009) or DAAM (Barko et al., 2010). In the presence of profilin, filaments in a control reaction behaved similarly to actin
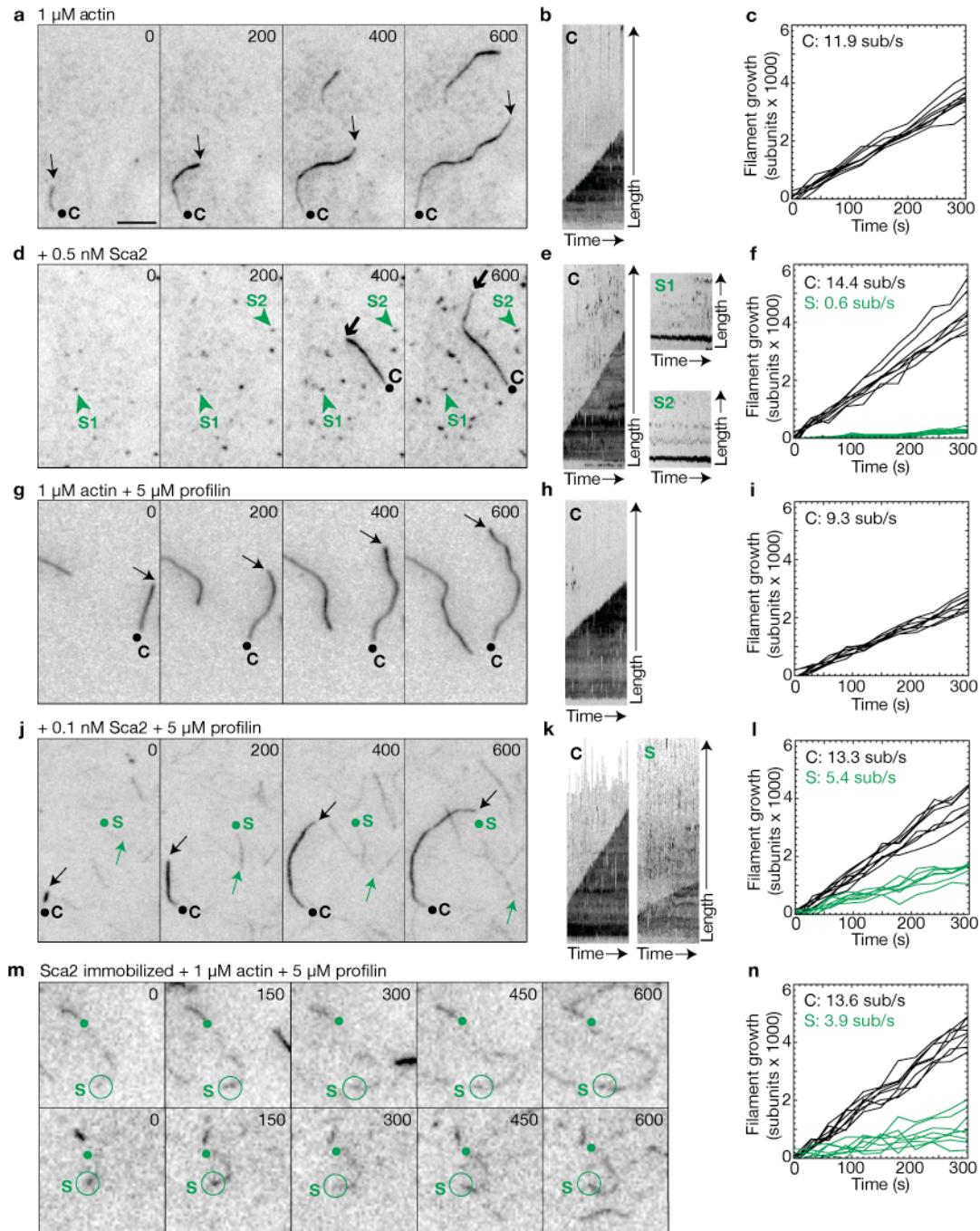
Figure 2.4. Sca2 processively associates with growing filament barbed ends, and elongation is accelerated by profilin. (**a**) Assembly of individual actin filaments imaged by timelapse TIRF microscopy. A black dot (C) marks the pointed end of a control filament, and an arrow marks the growing barbed end. Elapsed time in seconds in upper right corner. Scale bar and elapsed time apply to all images in this figure (unless indicated). (**b**) Kymograph showing growth of the filament depicted in **a**. (**c**) Plots of growth over time for eight individual filaments from the reaction pictured in **a**. Average growth rate is indicated at the top (subunits s^{-1}). (**d**) Filaments

34

imaged by TIRF microscopy as in **a**, but with GST-Sca2 (0.5 nM) included in the reaction. A control filament is labeled in black as in **a**. Green arrowheads (s1, s2) mark two Sca2-associated filaments. (**e**) Kymographs of the filaments marked in **d**. (**f**) Plots of growth over time for eight individual filaments per condition from the reaction pictured in **d**. Average growth rates are indicated at the top (subunits s^{-1}). (**g-i**) Timelapse images, kymograph, and growth plots of control filaments assembled in the presence of profilin. (**j-l**) Timelapse images, kymographs, and growth plots of filaments assembled in the presence of GST-Sca2 and profilin. Control filaments are labeled in black and Sca2 filaments in green; dots mark the pointed ends and arrows mark the growing barbed ends. (**m**) Timelapse TIRF microscopy images showing two examples of filaments buckling in the presence of immobilized GST-Sca2. Green dots mark the pointed ends and open circles (s) mark the barbed ends. (**n**) Growth plots of filaments assembled in the presence of immobilized GST-Sca2. Scale bars, 5 μm.
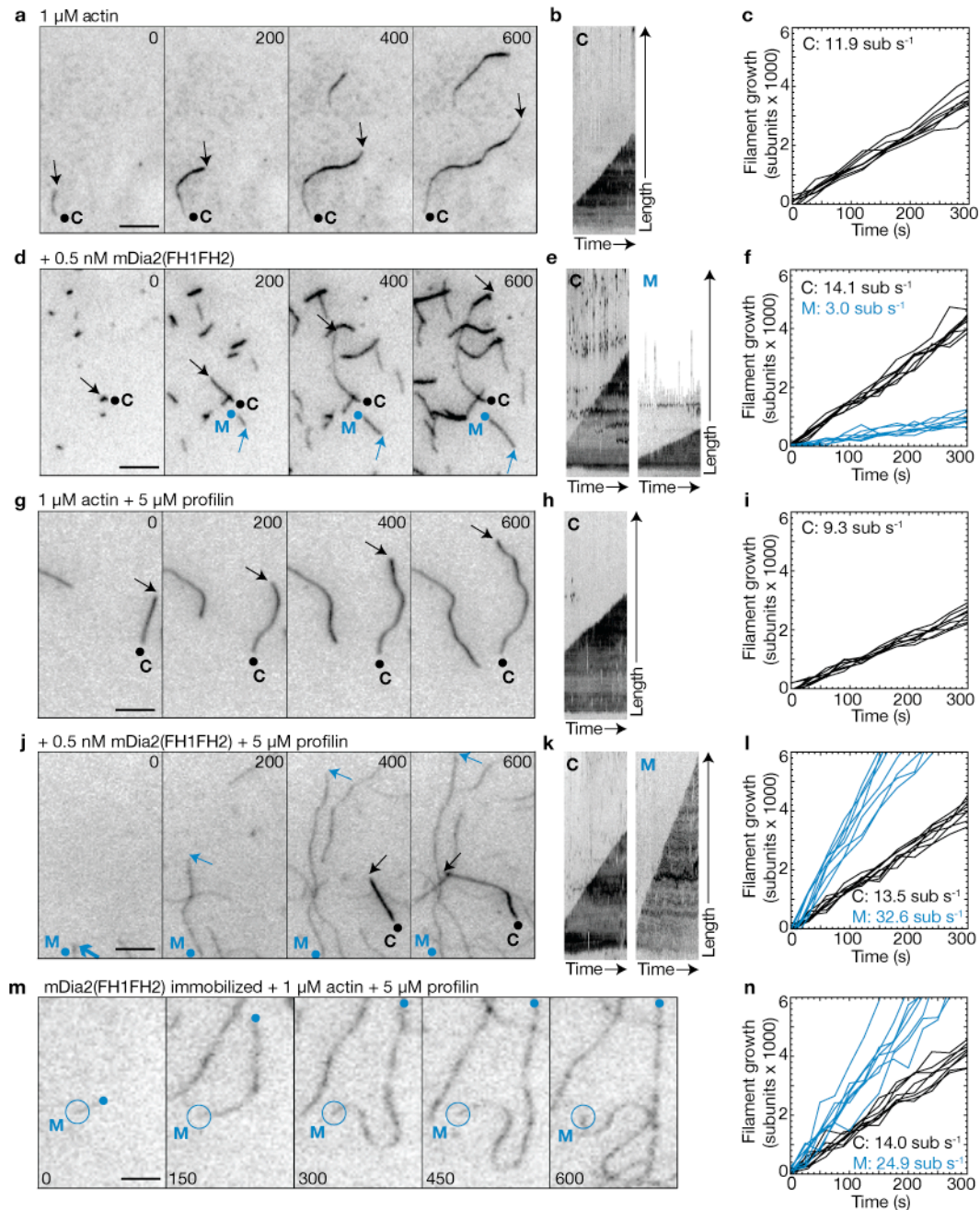
Figure 2.5. Processive association with growing actin filaments by the formin mDia2, for comparison to Sca2. (**a**) Assembly of individual actin filaments imaged by timelapse TIRF microscopy. A black dot (C) marks the pointed end of a control filament, and an arrow marks the growing barbed end. Elapsed time in seconds in upper right corner. (**b**) Kymograph showing growth of the filament indicated in **a**. (**c**) Plots of growth over time for eight individual filaments from the reaction pictured in **a**. Average growth rate is indicated at the top (subunits s^{-1}). (**d**) Filaments imaged by TIRF microscopy as in **a**, but with GST-mDia2(FH1FH2) included in the reaction. A control filament is labelled in black as in **a**. A blue dot (M) marks the pointed end of

an mDia2-associated filament, and a blue arrow marks the growing barbed end. (**e**) Kymographs of the filaments marked in **d**. Left, control filament; right, mDia2-associated filament. (**f**) Plots of growth over time for eight individual filaments per population from the reaction pictured in **d**. Average growth rates are indicated at the top (subunits s^{-1}). (**g-i**) Timelapse TIRF microscopy images (**g**), kymograph (**h**), and growth plots (**i**) of control filaments assembled in the presence of profilin. (**j-l**) Timelapse TIRF microscopy images (**j**), kymographs (**k**), and growth plots (**l**) of filaments assembled in the presence of GST-mDia2(FH1FH2) and profilin. A control filament is labelled in black and an mDia2 filament in blue, as in **d**. (**m**) Timelapse TIRF microscopy images of a filament buckling in the presence of immobilized GST-mDia2(FH1FH2). A blue dot marks the pointed end, and a blue open circle (M) marks the barbed end. Elapsed time in seconds in lower left corner. (**n**) Growth plots of filaments assembled in the presence of immobilized GST-mDia2(FH1FH2). Scale bars, 5 μm.

alone (Fig. 2.4g-i, Video 5). However, inclusion of profilin with subnanomolar amounts of Sca2 caused the Sca2-associated filaments to elongate significantly faster than in the absence of profilin (5.4 ± 0.6 subunits/sec, n = 8) (Fig. 2.4j-l, Video 6), confirming that Sca2-mediated elongation is activated by profilin. Additionally, Sca2-associated filaments were less intensely labeled than control filaments, a phenomenon that is observed with formins but not other actin nucleators (Kovar et al., 2006). Importantly, Sca2-bound filaments were rarely observed to switch from the dim slow-growing population to the bright fast-growing one, as was observed for mDia2 (Fig. 2.5j-l) and other formins (Kovar et al., 2006; Neidt et al., 2008), demonstrating that Sca2 associates with polymerizing barbed ends for extended periods of time (>10 min, >5000 subunits added).

To further confirm that Sca2 processively associates with barbed ends, we immobilized Sca2 on a coverslip and looked for buckling of actin filaments between their growing barbed ends and myosin anchor points along filament sides, a behaviour observed with mDia2 (Fig. 2.5m, Video 7) and other formins (Kovar and Pollard, 2004). Notably, in the presence of profilin and immobilized GST-Sca2, we observed actin filaments elongating and buckling (Fig. 2.4m, Video 8). Thus, Sca2 acts as a true functional mimic of host formins.

The activity of purified Sca2 in vitro suggests that it participates in the actin-based motility of *Rickettsia*, which would require it to localize to the bacterial surface. To test this, we raised antibodies that recognized Sca2 on Western blots of *R. parkeri*-infected cell lysates (Fig. 2.6a). Abundant Sca2 was detected by immunofluorescence microscopy on the surface of *R. parkeri* in infected Vero (Fig. 2.6b) or *Drosophila* S2R+ cells (Fig. 2.6c, d), independent of the presence of an actin tail. When a tail was present, Sca2 was generally enriched at actin-associated bacterial surfaces. Sca2 was not detected along the length of the tail, suggesting that it is not released from *Rickettsia* during movement.

To determine whether surface-bound Sca2 is sufficient to assemble actin in cell cytoplasm in the absence of other bacterial factors, we coated polystyrene beads (0.5-2.0 μm diameter) with GST-Sca2 and observed their ability to polymerize rhodamine-actin in Xenopus laevis egg extract. Sca2 beads tended to form clumps, from which dense starbursts of actin filaments assembled and lengthened over time (Fig. 2.6e, Video 9). Once formed, the actin starbursts persisted for extended periods (>30 min). This demonstrates that Sca2 is sufficient to nucleate actin filaments and promote their elongation in the context of cell cytoplasm.

Our results show that *Rickettsia* have evolved a mechanism of polymerizing host actin through functional mimicry of eukaryotic formins by the bacterial surface protein Sca2. The ability of Sca2 to nucleate unbranched filaments and protect them from capping corresponds with the long, parallel filament organization in *Rickettsia* tails (Gouin et al., 1999; Van Kirk et al., 2000). Moreover, the profilin-dependent elongation mechanism is consistent with the requirement for profilin for *Rickettsia* motility in cells (Serio et al., 2010). Although it has been proposed that both Sca2 and RickA might contribute to *Rickettsia* motility (Kleba et al., 2010), our work together with other evidence suggests that Sca2 plays a primary role. In particular, the observation that actin tails assemble independently of the host Arp2/3 complex (Harlander et al., 2003; Heinzen, 2003; Serio et al., 2010) argues against a RickA-dependent motility mechanism. Moreover, the recent discovery that a transposon insertion in *sca2* abolishes *R. rickettsii* actin tail formation indicates that Sca2 is required for actin assembly (Kleba et al., 2010). Further evidence comes from correlations between the *sca2* and *rickA* gene sequences and the properties of actin-based motility of various *Rickettsia* species (Fig. 2.7). Both *sca2* and *rickA* are intact and
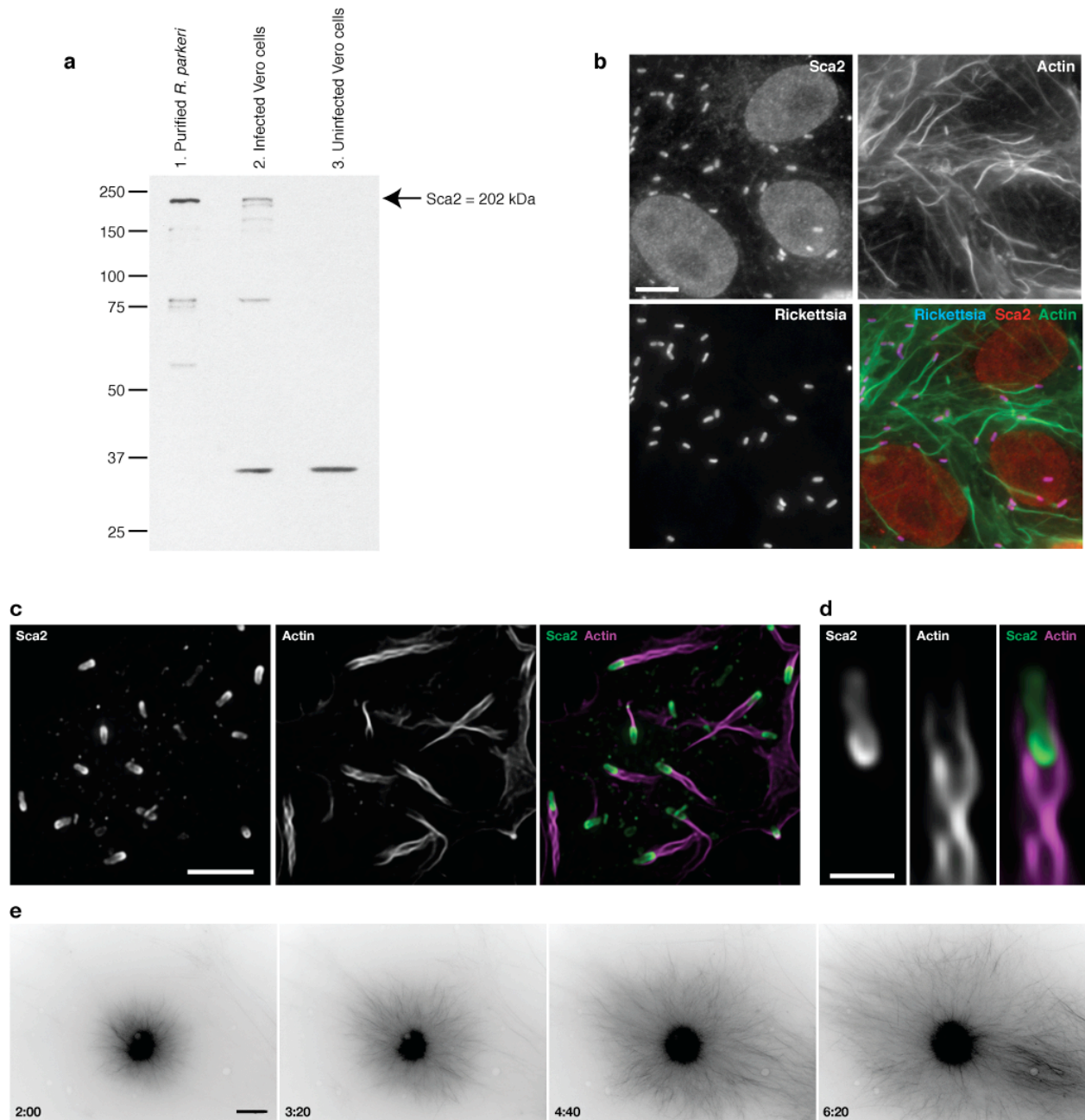
Figure 2.6. Sca2 localizes to actin-associated bacterial surfaces and is sufficient to promote actin polymerization in cell extracts. (**a**) Polyclonal anti-Sca2 antibody recognizes Sca2 on Western blots. Total protein lysate from purified *R. parkeri* (lane 1), and equal volumes of total protein lysates from *R. parkeri*-infected (lane 2) or uninfected (lane 3) Vero cells, were separated by SDS-PAGE and immunoblotted with antibodies raised against the N-terminal 500 amino acids of Sca2. The antibody recognizes a major band at the predicted size of full-length Sca2 (202 kDa), as well as likely degradation products (80 kDa and 58 kDa), and a Vero cell protein (34 kDa). (**b**) Sca2 (red in merge) and *Rickettsia* (blue in merge) stained by immunofluorescence,

and actin (green in merge) stained with Alexa 488-phalloidin in *R. parkeri*-infected Vero cells. Imaging was by epifluorescence microscopy. Scale bar 5 μm. (**c**) Sca2 (white or green) was stained by immunofluorescence and actin (white or purple) was stained with Alexa 488-phalloidin in *R. parkeri*-infected *Drosophila* melanogaster S2R+ cells. Imaging was by deconvolution microscopy. Scale bar 5 μm. (**d**) Magnified image of the bacterium in the lower left corner of a. Scale bar 1 μm. (**e**) Timelapse micrographs of polystyrene beads coated with GST-Sca2 and added to *Xenopus laevis* egg extract supplemented with rhodamine-actin. The elapsed time from adding beads to the extract is indicated (min:s). Scale bar 20 μm.
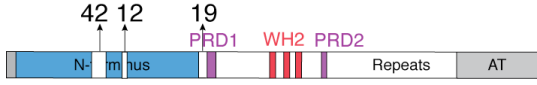
Species	Group	Motility	Actin tail structure	RickA	Sca2
R. parkeri, R. rickettsii, R. conorii, R. africae	Spotted fever	Yes	Long, bundled, unbranched	Intact	Yes
R. canadensis	Uncertain	No	None	Intact	Deletions (# of amino acids)
R. peacockii	Spotted fever	No	None	Transposon insertion	Deletions (# of amino acids)
R. prowazekii	Typhus	No	None	No	Truncated
R. typhi	Typhus	Yes	Short, not bundled	No	Yes
R. bellii	Ancestral	Yes	Unknown	Intact	Yes

Figure 2.7. The motility properties of *Rickettsia* species correlate with the sequence of the *sca2* gene and its protein product. Listed species are those for which whole genome sequence information is available and published reports document their capacity to undergo actin-based motility. For each species, the phylogenomic grouping (Gillespie et al., 2008), the capacity to undergo motility, the actin tail structure, and the presence or absence of genes encoding RickA and Sca2 are indicated. A diagram of the predicted domain organization of Sca2 is shown, based on comparison of each protein sequence with Sca2 from *R. parkeri*. Deletions of >10 amino acids are depicted (excluding the repetitive region). Accession numbers of protein or translated nucleotide records used in this figure are listed below.

	Sca2	RickA
R. africae str. ESF-5	YP_002844823	YP_002845437)
R. bellii str. RML369-C	YP_538450	YP_538025
R. canadensis str. McKiel 2678	YP_001491816 (misannotated as a secG subunit)	YP_001492102
R. conorii str. Malish 7	NP_359747	NP_360546
R. parkeri str. Portsmouth	HM055592	Not available
R. peacockii str. Rustic	ACR47349	Not applicable
R. prowazekii str. Madrid E	NP_220474	Not applicable
R. rickettsii str. Sheila Smith	YP_001494228	Extract from complete genome CP000848 that is 99.9% identical to str. Iowa YP_001650281
R. typhi str. Wilmington	YP_067021 (also known as *sca6* (Ngwamidiba et al., 2005))	Not applicable

their sequences are conserved in SFG species that undergo motility and polymerize long unbranched comet tails. However, in the typhus group species *R. typhi*, which undergoes actin-based motility but forms shorter actin tails of unknown filament organization (Heinzen, 2003; Van Kirk et al., 2000), *sca2* encodes a divergent protein, whereas *rickA* is absent. Conversely, in *R. canadensis*, a species of uncertain phylogeny (Gillespie et al., 2008) that does not undergo actin-based motility (Heinzen et al., 1993), the *rickA* gene is apparently intact, but *sca2* contains deletions compared with *R. parkeri*, the largest of which occurs in the N-terminal domain. The simplest interpretation of these observations is that Sca2 drives motility and RickA participates in host-cell invasion, a process that is thought to depend on the Arp2/3 complex (Martinez and Cossart, 2004).

We asked whether other cellular nucleators, such as formins or Spire, might contribute to *R. parkeri* motility. RNAi targeting of single nucleators in *Drosophila* S2R+ cells did not reveal significant defects in *R. parkeri* actin tail formation (Serio et al., 2010). Because *Drosophila* express six formins with varying levels of homology, functional redundancy could occur. We therefore tested whether RNAi targeted against multiple formins might reveal a defect in *R. parkeri* actin tail formation in *Drosophila* S2R+ cells (Fig. 2.8). First, we targeted formin pairs and triplets, grouped by homology. Because Spire cooperates with the formin cappucino (Rosales-Nieves et al., 2006), we also targeted these two nucleators simultaneously. None of the tested combinations resulted in a defect in *R. parkeri* actin tail formation (Fig. 2.8a). Next, we targeted all six formins simultaneously (Fig. 2.8b) and quantified actin tails as well as short actin nubs, presumed to be precursors to full tails. No reduction in actin nubs or tails was observed. Although the lack of antibodies specific for most *Drosophila* formins precluded quantification of RNAi depletion efficiency, depletion of the *Drosophila* formin diaphanous is known to interfere with cytokinesis and results in large, multinucleate cells (Rogers et al., 2003). Thus, cell morphology acts as a partial internal control. Large, multinucleate cells were observed in our samples in equivalent proportion whether we targeted diaphanous singly or in combination with other formins, suggesting that targeting multiple genes did not significantly reduce the efficiency of RNAi. Thus, although these experiments can not rule out a role for host formins in *Rickettsia* motility, they fail to provide evidence to support such a role.

The striking similarity between the activities of Sca2 and formins raises the question of whether Sca2 structurally mimics formins or acts by a divergent mechanism. The core structural and functional unit of formins is the formin homology 2 (FH2) domain, which forms an elongated, curved rod that assembles into donut-shaped dimers (Chesarone et al., 2010). Although Sca2 does not share significant primary sequence similarity with this domain, the predicted secondary structure of the Sca2 N-terminus is surprisingly similar to that of FH2 (Fig. 2.9). To predict whether the Sca2 N-terminus might resemble FH2 domains at the level of tertiary structure, we submitted the sequence of Sca2 amino acids 34-670 to the mGenThreader fold recognition server (McGuffin and Jones, 2003). This computational method did not identify a high-probability match between Sca2 and any known protein structure. However, of the thousands of structures scored, the third and fourth highest-ranked matches were the FH2 domains of *Saccharomyces cerevisiae* Bni1p and human DAAM1, suggesting that Sca2 might contain a similar fold. We attempted to determine the oligomerization state of the Sca2 N-terminus by size-exclusion chromatography of purified Sca2-670-His (monomer size 74 kDa). The apparent molecular weight of the eluted protein was 294 kDa, consistent with a tetrameric complex. Bni1p was also reported to form tetramers as determined by size-exclusion
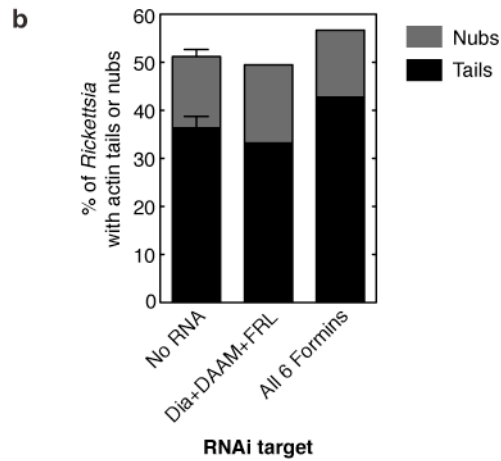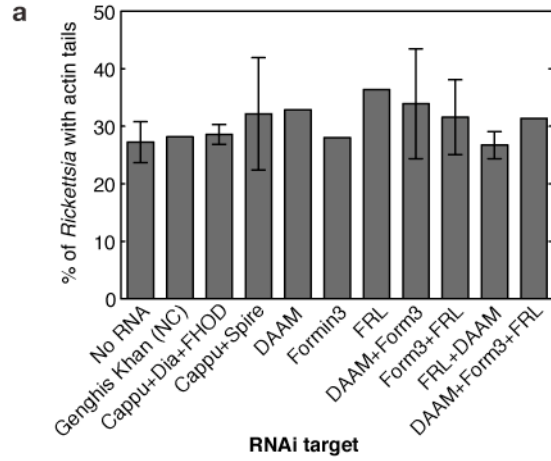
Figure 2.8. RNAi targeting cellular formins does not reduce the frequency of *R. parkeri* actin tail formation. Monolayers of *Drosophila* S2R+ cells were treated with double-stranded RNA (dsRNA) targeting the indicated proteins for 4 days, then infected with *R. parkeri* for 2 days. The percentage of *Rickettsia* associated with actin tails (and also actin nubs in **b**) was quantified and compared to negative controls (either untreated or treated with dsRNA against a target that does not affect tail formation [Serio et al., 2010]). (Cappu = cappucino, Dia = diaphanous.)
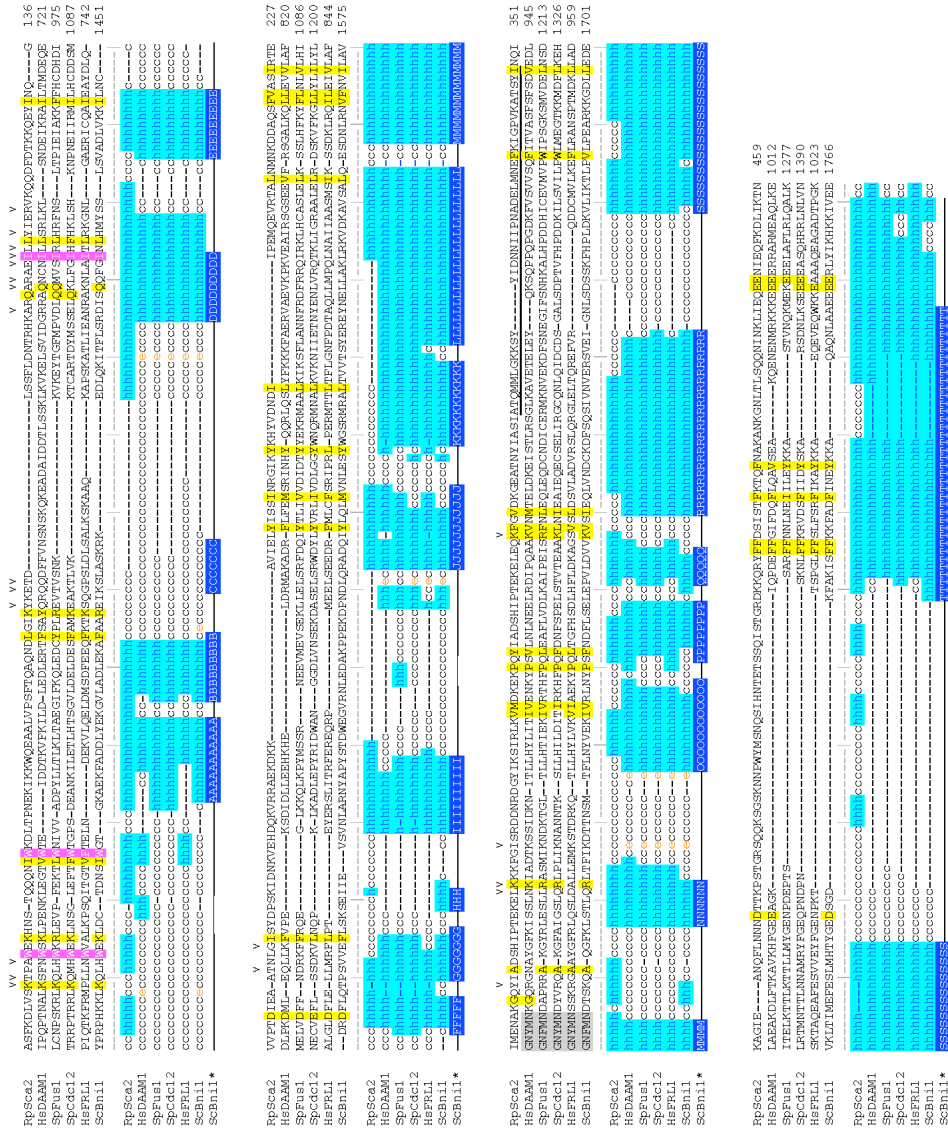
Figure 2.9. Sequence alignment of the N-terminus of Sca2 with FH2 domains of formins from various species. Sequences were aligned according to their predicted secondary structures, obtained from the Porter server (Pollastri and McLysaght, 2005). Structure predictions are shown below the primary sequences, with predicted helices (h) shaded light blue. The edges of predicted helices were aligned manually, while attempting to minimize gaps and preserve primary sequence alignment within the formins. The actual secondary structure and helix-lettering scheme of Bni1p (Xu et al., 2004) determined by x-ray crystallography is included in the last row and highlighted in dark blue. Residues that are conserved in diverse formins and also found in Sca2 are shaded yellow, or pink for those of known functional importance. The functionally important GNYMN motif is shaded in grey. Bni1p residues known to contact actin (Otomo et al., 2005) are marked with a caret (∨). Residues that are missing from *R. peacockii* Sca2 are underlined. (Hs = *Homo sapiens*, Sp = *Schizzosaccharomyces pombe*, Sc = *Saccharomyces cerevisiae*.)

44

chromatography and static light scattering (Zigmond et al., 2003), but the crystal structure suggests that it binds actin as a donut-shaped dimer (Otomo et al., 2005). Other FH2 domains, such as mammalian FRLα (Harris et al., 2004), elute earlier than expected from size-exclusion columns due to their elongated shape. Thus, additional experiments, such as analytical ultracentrifugation, are required to determine the oligomerization state of Sca2. Nonetheless, several lines of evidence suggest that the Sca2 N-terminus might be a structural mimic of the FH2 domain, which was previously thought to be exclusively eukaryotic (Chalkia et al., 2008). The domain organization of Sca2, with its putative FH2-like domain located N-terminal to its PRDs, is inverted compared to the arrangement in formins. We propose that the WH2s and/or PRDs deliver actin monomers to the N-terminal domain to enable efficient nucleation and elongation. The molecular details of Sca2-mediated actin assembly remain to be determined and will likely exhibit general similarities and interesting differences with eukaryotic formins.

Adaptation of a formin-dependent mechanism for actin assembly distinguishes *Rickettsia* from well-studied motile pathogens, which require the host Arp2/3 complex (and in some cases NPFs) for actin-based motility (Gouin et al., 2005; Stevens et al., 2006). This raises an intriguing question: what evolutionary advantage is conferred by each strategy? Mimicry of formins enables *Rickettsia* to bypass a requirement for host protein intermediates, which could be critical for a life cycle that involves infection of diverse species (arthropods and mammals) and cell types. A recent report suggests that cell-to-cell spread of *Shigella flexneri* is impacted by inactivation of the mammalian formins mDia1 and mDia2, although these proteins do not participate in actin-based motility (Heindl et al., 2010). Therefore, exploitation of formin-mediated actin assembly pathways might be a more widespread pathogenic strategy. In the future, *Rickettsia* motility can be exploited to further elucidate the cellular functions of formins and other factors that influence the assembly of long, parallel actin filaments.

Materials and Methods

Bioinformatics (WH2 homology search, sequence alignments, and structure prediction). The PHI-BLAST algorithm was used to search NCBI databases (restricted to *Rickettsia* taxid:780) using the query RxxLLxxIxxxxxxLKKV and the pattern L-[LM]-X (1,3)-I-X (3,8)-L-[KRH]-[KRHQSPN]-[VILATSG]. This query matched a motif in *R. felis* that was similar to motifs in *R. parkeri*. Putative WH2 motifs were aligned manually based on characteristics that include a short amphiphilic helix, a variable linker sequence, and an LKkv motif defined by invariant leucine, a basic residue, a basic or variable residue, and a small residue (Dominguez, 2007). These motifs differ from the WH2 sequences defined by Kleba et al. (Kleba et al., 2010). Similarity scoring was done in Geneious software v4.5.4 (Biomatters) using the Blosum45 matrix and default settings. Alignment of Sca2 and RickA protein or translated nucleotide sequences was performed in Geneious using the Geneious Align algorithm and corrected manually. Tertiary structure prediction was performed using mGenThreader v8.1 (McGuffin and Jones, 2003). Our sequence of full-length *R. parkeri* Sca2 is deposited in GenBank under accession number HM055592.

Molecular biology. The sca2 gene was amplified by PCR from boiled *R. parkeri* and subcloned into either pGEX4T-2, using BamHI and XhoI sites (GST constructs), or pET22b, using NcoI and XhoI sites. The primers are listed below.

GST-Sca2	5'-GCGTGTGGATCCGCAAGCTTTAAAGATTTAGTTAGTAAAACC-3' 5'-GAATAGCTCGAGTTCATCACCGGCCCC-3'
GST-Sca2-1106	5'-GCGTGTGGATCCGCAAGCTTTAAAGATTTAGTTAGTAAAACC-3' 5'-GAATAGCTCGAGAGATGTGGATTTAGTAATCCCTAAAC-3'
GST-Sca2-670	5'-GCGTGTGGATCCGCAAGCTTTAAAGATTTAGTTAGTAAAACC-3' 5'-GAATAGCTCGAGGTCGCTTTGTGTTTGTTCTAAATT-3'
GST-Sca2-646-1106	5'-GCGTGTGGATCCAGCAATGAAGCCAATAAAATTTTAG-3' 5'-GAATAGCTCGAGAGATGTGGATTTAGTAATCCCTAAAC-3'
Sca2-670-His	5'-GCGTGTCCATGGTCCATATGGCAAGCTTTAAAGATTTAGTTAGTAAAACC-3' 5'-GAATAGCTCGAGGTCGCTTTGTGTTTGTTCTAAATT-3'
Sca2-500-His (antibody production)	5'-GCGTGTCCATGGTCCATATGGCAAGCTTTAAAGATTTAGTTAGTAAAACC-3' 5'-GAATAGCTCGAGTAATTGTCCCGTATCATTGGTAAA-3'
Full-length Sca2	5'-GCGTGTCCATGGCTCATATGAATTTACAAAATTCCCACTCA-3' 5'-GAATAGCTCGAGTAGCCCTTGATGGCTTTGATAC-3'

Protein purification. Sca2 derivatives were expressed in E. coli BL21-CodonPlus (DE3)-RIPL cells (Stratagene) induced with 1 mM IPTG overnight at 16˚C. Cell suspensions were sonicated, and proteins were isolated using Glutathione Sepharose 4B (GE Healthcare) or Ni-NTA agarose (Qiagen) affinity chromatography. For some experiments the GST tag was cleaved by incubation with thrombin for 2.5 h at room temperature. Eluted proteins were further purified by gel-filtration chromatography on a Superdex 200 10/300 GL column (GE Healthcare) into protein storage buffer (20 mM MOPS pH 7.0 or HEPES pH 7.4, 100 mM KCl, 5 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 10% glycerol). GST-mDia2 (FH1FH2), encompassing amino acids 521-1171, was also expressed in E. coli and purified by glutathione affinity chromatography, but eluted protein was further purified by chromatography on a Source Q column (GE Healthcare) and dialyzed against mDia2 storage buffer (5 mM NaH_2PO_4, pH 7.0, 150 mM NaCl, 0.5 mM EGTA, 0.5 mM DTT). GST-RickA purification was described previously (Jeng et al., 2004). Human platelet profilin was purified by passing platelet extracts over a poly-L-proline affinity

column as described previously (Janmey, 1991), washing in wash buffer (20 mM PIPES pH 6.8, 150 mM KCl, 0.2 mM ATP, 0.2 mM DTT, 3 M urea), and then eluting with wash buffer plus 7 M urea. Profilin was dialyzed into storage buffer (20 mM HEPES pH 7.7, 20 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM ATP, 0.2 mM DTT, 100 mM sucrose). Proteins were flash frozen in liquid N_2 and stored at –80°C, except GST-mDia2 (FH1FH2) was kept at 4° C (however, contrary to a previous report (Li and Higgs, 2005), the activity of flash-frozen GST-mDia2 (FH1FH2) was identical to aliquots stored at 4°).

Pyrene-actin polymerization assays. Rabbit muscle actin and pyrene-labeled actin (Cytoskeleton, Inc.) were resuspended in G buffer (5 mM Tris pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT), dialyzed into G buffer for >16 h, and stored at 4°C. Polymerization assays included 1 μM actin monomers, 10% pyrene-labeled. Polymerization was initiated by adding 10x initiation buffer (10 mM $MgCl_2$, 10 mM EGTA, 5 mM ATP, 500 mM KCl), bringing final buffer components to 1 mM $MgCl_2$, 2.2 mM EGTA, 0.7 mM ATP, 0.5 mM DTT, 75 mM KCl. In experiments with GST-Sca2-1106 and GST-Sca2-670, KCl was omitted from initiation buffer, and the final KCl concentration was 25 mM. For kinetic experiments, fluorescence was detected at 20 s intervals on a Fluorolog-3 model FL3-11 spectrofluorometer (Horiba Jobin Yvon Inc.) at 365 nm excitation and 407 nm emission, using SpectrAcq v5.20 and DataMax v2.2.12B software. Data were normalized (min = first value in each data set, max = highest value from complete experiment) and graphed using Prism v5.0b (GraphPad Software). Initial polymerization rates were calculated from the first 60 s or 100 s of normalized data. Elongation assays were derived from Moseley et al. (Moseley et al., 2006). Briefly, unlabeled actin filaments were added to proteins of interest in F buffer (5 mM Tris pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.7 mM ATP, 0.2 mM DTT) and sheared 5x through a 27G syringe. Half of this mixture was added to actin monomers (final concentration 0.4 or 0.5 μM, 10% pyrene-labeled), then transferred to a cuvette containing 10x initiation buffer. For competition experiments, proteins (Sca2, CapZ, and profilin) were mixed with F buffer before adding actin seeds. Affinity of Sca2 for barbed ends was estimated from initial elongation rates using Prism software with default "One-site binding" parameters. Depolymerization was induced by diluting preformed actin filaments (1 μM, 50% pyrene labeled) 1:10 into polymerization buffer containing Sca2. To determine the effect of Sca2 on actin critical concentration (Cc), actin monomers (10% pyrene labeled, 1 μM final concentration) were mixed with a range of Sca2 concentrations and initiation buffer, then incubated overnight. Pyrene fluorescence readings were collected on a PerkinElmer Victor X3 plate reader with 355 nm excitation and 405/410 nm emission filters. Control reactions established the baseline fluorescence of pyrene-actin monomers and confirmed that the Cc was 0.1 μM as expected. Baseline-corrected fluorescence data were converted to units of actin polymer.

Epifluorescence and TIRF microscopy to visualize actin filaments. For epifluorescence microscopy, pyrene-actin polymerization reactions were stabilized by mixing with equimolar rhodamine-phalloidin (Invitrogen), diluted 1:50 into fluorescence buffer (50 mM KCl, 1 mM $MgCl_2$, 3 mg/ml dextrose, 10 mM imidazole pH 7.0, 10 mM DTT, 0.5% methylcellulose, 20 μg/ml catalase and 25 U/ml glucose oxidase) and observed using an Olympus IX71 microscope with a 100x (1.35 NA) PlanApo objective and a Photometrics CoolSNAP HQ camera controlled through MetaMorph v5.0r7 (Molecular Devices). Brightness and contrast were adjusted,

greyscale inverted, and dimensions set to 300 dpi in Photoshop CS4 (Adobe). Filament lengths were measured for at least 200 filaments (buffer control, n = 415; profilin, n = 209; Sca2, n = 356; Sca2 plus profilin, n = 351) from 6-10 random fields per condition using the ImageJ NeuronJ plugin (Meijering et al., 2004). Pixels were converted to microns in Excel (Microsoft). Statistical significance was assessed in Prism using the Kruskal-Wallis test.

TIRF microscopy was performed essentially as described previously (Kovar et al., 2006; Neidt et al., 2008). The growth of 8 filaments was measured over the course of 300-600 s for each condition and population using custom ImageJ plugins and Excel macros (Kuhn and Pollard, 2005). The minimal growth of Sca2-associated filaments in the absence of profilin made it impossible to distinguish barbed from pointed end, so total filament length was measured. Immobilization experiments were performed as described previously (Kovar and Pollard, 2004). Images and videos were prepared as described previously (Kovar et al., 2006).

Tissue culture and bacterial growth. *R*. *parkeri* str. Portsmouth was a gift from C. Paddock (Centers for Disease Control and Prevention) (Paddock et al., 2004). Tissue culture conditions were described previously (Serio et al., 2010). For infections, *R*. *parkeri* was purified by Renografin density gradient centrifugation (Hackstadt et al., 1992) and added to the media of cells growing on glass coverslips, and infection continued at 33° C for 2 d.

Detection of Sca2 by Western blot and immunofluorescence. The purified N-terminal 500 amino acids of Sca2 with a 6x His tag (Sca2-500-His) was used to raise antisera in rabbits (Covance Inc). Affinity purification was performed by standard methods using GST-Sca2-670 conjugated to Affigel-10 (Bio-Rad). Eluted antibodies were dialyzed into antibody storage buffer (10 mM MOPS pH 7.0, 150 mM NaCl, 35% glycerol). For Western blots, proteins were separated by SDS-PAGE, transferred to nitrocellulose, incubated with anti-Sca2 antibodies (0.2 μg/ml) for 2 h, probed with anti-rabbit HRP secondary antibodies for 1 h, then detected with Lumigen TMA-6 (Lumigen). For immunofluorescence, Vero or *Drosophila* S2R+ cells were fixed 2 d post-infection with 4% formaldehyde in PBS and blocked with PBS + 2% BSA + 0.1% Triton X-100. Anti-Sca2 antibodies were used at 2 μg/ml. *Rickettsia* were detected with anti-rOmpA 14-13 monoclonal antibodies (Anacker et al., 1987) diluted 1:300, and filamentous actin was detected with Alexa 488-phalloidin at 0.5 U/ml. Secondary antibodies were Alexa 568 goat anti-rabbit (Invitrogen) and AMCA donkey anti-mouse (Jackson). Coverslips were mounted with ProLong Gold (Invitrogen). S2R+ cells were imaged on an Applied Precision DeltaVision 4 Spectris deconvolution microscope with a 100x (1.4 NA) PlanApo objective and a Photometrics CH350 CCD camera. Images were captured using SoftWoRx v3.3.6 (Applied Precision) and deconvolved with Huygens Professional v3.1.0p0 (Scientific Volume Imaging). Slices covering 4 μm of depth were merged using ImageJ's ZProjection at Max Intensity. Brightness was adjusted and dimensions set to 300 dpi in Photoshop CS4. Vero cells were imaged by epifluorescence microscopy (described above).

Actin polymerization on beads in Xenopus extract. Polystyrene microspheres (Polysciences, Inc., 0.5 μm and 2 μm, non-functionalized) were incubated on ice with 1-10 μM Sca2 for 1 h before adding BSA to 5 mg/ml and incubating 15 min. Beads were washed in CSF-XB (10 mM HEPES pH 7.7, 2 mM MgCl$_2$, 0.1 mM CaCl$_2$, 100 mM KCl, 5 mM EGTA, 50 mM sucrose) and kept at 4°C. Xenopus laevis egg extract was provided by the laboratory of R. Heald. To 8 μl of extract, 1

μl of actin (3 μM, 20% rhodamine-labeled) and 1 μl of beads were added. 2-3 μl was placed between a slide and coverslip and observed immediately by epifluorescence microscopy (described above), or sealed with nail polish and observed after 2-30 min incubation. For Video 9, 1 μl of 1% Triton X-100 was also included. Images were recorded every 20 s (Fig. 6e) or 15 s (Video 9). ImageJ was used to adjust brightness and contrast and export QuickTime movies.

RNA synthesis and RNAi. RNAi in *Drosophila* S2R+ cells was performed as described previously (Serio et al., 2010), except that final RNA concentration for the experiment shown in Fig. 2.8b was 10 μg/ml.

References

Anacker, R.L., R.E. Mann, and C. Gonzales. 1987. Reactivity of monoclonal antibodies to *Rickettsia rickettsii* with spotted fever and typhus group rickettsiae. *J Clin Microbiol*. 25:167-71.

Barko, S., B. Bugyi, M.-F. Carlier, R. Gombos, T. Matusek, J. Mihaly, and M. Nyitrai. 2010. Characterization of the biochemical properties and biological function of the formin homology domains of *Drosophila* DAAM. *J Biol Chem*. 285:13154-13169.

Campellone, K.G., and M.D. Welch. 2010. A nucleator arms race: cellular control of actin assembly. *Nat Rev Mol Cell Biol*. 11:237-251.

Cardwell, M.M., and J.J. Martinez. 2009. The Sca2 autotransporter protein from *Rickettsia conorii* is sufficient to mediate adherence to and invasion of cultured mammalian cells. *Infect Immun*. 77:5272-80.

Chalkia, D., N. Nikolaidis, W. Makalowski, J. Klein, and M. Nei. 2008. Origins and evolution of the formin multigene family that is involved in the formation of actin filaments. *Mol Biol Evol*. 25:2717-33.

Chesarone, M.A., A.G. DuPage, and B.L. Goode. 2010. Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol*. 11:62-74.

Dominguez, R. 2007. The beta-thymosin/WH2 fold: multifunctionality and structure. *Ann NY Acad Sci*. 1112:86-94.

Gillespie, J.J., K. Williams, M. Shukla, E.E. Snyder, E.K. Nordberg, S.M. Ceraul, C. Dharmanolla, D. Rainey, J. Soneja, J.M. Shallom, N.D. Vishnubhat, R. Wattam, A. Purkayastha, M. Czar, O. Crasta, J.C. Setubal, A.F. Azad, and B.S. Sobral. 2008. *Rickettsia* phylogenomics: unwinding the intricacies of obligate intracellular life. *PLoS ONE*. 3:e2018.

Gouin, E., C. Egile, P. Dehoux, V. Villiers, J. Adams, F. Gertler, R. Li, and P. Cossart. 2004. The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex. *Nature*. 427:457-61.

Gouin, E., H. Gantelet, C. Egile, I. Lasa, H. Ohayon, V. Villiers, P. Gounon, P.J. Sansonetti, and P. Cossart. 1999. A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia conorii*. *J Cell Sci*. 112:1697-708.

Gouin, E., M.D. Welch, and P. Cossart. 2005. Actin-based motility of intracellular pathogens. *Curr Opin Microbiol*. 8:35-45.

Hackstadt, T., R. Messer, W. Cieplak, and M.G. Peacock. 1992. Evidence for proteolytic cleavage of the 120-kilodalton outer membrane protein of rickettsiae: identification of an avirulent mutant deficient in processing. *Infect Immun*. 60:159-65.

Harlander, R.S., M. Way, Q. Ren, D. Howe, S.S. Grieshaber, and R.A. Heinzen. 2003. Effects of ectopically expressed neuronal Wiskott-Aldrich syndrome protein domains on *Rickettsia rickettsii* actin-based motility. *Infect Immun*. 71:1551-6.

Harris, E.S., F. Li, and H.N. Higgs. 2004. The mouse formin, FRLalpha, slows actin filament barbed end elongation, competes with capping protein, accelerates polymerization from monomers, and severs filaments. *J Biol Chem*. 279:20076-87.

Heindl, J.E., I. Saran, C.R. Yi, C.F. Lesser, and M.B. Goldberg. 2010. Requirement for formin-induced actin polymerization during spread of *Shigella flexneri*. *Infect Immun*. 78:193-203.

Heinzen, R.A. 2003. Rickettsial actin-based motility: behavior and involvement of cytoskeletal regulators. *Ann NY Acad Sci*. 990:535-47.

Heinzen, R.A., S.F. Hayes, M.G. Peacock, and T. Hackstadt. 1993. Directional actin polymerization associated with spotted fever group *Rickettsia* infection of Vero cells. *Infect Immun*. 61:1926-35.

Holt, M.R., and A. Koffer. 2001. Cell motility: proline-rich proteins promote protrusions. *Trends Cell Biol*. 11:38-46.

Janmey, P.A. 1991. Polyproline affinity method for purification of platelet profilin and modification with pyrene-maleimide. *Methods Enzymol*. 196:92-9.

Jeng, R.L., E.D. Goley, J.A. D'Alessio, O.Y. Chaga, T.M. Svitkina, G.G. Borisy, R.A. Heinzen, and M.D. Welch. 2004. A *Rickettsia* WASP-like protein activates the Arp2/3 complex and mediates actin-based motility. *Cell Microbiol*. 6:761-9.

Jewett, T.J., E.R. Fischer, D.J. Mead, and T. Hackstadt. 2006. Chlamydial TARP is a bacterial nucleator of actin. *Proc Natl Acad Sci U S A*. 103:15599-604.

Jewett, T.J., N.J. Miller, C.A. Dooley, and T. Hackstadt. 2010. The conserved Tarp actin binding domain is important for chlamydial invasion. *PLoS Pathog*. 6:e1000997.

Kleba, B., T.R. Clark, E.I. Lutter, D.W. Ellison, and T. Hackstadt. 2010. Disruption of the *Rickettsia rickettsii* Sca2 autotransporter inhibits actin-based motility. *Infect Immun*. 78:2240-7.

Kovar, D.R., E.S. Harris, R. Mahaffy, H.N. Higgs, and T.D. Pollard. 2006. Control of the assembly of ATP- and ADP-actin by formins and profilin. *Cell*. 124:423-35.

Kovar, D.R., J.R. Kuhn, A.L. Tichy, and T.D. Pollard. 2003. The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. *J Cell Biol*. 161:875-87.

Kovar, D.R., and T.D. Pollard. 2004. Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. *Proc Natl Acad Sci USA*. 101:14725-30.

Kuhn, J.R., and T.D. Pollard. 2005. Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophys J*. 88:1387-402.

Li, F., and H.N. Higgs. 2005. Dissecting requirements for auto-inhibition of actin nucleation by the formin, mDia1. *J Biol Chem*. 280:6986-92.

Liverman, A.D.B., H.-C. Cheng, J.E. Trosky, D.W. Leung, M.L. Yarbrough, D.L. Burdette, M.K. Rosen, and K. Orth. 2007. Arp2/3-independent assembly of actin by *Vibrio* type III effector VopL. *Proc Natl Acad Sci USA*. 104:17117-22.

Martinez, J.J., and P. Cossart. 2004. Early signaling events involved in the entry of *Rickettsia conorii* into mammalian cells. *J Cell Sci*. 117:5097-106.

McGuffin, L.J., and D.T. Jones. 2003. Improvement of the GenTHREADER method for genomic fold recognition. *Bioinformatics*. 19:874-81.

Meijering, E., M. Jacob, J.C. Sarria, P. Steiner, H. Hirling, and M. Unser. 2004. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry A*. 58:167-76.

Moseley, J.B., S. Maiti, and B.L. Goode. 2006. Formin proteins: purification and measurement of effects on actin assembly. *Meth Enzymol*. 406:215-34.

Neidt, E.M., C.T. Skau, and D.R. Kovar. 2008. The cytokinesis formins from the nematode worm and fission yeast differentially mediate actin filament assembly. *J Biol Chem*. 283:23872-83.

Ngwamidiba, M., G. Blanc, H. Ogata, D. Raoult, and P. Fournier. 2005. Phylogenetic study of *Rickettsia* species using sequences of the autotransporter protein-encoding gene *sca2*. *Ann NY Acad Sci*. 1063:94-99.

Otomo, T., D.R. Tomchick, C. Otomo, S.C. Panchal, M. Machius, and M.K. Rosen. 2005. Structural basis of actin filament nucleation and processive capping by a formin homology 2 domain. *Nature*. 433:488-94.

Paddock, C.D., J.W. Sumner, J.A. Comer, S.R. Zaki, C.S. Goldsmith, J. Goddard, S.L.F. McLellan, C.L. Tamminga, and C.A. Ohl. 2004. *Rickettsia parkeri*: a newly recognized cause of spotted fever rickettsiosis in the United States. *Clin Infect Dis*. 38:805-11.

Pollastri, G., and A. McLysaght. 2005. Porter: a new, accurate server for protein secondary structure prediction. *Bioinformatics*. 21:1719-20.

Rogers, S.L., U. Wiedemann, N. Stuurman, and R.D. Vale. 2003. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J Cell Biol*. 162:1079-88.

Romero, S., C. Le Clainche, D. Didry, C. Egile, D. Pantaloni, and M.-F. Carlier. 2004. Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell*. 119:419-29.

Rosales-Nieves, A.E., J.E. Johndrow, L.C. Keller, C.R. Magie, D.M. Pinto-Santini, and S.M. Parkhurst. 2006. Coordination of microtubule and microfilament dynamics by *Drosophila* Rho1, Spire and Cappuccino. *Nat Cell Biol*. 8:367-76.

Serio, A.W., R.L. Jeng, C.M. Haglund, S.C. Reed, and M.D. Welch. 2010. Defining a core set of actin cytoskeletal proteins critical for actin-based motility of *Rickettsia*. *Cell Host Microbe*. 7:388-98.

Skau, C.T., E.M. Neidt, and D.R. Kovar. 2009. Role of tropomyosin in formin-mediated contractile ring assembly in fission yeast. *Mol Biol Cell*. 20:2160-73.

Stevens, J.M., E.E. Galyov, and M.P. Stevens. 2006. Actin-dependent movement of bacterial pathogens. *Nat Rev Microbiol*. 4:91-101.

Tam, V.C., D. Serruto, M. Dziejman, W. Brieher, and J.J. Mekalanos. 2007. A type III secretion system in *Vibrio cholerae* translocates a formin/spire hybrid-like actin nucleator to promote intestinal colonization. *Cell Host Microbe*. 1:95-107.

Van Kirk, L.S., S.F. Hayes, and R.A. Heinzen. 2000. Ultrastructure of *Rickettsia rickettsii* actin tails and localization of cytoskeletal proteins. *Infect Immun*. 68:4706-13.

Xu, Y., J.B. Moseley, I. Sagot, F. Poy, D. Pellman, B.L. Goode, and M.J. Eck. 2004. Crystal structures of a Formin Homology-2 domain reveal a tethered dimer architecture. *Cell*. 116:711-23.

Zigmond, S.H., M. Evangelista, C. Boone, C. Yang, A.C. Dar, F. Sicheri, J. Forkey, and M. Pring. 2003. Formin leaky cap allows elongation in the presence of tight capping proteins. *Curr Biol*. 13:1820-3.

CHAPTER 3

Future Directions

Future Directions

R. parkeri Sca2 acts at the crossroads of eukaryotic and bacterial biology, and thus offers avenues for discovery in both host cytoskeletal dynamics and microbial pathogenesis. On the eukaryotic side, a pressing question is whether the tertiary structure of *R. parkeri* Sca2 is similar to that of formins. Knowing the structure of Sca2 would facilitate dissection of its molecular interactions with actin and profilin and permit detailed comparison to formins. It will also be important to ask whether Sca2 interacts with other host proteins or possesses additional activities, as do some formins (Chesarone et al., 2010). On the microbial side, an important question is whether the mechanism of SFG *Rickettsia* motility provides particular advantages during infection compared to that of *Listeria* and *Shigella*. Understanding the advantages of Sca2-mediated motility might also shed light on endogenous formin-mediated motility processes. A final intriguing question is whether the divergent Sca2 proteins encoded by non-SFG *Rickettsia* possess formin-like activity or perhaps assemble actin by a different mechanism. Analysis of divergent Sca2 proteins could illuminate how actin-based motility evolved in the *Rickettsia* genus.

Structure and molecular mechanism of Sca2

Obtaining a crystal structure of the *R. parkeri* Sca2 N-terminal domain complexed with actin would be a significant advance towards understanding its molecular mechanism of action. Our prediction is that the N-terminal domain of Sca2 forms an elongated, curved rod that assembles into doughnut-shaped dimers, analogous to formin FH2 domains. Crystal structures of several FH2 domains in complex with actin have been determined (Lu et al., 2007; Otomo et al., 2005; Xu et al., 2004), which could facilitate solving the structure of Sca2 and/or identifying functionally important amino acid residues.

  Before attempting to crystalize the Sca2 N-terminus, it is necessary to determine the minimal region sufficient for FH2-like activity. Our experiments so far have used a truncation derivative consisting of the first 670 amino acids of Sca2 (minus the predicted 33-amino-acid secretion signal). We encounter a moderate level of degradation during purification of this derivative, suggesting it is not optimally stable. Comparison of the Sca2 amino acid sequence to FH2 domains suggests that the active domain might consist of only the first ~450 residues of Sca2. A derivative of this length, containing the minimal nucleation domain, might be more stable and increase the chance of successful crystallization.

  A minor technical hurdle in defining the minimal nucleation domain is the validation of an optimal fusion partner for Sca2 derivatives. In previous experiments, we used Sca2 derivatives tagged with 6xHis or GST, or with the GST tag removed by thrombin treatment. Each of these constructs has a drawback. The 6xHis fusions had poor solubility and low yield. The GST tag dimerizes, precluding the identification of truncations that abrogate the presumed inherent dimerization of Sca2. Lastly, cleavage of the GST tag adds an extra step to the purification, which could be cumbersome when evaluating a series of derivatives. Fortunately, N-terminal fusion tags do not appear to alter Sca2 activity (chapter 2). We have begun to test a new tag, SUMO, which improves the solubility of fusion partners but does not dimerize. We constructed (in collaboration with QB3 MacroLab) a SUMO-tagged Sca2 passenger domain. If we achieve good yields of SUMO-Sca2 and confirm that its in vitro activity is comparable to

untagged Sca2, we will generate a series of truncation derivatives to identify the minimal N-terminal domain that is sufficient for nucleation.

Using the minimal domain, we plan to collaborate with a crystallographer to obtain the tertiary structure of the Sca2 N-terminus complexed with actin. The solved structure would facilitate identification of actin-binding interfaces and presumed dimerization regions. This knowledge would support molecular dissection of Sca2 activity, which we predict will broaden our understanding of formin biochemistry. It would also allow us to generate mutations that alter polymerization properties, which could be used to answer interesting questions about the contribution of Sca2 activity to *R. parkeri* biology (discussed below). Alternatively, it is possible the crystal structure of Sca2 might reveal little or no similarity to eukaryotic formins – such an unexpected result would open a window onto a completely novel mechanism of actin assembly.

Host factors required for Sca2 activity and Rickettsia motility

The importance of host profilin for Sca2 activity (chapter 2) and for *R. parkeri* motility (Serio et al., 2010) is clear, but the molecular details of the interaction between Sca2 and profilin are unknown. For instance, does profilin bind both of the PRDs in Sca2? If so, are there differences in its affinity for the two domains? Why does Sca2 have two separate PRDs when most formins have only one? It is conceivable that the two PRDs could be involved in different aspects of Sca2 activity; for example one might be predominantly involved in nucleation, while the other is more important during elongation. These questions could be addressed by site-directed mutagenesis of proline residues within the PRDs, which should abrogate profilin binding. Another intriguing question is whether sequence differences between tick and mammalian profilins affect Sca2 activity. Profilins from diverse organisms – or even profilin isoforms from the same species – when complexed with actin can interact differently with formins in vitro, altering actin polymerization rates (Ezezika et al., 2009; Neidt et al., 2009). Profilin is poorly conserved across the evolutionary distance from arthropods to mammals (e.g. <20% identical, <30% similar between human and *Drosophila melanogaster* profilins). The experiments in chapter 2 were performed with human profilin purified from platelets. It would be interesting to compare the activity of tick versus human profilin in Sca2-mediated actin polymerization assays. A dramatically different polymerization rate with tick profilin could suggest that actin-based motility plays a different role in arthropods compared to mammals. Additionally, it is possible that Sca2 differentially utilizes human profilin isoforms, which could affect the spread of *Rickettsia* within different cell types, but this seems less likely given that the main human isoform, profilin 1, is ubiquitously expressed (Jockusch et al., 2007).

Depletion experiments in insect and mammalian cells showed that, in addition to profilin, the host protein fimbrin (T-plastin in mammals) is specifically important for the motility of *R. parkeri* but not *L. monocytogenes* (Serio et al., 2010). Investigating why fimbrin depletion interferes with *R. parkeri* motility could shed light on fimbrin function in cellular actin structures. Evidence from fission yeast demonstrates that fimbrin controls actin filament dynamics by competing with tropomyosin for binding to actin filament sides, which regulates access to filaments by proteins involved in severing, nucleation, and contractility (Clayton et al., 2010; Skau and Kovar, 2010). Actin structures rich in fimbrin recruit a partially distinct set of proteins compared to structures rich in tropomyosin, and the two types of structures have different kinetic properties (Skau and Kovar, 2010). This role of fimbrin as an access-controller has not yet been investigated in other organisms. SFG *Rickettsia* tails might be an example of the

"fimbrin-rich" class of structures – conversely, they contain little tropomyosin (Van Kirk et al., 2000). Thus, *Rickettsia* tails could serve as a model to understand how the fimbrin-rich state is established. A starting point could be to ask if Sca2 directly interacts with fimbrin, or if fimbrin is recruited to *Rickettsia* tails by another mechanism.

The role of the host Arp2/3 complex and its rickettsial activator RickA in SFG *Rickettsia* motility is unsettled (Gouin et al., 2004; Gouin et al., 1999; Harlander et al., 2003; Heinzen, 2003; Jeng et al., 2004; Serio et al., 2010). A significant technical hurdle in the quest to resolve this issue is the difficulty of performing targeted genetic deletions in *Rickettsia*. Recent progress in the development of genetic tools for *Rickettsia*, including directed mutagenesis (Driskell et al., 2009) and plasmids (Baldridge et al., 2010), put this goal within reach. Targeted knockout of the *R. parkeri rickA* gene would provide unambiguous data, which was unobtainable by previous methods, finally answering whether RickA is required for *R. parkeri* actin-based motility, invasion, or both. If RickA is required for *R. parkeri* motility, it will be crucial to investigate how Arp2/3 and Sca2 nucleation activities are coordinated during *Rickettsia* infection. For instance, it has been proposed that Arp2/3-mediated generation of Y-branches could be required to initiate motility, and that elongation activity by another factor (such as Sca2) could predominate at a later stage (Jeng et al., 2004). This model is similar to the convergent-elongation model of filopodia formation (Svitkina et al., 2003; Vignjevic et al., 2003) and thus could potentially shed light on the transition from nucleation to elongation phases in filopodia.

Within cells, actin can potentially interact with hundreds of proteins that regulate filament nucleation, elongation, capping, cross-linking, contractility, severing, and monomer recycling. Aside from actin and profilin, potential direct interactions between Sca2 and other host proteins have not been investigated. Pull-down experiments might identify additional interacting partners of Sca2. Whether or not additional Sca2-binding partners are found, many host proteins localize to SFG *Rickettsia* actin tails (Gouin et al., 1999; Serio et al., 2010; Van Kirk et al., 2000) and contribute to motility (Serio et al., 2010), and characterizing their roles will provide a more complete understanding of Sca2-driven propulsion. Given that many formins possess additional activities – such as actin filament bundling, severing, or microtubule binding – and are regulated by diverse upstream signals (reviewed in (Chesarone et al., 2010), it would also be worthwhile to investigate whether Sca2 shares any of these properties. Characterizing host proteins that interact with Sca2 or are required for SFG *Rickettsia* motility might ultimately permit in vitro reconstitution of Sca2-mediated motility using purified proteins, as has been achieved for motility mediated by the Arp2/3 complex (Loisel et al., 1999) or formin FH1-FH2 domains (Romero et al., 2004).

Biological roles of formin-mediated motility

An essential outstanding question about Sca2-mediated motility is what advantage(s) it affords SFG *Rickettsia* compared to the Arp2/3-based motility mechanisms of other bacterial pathogens. As mentioned in chapter 2, one potential advantage lies in bypassing the requirement for a host nucleator, which might facilitate infection in diverse species and cell types. Another possible advantage is suggested by recent work showing that cell-to-cell spread of *Shigella* is diminished following depletion of the mammalian formins mDia1 and mDia2 (Heindl et al., 2010). As described in chapter 1, the structure and protein composition of *Shigella* and *Listeria* actin tails changes after they contact the plasma membrane and form protrusions into neighboring cells. Actin filaments in *Listeria* or *Shigella* tails within protrusions tend to be longer and less

branched than filaments in cytoplasmic tails (Gouin et al., 1999; Sechi et al., 1997). Thus, it seems plausible that formins contribute to the structure of *Listeria* and *Shigella* actin tails within protrusions. Together, this evidence suggests that formin-mediated actin polymerization at the plasma membrane can enhance the efficiency of protrusion formation and cell-to-cell spread by actin-motile pathogens. *Listeria* and *Shigella* tails undergo a gradual transition from cytoplasmic, Arp2/3-mediated dendritic structures to rigid, membrane-bound protrusions (reviewed in chapter 1). Because SFG *Rickettsia* encode their own formin-like nucleator and appear to express it constitutively, they might form protrusions more quickly and spread more efficiently. As mentioned above, dissecting the molecular mechanism of Sca2 and generating mutants with altered activity would enable us to test how particular aspects of Sca2-mediated actin assembly affect cell-to-cell spread. For instance, is spreading efficiency or protrusion formation affected by the motility rate? By compactness of the bundles within tails? By the recruitment of accessory proteins? Do any mutations have differential effects on cell-to-cell spread compared to cytoplasmic motility? These experiments would require genetic manipulation of *Rickettsia* (or expression in a heterologous organism), but, as mentioned in the previous section, these manipulations are now becoming possible.

The flip side of the question of biological advantages is whether formin-mediated motility has drawbacks compared to Arp2/3-mediated motility. Presumably some trade-offs exist; otherwise, it seems likely that more pathogens would have evolved a formin-like mechanism (discussed in the next section). Defining the unique pros and cons of formin-mediated versus Arp2/3-mediated bacterial motility could shed light on the roles of these modes of motility in endogenous cellular processes. Arp2/3-mediated propulsion is implicated in vesicle rocketing (Marchand et al., 1995; Merrifield et al., 1999; Taunton et al., 2000) and lamellipodia protrusion (Borisy and Svitkina, 2000; Bugyi et al., 2008; Pollard and Borisy, 2003), whereas formin-mediated motility is implicated in the migration of meiotic chromosomes in oocytes (Li et al., 2008). Why certain cellular processes employ different nucleators during actin-based motility is unknown, but the contrast between SFG *Rickettsia* and *Listeria* motility could help address this question.

Evolution of Rickettsia *actin-based motility*

SFG *Rickettsia* are currently the only pathogens known to employ a formin-based motility mechanism. Other pathogens that employ actin-based motility – including species of *Listeria*, *Shigella*, *Burkholderia*, *Mycobacteria*, vaccinia virus, and baculovirus – all appear to exploit the Arp2/3 complex. Many factors could contribute to this skewed distribution. For instance, sampling error might play a part (instances of formin-mediated motility might exist among bacterial species that have not been studied). Alternatively, Arp2/3-mediated motility could be more prevalent in nature. The Arp2/3 complex might provide unknown biophysical or biological advantages over formin-mediated motility. Or, given that NPFs are smaller and structurally simpler than formins, it seems plausible that they would arise more frequently in bacterial genomes by chance. Moreover, because Arp2/3 is a central player in phagocytosis and bacterial invasion, NPFs that evolve to promote entry could be repurposed for actin-based motility, facilitating acquisition of this trait. We hypothesize that a rickettsial ancestor originally evolved an Arp2/3-based motility mechanism, perhaps mediated by a precursor to RickA or Sca2, and that the formin-like N-terminal domain of Sca2 was acquired later, during the evolution of the SFG clade. Testing this hypothesis directly on rickettsial ancestors is of course impossible, but
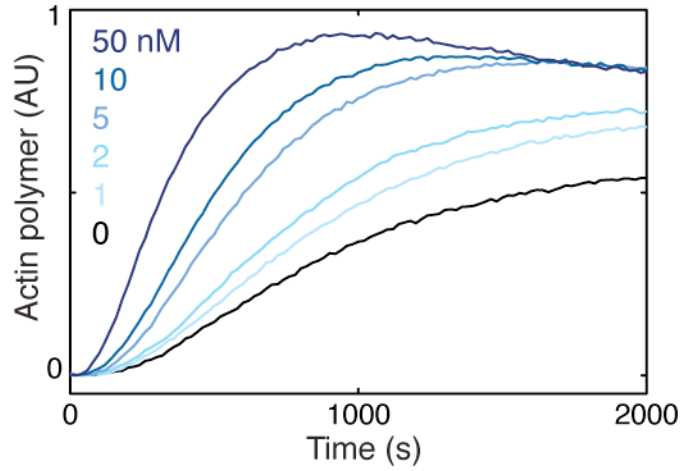
Figure 3.1: *R. bellii* Sca2 promotes actin polymerization. Polymerization of pyrene-actin (1 μM, 10% pyrene labeled) over time with the indicated concentrations of *R. bellii* Sca2 passenger domain fused to GST (nM).

insight might be provided by comparing the activities of Sca2 from diverse extant *Rickettsia*. Two early-branching species, *R. bellii* and *R. typhi*, have highly divergent *sca2* genes compared to SFG species and to each other, yet both undergo actin-based motility. Comparing their activities to *R. parkeri* Sca2 might illuminate how actin-based motility evolved in the *Rickettsia* genus, and could also inform broader questions about how pathogens acquire this virulence trait.

     R. bellii is the earliest-branching species of *Rickettsia* (Ogata et al., 2006). As yet, only short tails have been reported for *R. bellii* (Ogata et al., 2006), but the experimental conditions (a relatively brief 6 hour infection at high MOI) might not have been conducive to long tails. The tails were observed by fluorescence microscopy, which can not resolve filament architecture, so it remains unknown whether filaments are branched or linear. The *R. bellii sca2* gene encodes a 896 amino acid protein (Ogata et al., 2006), much smaller than the 1,816 amino acids encoded by *R. parkeri*. We did not detect homology between *R. bellii* Sca2 and the N-terminal domain of *R. parkeri* Sca2, suggesting it does not possess formin-like activity. Overall, the two passenger domains share only 21% identity (the autotransporters are 65% identical). The predicted amino acid sequence of *R. bellii* Sca2 contains a single PRD, three sequences with partial similarity to WH2 motifs, and two acidic stretches that contain a tryptophan, similar to the acidic domain of NPFs. Thus, the primary sequence suggests that *R. bellii* Sca2 might function as an NPF, or perhaps as a tandem monomer-binding nucleator. We have begun to investigate the biochemistry of *R. bellii* Sca2. We purified the *R. bellii* Sca2 passenger domain fused to GST (using the same methods as for *R. parkeri* Sca2) and tested its activity in pyrene-actin polymerization assays. *R. bellii* Sca2 was sufficient to accelerate actin polymerization on its own, in the absence of the Arp2/3 complex (Fig. 3.1). This preliminary data suggests that *R. bellii* Sca2 might function as a tandem monomer-binding nucleator. We have not yet investigated whether *R. bellii* Sca2 possesses formin-like properties. Such a result would be surprising, given the lack of sequence similarity to the *R. parkeri* N-terminal domain or to eukaryotic formins.

     R. typhi is a member of the typhus group (TG), which split from the SFG after the divergence of *R. bellii* (Gillespie et al., 2008). In infected cells, *R. typhi* forms short, curved actin tails (Heinzen, 2003; Van Kirk et al., 2000), reminiscent of *Listeria* tails, suggesting an Arp2/3-mediated mechanism. However, the ultrastructure of *R. typhi* tails is unknown. *R. typhi sca2* encodes a 1,533 amino acid protein (McLeod et al., 2004) with 23% identity to *R. parkeri* in the passenger domain (82% in the autotransporter). Like *R. bellii*, the predicted *R. typhi* Sca2 protein lacks significant homology to the *R. parkeri* N-terminal domain, suggesting that it does not possess formin-like activity. *R. typhi* Sca2 contains a single PRD and seven sequences with full or partial similarity to WH2 motifs, supporting the hypothesis that it interacts with actin. However, we have not yet been able to purify recombinant *R. typhi* Sca2 to test this prediction.

     Characterization of *R. bellii* and *R. typhi* Sca2 will likely demonstrate whether the mechanism of actin nucleation by Sca2 has changed during the course of *Rickettsia* evolution. If differences are found, it will be particularly illuminating to test how these differences affect the motility properties of *Rickettsia* species during infection.

     Study of intracellular pathogens has yielded a wealth of knowledge about both microbial pathogenesis and eukaryotic cellular processes. Bacterial actin-based motility has been a particularly rich source of insight. Because SFG *Rickettsia* evolved a different solution to the engineering problem posed by actin-based motility, they provide a unique, alternative system to compare and contrast with Arp2/3-mediated pathogen motility. Future studies should delineate the relative advantages of the two motility mechanisms. Similarly, *R. parkeri* Sca2 represents a

unique outgroup that can be productively compared and contrasted to eukaryotic formins, generating new understanding about the molecular requirements for processive actin filament assembly.

References

Baldridge, G.D., N.Y. Burkhardt, M.B. Labruna, R.C. Pacheco, C.D. Paddock, P.C. Williamson, P.M. Billingsley, R.F. Felsheim, T.J. Kurtti, and U.G. Munderloh. 2010. Wide dispersal and possible multiple origins of low-copy-number plasmids in *Rickettsia* species associated with blood-feeding arthropods. *Appl Environ Microbiol*. 76:1718-31.

Borisy, G.G., and T.M. Svitkina. 2000. Actin machinery: pushing the envelope. *Curr Opin Cell Biol*. 12:104-12.

Bugyi, B., C. Le Clainche, G. Romet-Lemonne, and M.F. Carlier. 2008. How do in vitro reconstituted actin-based motility assays provide insight into in vivo behavior? *FEBS Lett*. 582:2086-92.

Chesarone, M.A., A.G. DuPage, and B.L. Goode. 2010. Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol*. 11:62-74.

Clayton, J.E., M.R. Sammons, B.C. Stark, A.R. Hodges, and M. Lord. 2010. Differential regulation of unconventional fission yeast myosins via the actin track. *Curr Biol*. 20:1423-31.

Driskell, L.O., X.-J. Yu, L. Zhang, Y. Liu, V.L. Popov, D.H. Walker, A.M. Tucker, and D.O. Wood. 2009. Directed mutagenesis of the *Rickettsia prowazekii* pld gene encoding phospholipase D. *Infect Immun*. 77:3244-8.

Ezezika, O.C., N.S. Younger, J. Lu, D.A. Kaiser, Z.A. Corbin, B.J. Nolen, D.R. Kovar, and T.D. Pollard. 2009. Incompatibility with formin Cdc12p prevents human profilin from substituting for fission yeast profilin: insights from crystal structures of fission yeast profilin. *J Biol Chem*. 284:2088-97.

Gillespie, J.J., K. Williams, M. Shukla, E.E. Snyder, E.K. Nordberg, S.M. Ceraul, C. Dharmanolla, D. Rainey, J. Soneja, J.M. Shallom, N.D. Vishnubhat, R. Wattam, A. Purkayastha, M. Czar, O. Crasta, J.C. Setubal, A.F. Azad, and B.S. Sobral. 2008. *Rickettsia* phylogenomics: unwinding the intricacies of obligate intracellular life. *PLoS ONE*. 3:e2018.

Gouin, E., C. Egile, P. Dehoux, V. Villiers, J. Adams, F. Gertler, R. Li, and P. Cossart. 2004. The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex. *Nature*. 427:457-61.

Gouin, E., H. Gantelet, C. Egile, I. Lasa, H. Ohayon, V. Villiers, P. Gounon, P.J. Sansonetti, and P. Cossart. 1999. A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia conorii*. *J Cell Sci*. 112 (Pt 11):1697-708.

Harlander, R.S., M. Way, Q. Ren, D. Howe, S.S. Grieshaber, and R.A. Heinzen. 2003. Effects of ectopically expressed neuronal Wiskott-Aldrich syndrome protein domains on *Rickettsia rickettsii* actin-based motility. *Infect Immun*. 71:1551-6.

Heindl, J.E., I. Saran, C.R. Yi, C.F. Lesser, and M.B. Goldberg. 2010. Requirement for formin-induced actin polymerization during spread of *Shigella flexneri*. *Infect Immun*. 78:193-203.

Heinzen, R.A. 2003. Rickettsial actin-based motility: behavior and involvement of cytoskeletal regulators. *Ann NY Acad Sci*. 990:535-47.

Jeng, R.L., E.D. Goley, J.A. D'Alessio, O.Y. Chaga, T.M. Svitkina, G.G. Borisy, R.A. Heinzen, and M.D. Welch. 2004. A *Rickettsia* WASP-like protein activates the Arp2/3 complex and mediates actin-based motility. *Cell Microbiol*. 6:761-9.

Jockusch, B.M., K. Murk, and M. Rothkegel. 2007. The profile of profilins. *Rev Physiol Biochem Pharmacol*. 159:131-49.

Li, H., F. Guo, B. Rubinstein, and R. Li. 2008. Actin-driven chromosomal motility leads to symmetry breaking in mammalian meiotic oocytes. *Nat Cell Biol*. 10:1301-8.

Loisel, T.P., R. Boujemaa, D. Pantaloni, and M.F. Carlier. 1999. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature*. 401:613-6.

Lu, J., W. Meng, F. Poy, S. Maiti, B.L. Goode, and M.J. Eck. 2007. Structure of the FH2 domain of Daam1: implications for formin regulation of actin assembly. *J Mol Biol*. 369:1258-69.

Marchand, J.B., P. Moreau, A. Paoletti, P. Cossart, M.F. Carlier, and D. Pantaloni. 1995. Actin-based movement of *Listeria monocytogenes*: actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. *J Cell Biol*. 130:331-43.

McLeod, M.P., X. Qin, S.E. Karpathy, J. Gioia, S.K. Highlander, G.E. Fox, T.Z. McNeill, H. Jiang, D. Muzny, L.S. Jacob, A.C. Hawes, E. Sodergren, R. Gill, J. Hume, M. Morgan, G. Fan, A.G. Amin, R.A. Gibbs, C. Hong, X.J. Yu, D.H. Walker, and G.M. Weinstock. 2004. Complete genome sequence of *Rickettsia typhi* and comparison with sequences of other rickettsiae. *J Bacteriol*. 186:5842-55.

Merrifield, C.J., S.E. Moss, C. Ballestrem, B.A. Imhof, G. Giese, I. Wunderlich, and W. Almers. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. *Nat Cell Biol*. 1:72-4.

Neidt, E.M., B.J. Scott, and D.R. Kovar. 2009. Formin differentially utilizes profilin isoforms to rapidly assemble actin filaments. *J Biol Chem*. 284:673-84.

Ogata, H., B. La Scola, S. Audic, P. Renesto, G. Blanc, C. Robert, P.-E. Fournier, J.-M. Claverie, and D. Raoult. 2006. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. *PLoS Genet*. 2:e76.

Otomo, T., D.R. Tomchick, C. Otomo, S.C. Panchal, M. Machius, and M.K. Rosen. 2005. Structural basis of actin filament nucleation and processive capping by a formin homology 2 domain. *Nature*. 433:488-94.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-65.

Romero, S., C. Le Clainche, D. Didry, C. Egile, D. Pantaloni, and M.-F. Carlier. 2004. Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell*. 119:419-29.

Sechi, A.S., J. Wehland, and J.V. Small. 1997. The isolated comet tail pseudopodium of *Listeria monocytogenes*: a tail of two actin filament populations, long and axial and short and random. *J Cell Biol*. 137:155-67.

Serio, A.W., R.L. Jeng, C.M. Haglund, S.C. Reed, and M.D. Welch. 2010. Defining a core set of actin cytoskeletal proteins critical for actin-based motility of *Rickettsia*. *Cell Host Microbe*. 7:388-98.

Skau, C.T., and D.R. Kovar. 2010. Fimbrin and tropomyosin competition regulates endocytosis and cytokinesis kinetics in fission yeast. *Curr Biol*. 20:1415-22.

Svitkina, T.M., E.A. Bulanova, O.Y. Chaga, D.M. Vignjevic, S.-i. Kojima, J.M. Vasiliev, and G.G. Borisy. 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *J Cell Biol*. 160:409-21.

Taunton, J., B.A. Rowning, M.L. Coughlin, M. Wu, R.T. Moon, T.J. Mitchison, and C.A. Larabell. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. *J Cell Biol*. 148:519-30.

Van Kirk, L.S., S.F. Hayes, and R.A. Heinzen. 2000. Ultrastructure of *Rickettsia rickettsii* actin tails and localization of cytoskeletal proteins. *Infect Immun*. 68:4706-13.

Vignjevic, D., D. Yarar, M.D. Welch, J. Peloquin, T. Svitkina, and G.G. Borisy. 2003. Formation of filopodia-like bundles in vitro from a dendritic network. *J Cell Biol*. 160:951-62.

Xu, Y., J.B. Moseley, I. Sagot, F. Poy, D. Pellman, B.L. Goode, and M.J. Eck. 2004. Crystal structures of a Formin Homology-2 domain reveal a tethered dimer architecture. *Cell*. 116:711-23.