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Novel Roles of Actin Binding Proteins in Listeria monocytogenes Actin-Based Motility Revealed Within a Cellular Context

by

Raphael Hector Domingo Buencamino

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
This work is dedicated to the memory of Jesusa Alonzo Mossesgeld Buencamino and Teoducia Antonio Domingo. In life, they held my hand and led me through my first challenges. Now I persevere through the toughest trials carried upon the shoulders of their inspiration.
Preface

I first would like to thank my PhD thesis advisor, Ron Vale, for giving me the opportunity and freedom to do this work. I have enjoyed working with Ron because of his constant flow of creative ideas combined with his practical approach to science. My own work was borne out of a series of ideas that Ron had presented to our lab and later led it to its fruition. Shortly before I started in lab Ron had just started exploring *Drosophila* cells as a vehicle to use RNAi and also develop as a tool that we could combine with our strength in microscopy. As often happened with projects that Ron brought to the lab, this led to an enormous surge of new amazing work in his lab that even spread to several other labs at UCSF and across the United States. Ron, throughout my time in his lab continued to present to me many ideas to work on while also giving me enormous support in working on my own ideas. Ron as my advisor has always supported me without hesitation on new endeavors such as my application to medical school. And he has always understanding during some of the more difficult times in my life that coincided during my time in graduate school. Most importantly, Ron has tirelessly taught me how to be a good scientist. These are lessons I will never forget.

Dave Agard, my mentor and thesis committee member, has played a vital part in my development as a scientist. Starting from the summer that I spent in his lab as an undergraduate from Missouri, Dave has taught me so much about how to be a scientist, how to develop new ideas and how to succeed in what ever endeavor I take on. My first exposure to the type of ingenious ideas that were possible started in his lab and through our first discussions. My ever-growing love for physical questions in figuring out the
intricate mechanisms in how biology moves and my notion to always look beyond the conventional answers originates from our enthusiastic conversations on science that continue to this day. He played a large part in my decision to not only come to UCSF but in even entering graduate school. Beyond his role as a teacher he has served as a mentor to me starting from the time that I met him as a naïve undergrad. During some of the most troubled times he has been there to not only support me but without request he has looked out for me; a gift I cannot thank him enough for something so valuable to me. The success that I have earned thus far is greatly due to his role as a teacher, as a mentor and through his unwavering encouragement and support. For this, I owe him much.

Dyche Mullins, one of my thesis committee members, has in many ways been an additional PhD advisor especially because of the long-standing partnership our lab and his lab have continued throughout my graduate studies. Dyche has been indispensable for my project, helping to lead me in the right direction. I am quite lucky to have one of the foremost leaders in the actin field as a source of advice and information for my project. The bulk of what I know about the actin field has originated from my interactions with Dyche. Beyond the actin field he has taught me through my participation in their journal clubs and our weekly joint lab meetings how to interpret, critically read and form sound hypotheses based on problems and information garnered from the literature and other sources. Moreover, Dyche has been a constant calm support and mentor, of which I found to be absolutely valuable during my time here at UCSF.
It is unusual to have a forth thesis committee member, but when I asked around for advice I was always told that Wendell Lim is definitely a faculty you want on a thesis committee. These statements were correct since at every meeting Wendell manages to focus and calm the thesis committee with direct and simple question, while also providing sound and useful advice. Beyond my thesis committee there have been many excellent faculty that have offered me valuable advice and provided ideas in my graduate studies.

In lab, I want to especially acknowledge Steve Rogers. When I entered Ron’s lab I was extremely new cell biology, coming from a background of enzyme kinetics, structural biology, and genetics/microbiology none of which has much remote similarities to cell biology. Steve taught me every technique and approach to cell biology that I currently know. As a post-doc in the lab working also with S2 cells we worked closely together and will be ever thankful for his constant understanding in my constant barrage of questions. Steve also pioneered the model system that I use in this work and wouldn’t have this thesis without him.

I also want to acknowledge Nick Endres and Antonina Roll-Mecak my benchmates. Nick has always been a great supportive ear and fellow graduate student who I could vent my frustrations that only he could currently understand. Antonina has been a constant source of laughter, knowledge, and advice. Having entered as a post-doc halfway through my graduate studies when she arrived in lab we immediately became
friends and will be forever thankful for being my fellow grave-shift company. She has been a great confidant and never faltered to put me back on track when I needed it.

I want to thank the rest of the members of the Vale Lab, a group of the most intelligent, friendly, quirky, and ambitious people that I know. They have been extremely helpful at every step of my education and research. I had some of the best scientific interactions with them in lab and I will miss that greatly.

Much of this work is for my family, Potenciano (Dad), Aida (Mom), Isabel, Michael Buencamino. Through every step they have been there to support me, encourage me, love and accept me no matter the outcome or situation. My Dad, Potenciano, is one of my biggest inspirations for entering graduate school, from his sharp intelligence to his love of knowledge. He is the one person in the world for which I work so hard in order to accomplish something amazing for him like this doctoral thesis. I hope I have lived up to his expectations in this. Most of all I am happy that he is here to see me graduate especially after a time when we weren’t sure about the outcome of the future. We both did it and made it through. My Mom, Aida, she keeps the family together and keeps me together especially through all the hardship of graduate school and life. From her I received my tenacious character to never give up even when things look very bleak. I wouldn’t have accomplished this without her. My older sister, Isabel “Bessie”, it seems after locking me in my room as a baby she made some vow to always protect me and be there for me. In many times of trouble I always took comfort in her words of support. My brother, Michael “Mikey”, my best friend always there to joke around, listen, or just keep
me company in the times I needed it the most. He is the most understanding little brother I have. I know that one day he will surpass this achievement and I can’t wait for that moment.

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I finally want to thank my graduate school classmates we were and have become an amazing bunch. I thank them for all the encouragement and inspiration they have given me all through these years.

UCSF and graduate school is an amazing experience. It is astounding to know that I am taking my next steps forward.
Novel Roles of Actin Binding Proteins in *Listeria monocytogenes* Actin-Based Motility Revealed Within a Cellular Context

By

Raphael H. Buencamino

ABSTRACT

Actin-based motility is essential in many biological processes from fighting harmful organisms to the development of our bodies in the correct configuration. It has been dissected to its basic parts, finding the minimal set of actin binding proteins needed to produce movement. In contrast, actin-based motility in cells allows movement at a fast pace with a diverse set of activities and structures based on the cell’s immediate function within the body.

We dissected the mechanism of actin-based motility *in vivo* and found a set of proteins whose depletion drastically changes the character and efficiency of actin based movement. We accomplished this by using *Listeria monocytogenes*, a pathogen that expresses one bacterial protein, ActA, that allows *Listeria* to hijack the host proteins necessary to move within the cell using actin-based motility. To investigate the function and role of these host actin-binding proteins in actin-based motility, *in vivo*, we used a form of molecular “fractionation”, a powerful technique only previously available for *in vitro* studies. RNAi interference in the *Drosophila* S2 cell line provided us this form of *in vivo* “fractionation” by allowing selective depletion of any protein whose cellular function we wished to examine.
We first examined two proteins, profilin and ADF/cofilin, whose functions have been well documented through biochemical assays and *in vitro* systems. We also sought to dissect actin-based motility further and determine if other actin-binding proteins are involved in its mechanism. Our hypothesis is that with over 90 actin-binding proteins implicated in actin-based motility there are other key proteins that remain uncharacterized beyond the five in the current model. Our experiments identified that CAP is an essential protein for actin-based motility *in vivo*. Through our investigation we also discovered that fascin and other crosslinking proteins, though not essential for motility, might serve a new unique role in regulating the speed and dynamics of *Listeria* and its actin tail. Our observations have allowed us to identify and dissect the protein make-up of the actin-based motility machine.
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Introduction
For most animal cells, the actin cytoskeleton and its associated actin-binding proteins control both the shape and movements of the cell surface. Depending on the cellular response, a specific set of actin-binding proteins will work together to modulate actin and the mechanical forces it provides, resulting in one of a variety of possible cellular structures and motions. One of the more ubiquitous examples of these activities in nature is actin-based motility. In particular, a hallmark of actin-based motility is its tight control such that initiation and maintenance of action requires a particular set of cues: nucleotide state, conformational changes, time/age, cellular location, protein-protein interactions, monomer concentration and mechanical force (Hall 1998; Pollard and Borisy 2003). A unique set of proteins carry out these cues such that propulsion will not occur in the absence of any of these critical components (Loisel et al. 1999). Initially progress in understanding this process was difficult. Even though there were abundant examples of actin-based motility in nature, the components remained biochemically intractable. The discovery of the gram-positive bacteria *Listeria monocytogenes*, a discrete unit that could activate actin-based motility both *in vivo* and *in vitro* accelerated our understanding of actin propulsion (Tilney and Portnoy 1989; Theriot et al. 1992; Mullins et al. 1997; Welch et al. 1997). The questions that actin propulsion presents have continued to fascinate scientists across fields from physics to cell biology. Thus a variety of angles have been used to tackle the elegant complexity of actin-based motility.

**Assembly of the Actin Cytoskeleton**

The assembly of the actin cytoskeleton is essential for the formation of cellular ultra-structure and motion. The resulting actin fibers can be further characterized as
stable or in flux. As a result the cells and the activities they perform can be segmented by
the permanence of the actin fibers. In skeletal muscle it has been long understood that
contraction occurs through the ATP dependant interaction between an actin filament tract
and myosin. This myofibril apparatus remains relatively stable after its initial assembly.
(Gilev 1962; Barany 1967; Panner and Honig 1967).

The initial hypothesis was that actin-based cellular motion followed only one
consistent strategy, actin-myosin contractility (Taylor et al. 1973; Spudich 1974; Allen
and Taylor 1975; Pollard 1975). Instead, closer inspection of non-muscle cells found that
the controls that underlie their motility and even shape were found to be completely
different. An evident characteristic that was described in non-muscle cells is the
heightened flux of its actin cytoskeleton. The key prediction was that the motile events
observed in non-muscle cells were primarily the result of the assembly and disassembly
of its actin fiber matrix (Tilney et al. 1973; Pollard 1975; Tilney 1975; Tilney 1975).

A closer look at the actin filaments reveals a right-handed double-helical filament
with a diameter of 7.5nm. The helical repeat length is 74nm and a 180° strand crossover
distance of 38nm (Heuser and Kirschner 1980; Fowler and Aebi 1983; Egelman 1985).
Actin filaments decorated with heavy meromyosin display polarity in the structure of the
filament, showing a barbed end and a pointed end (Huxley 1963).

The actin elongation reaction at the barbed end is determined by the rate of
monomers colliding with the end, otherwise known as diffusion limited. Thus, the rate of
elongation is directly dependant on the concentration of actin monomers depicted by the rate equation of: \( R = k^+ [A] - k^- \) where \( k^+ \) is the association rate constant, \( k^- \) is the disassociation rate constant and \( A \) is the actin monomer concentration. At equilibrium:

\[ [A] = \frac{k^-}{k^+} \]

This is otherwise known as the critical concentration for actin polymerization, such that above the critical concentration actin monomers polymerize and below the critical concentration actin filaments depolymerize. The actin filaments act as a buffer for actin monomers, keeping monomer concentration to the critical concentration (Drenckhahn and Pollard 1986; Pollard 1986).

The rates of elongation at the barbed and pointed ends were measured by electron microscopy using the actin filament nuclei of the acrosomal process of a Limus sperm. At the barbed end the rate of elongation with Mg-ATP monomers is 11.6 \( \mu \text{M}^{-1}\text{s}^{-1} \) and at the pointed end the rate of elongation is 1.3 \( \mu \text{M}^{-1}\text{s}^{-1} \) (Pollard 1986). As research groups began to closely study the actin polymerization reaction and its role in biology it became increasingly clear that actin polymerization alone, in the absence of myosin, could provide the force needed for actin-based propulsion. An important demonstration of this postulate was shown at the membrane-filament interface of acrosomal vacuoles of Limus sperm: the actin filaments elongate at the membrane associated ends (Tilney et al. 1981).

**The Brownian Ratchet Model and Motility**

Fluorescence recovery after photobleaching (FRAP) of the leading edge of gerbil IMR-33 cells demonstrated further that the phenomenon of actin monomers addition at the membrane-filament interface occurred in motile cells. It was further postulated that
during actin polymerization at the membrane-filament interface the actin filament would either move toward the center of the cell or force the membrane to protrude and provide the extra space needed for monomer addition: this would later be refined into the “Brownian ratchet” model (Wang 1985; Peskin et al. 1993; Mogilner and Oster 1996).

The “Brownian ratchet” model continues to be modified and remain controversial. One of the latest forms of this model the “tethered ratchet” consists of four main characteristics: a polymerizing filament tip, a rigid actin network base, an elastic filament, and a population of tethered filaments (Peskin et al. 1993; Mogilner and Oster 1996; Mogilner and Oster 2003; Mogilner and Oster 2003).

The discovery of an intracellular pathogen, *Listeria monocytogenes*, that hijacks the cell’s actin cytoskeleton and propels itself through the cellular cytoplasm helped to provide much of the evidence for the first model of actin-based motility the “Brownian ratchet”. Electron micrographs illustrated that as the *Listeria* bacterium moves it trails a long tail of polymerized actin forming a solid meshwork of short cross-linked actin fibers (Tilney and Portnoy 1989; Tilney et al. 1992; Tilney et al. 1992). This matrix of cross-linked actin fibers forms the solid base or “ratchet,” preventing the backward diffusion of the load. Furthermore, these studies demonstrated that the actin filaments nucleate and polymerize at the filament-bacterial surface interface. Thus the “Brownian ratchet” motility model can be described as the net forward motion of an object caused by the combination of Brownian motion which provides space for monomer addition at the filament ends, and a rigid actin matrix base which prevents backward diffusion (Theriot et al. 1992; Tilney et al. 1992; Tilney et al. 1992; Peskin et al. 1993).
The actin-based motility of *Listeria* is not dependant on the Brownian motions of the bacterium body, rather the propulsion velocity of the bacterial body is driven by rectifying the elastic thermal fluctuations of the actin filament ends: an “elastic Brownian ratchet” model (Mogilner and Oster 1996). The velocity of *Listeria* propulsion is not dependant on size of the bacterium. Bacteria of different sizes, *Listeria* and *Shigella flexneri*, move at the same rate in potoroo kidney epithelial (PtK2) cells and *Escherichia coli* expressing IcsA on its surface moves in *Xenopus* extract at speeds faster than the smaller sized *Listeria* (Goldberg and Theriot 1995; Theriot 1995). Further support for the elastic Brownian ratchet model is shown in PtK2 cells where the speed of *Listeria* motility matches the rate of actin polymerization (Theriot et al. 1992).

Using experimentally determined parameters a new model, an “elastic Brownian ratchet,” was calculated. In these calculations it was determined that based on the diffusion coefficient for *Listeria* the “ideal ratchet velocity” is \( V_b = 0.05 \, \mu\text{m/sec} \), while the velocity based on the diffusion coefficient for a filament tip is \( V_f = 2500 \, \mu\text{m/sec} \) (Mogilner and Oster 1996). Using the equation for the free polymerization rate of an actin filament \( V_m = k'[A] - k \) where \( k' \) and \( k \) equal the rate of monomer addition and removal at the barbed end, respectively and \( [A] = 10\mu\text{m} \): \( V_m = 0.3 \, \mu\text{m/sec} \) (Pollard 1986). Where \( V_m \) closely matches the actual velocity of *Listeria* motility, \( V_b < V_m \) and \( V_f >> V_m \), demonstrating that while velocity determined from the diffusion constant of *Listeria* cannot account for the speeds seen in nature, the velocity of motility determined
from the diffusion coefficient of the flexible filament tip more than accounts for the fastest observed velocities of *Listeria* (Mogilner and Oster 1996).

**The Tethered Ratchet Model and F-Actin Attachment to the Surface of Pathogens**

The *Listeria* bacterial cell is not sensitive to the local viscoelasticity. When compared to adjacent lipid droplets in COS7 cells, lipid droplets fluctuate 20-fold more than the bacterial cell even when you compensate for size and shape differences. One clear explanation for this observation is that the bacterial cell is attached to its actin tail. Upon further examination, using high-resolution tracking technology, *Listeria* motility exhibits frequent pauses and step-like motion at discrete distances of 5.4nm, which corresponds to the spatial frequency of filamentous actin. This study led to the proposal of a new model for the mechanics of actin-based motility. In this model a population of actin filaments are attached to the bacterial surface and *Listeria* motility propels forward in steps by sliding along the actin filament from one actin monomer to another (Kuo and McGrath 2000).

Further evidence was found when optical traps demonstrated that 10pN of force is needed to separate a bacterial cell from its actin tail (Gerbal et al. 2000). Electron micrographs of actin tails demonstrated transient attachments of individual actin fibers to the surface of the load (Cameron et al. 2001). Each study prompted new models. One of these models the “tethered ratchet” model predicts there is a balance between hundreds of “working” free filaments that push the bacteria forward against transiently tethered filaments that are pulled taught and resist forward movement of the load. Three
interesting theories evolve from this model. One theory is the velocity increases with an increasing density of filaments. The second theory states the number of “working” filaments are unaffected by changes in the load, but the number of attached filaments increases with increasing load. The third theory aims to describe the biphasic nature of *Listeria’s* force-velocity relation. At small loads (0 to ~20 pN) the velocity decreases quickly. The postulation states that at this phase the velocity is sensitive to the number of attached filaments. In contrast, at greater loads the attachments break with the free disassociation rate, where the force per attachments begins to decrease with decreasing velocity; the external load’s resistance contribution begins to dominate. As a result, at the second phase the velocity decreases slowly as the load increases (Mogilner and Oster 2003).

**Dendritic Nucleation**

*Listeria* is motile for two main reasons: one is the polymerization of the actin cytoskeleton and the second is its ability to nucleate new actin filaments. *Listeria* expresses one extracellular protein on its cell surface to hijack the actin cytoskeleton, ActA (Domann et al. 1992; Kocks et al. 1992). Expression of ActA in potoroo, rat kangaroo, kidney epithelial cells (PtK₂) resulted in its localization at the mitochondrial membrane and the formation of actin at these sites. This demonstrated that ActA expression in *Listeria* alone could hijack the host actin cytoskeleton machinery (Pistor et al. 1994). However, bacteria expressing ActA incubated with G-actin in solution could not nucleate actin filaments (Tilney and Portnoy 1989; Tilney et al. 1992). This result
suggested that actin filament nucleation and tail formation required one or more host proteins.

When *Listeria* is added to cytoplasmic extract (*Xenopus laevis* eggs or human platelet) the bacteria begin forming actin tails and start moving, the same activity observed within intact cells. Fractionating this extract resulted in the discovery of a protein complex of eight polypeptides that produced actin filaments (Welch et al. 1997). A homologue of this complex was initially isolated from *Acanthamoeba castellanii* and named Arp2/3 (Machesky et al. 1994; Mullins et al. 1997). Arp2/3 functions in the cell by first binding the sides of existing actin filaments and nucleating a new actin filament angled at 70°. Arp2/3 essentially creates a network of crosslinked actin filaments with a specific structure of 70° angled branches and a capped pointed end, creating an ideal structure for motility. This description of Arp2/3 function is known as the “dendritic nucleation” model (Mullins et al. 1998; Amann and Pollard 2001).

**The in vitro reconstituted Minimum Requirement**

The discovery of Arp2/3 prompted attempts to investigate the system of proteins responsible for the dynamic actin cytoskeleton seen in motile cells and pathogens. Using purified proteins and the pathogen *Listeria*, actin-based motility can be reconstituted. Thus, *in vitro*, the minimum requirement for motility is an Arp2/3 activator such as ActA, Arp2/3, actin, ADF/cofilin and capping protein. For *Listeria* actin-based motility VASP and profilin were not essential but are nonetheless important for motility. For *E. coli* expressing IcsA, profilin but not VASP was found to be important. In this study the effect
of an actin crosslinker $\alpha$-actinin was also investigated and was found to have no strong role in motility or even the rate of motility (Loisel et al. 1999).

Several important conclusions emerged from this study. First, actin-based motility requires a specific set of proteins each with discrete roles. Second, the quality of motility can be affected by adding other actin-associated proteins or by changing the concentrations of these proteins. Thus, this finding provided direct evidence as to the essential role of these previously characterized proteins (Loisel et al. 1999; Pollard and Borisy 2003).

The Actin-Based Motility Prototype

The combination of the dendritic nucleation model with the identification of the minimal set of proteins required for reconstituted actin-based motility established the theoretical base upon which a unifying hypothesis for actin-based motility could be constructed. Most recently called the “dendritic nucleation/array treadmilling hypothesis,” this model begins to describe the interplay of the “minimal set” of actin-binding proteins in the construction, remodeling and deconstruction of the actin cytoskeleton (Pollard and Borisy 2003). Each component has evolved a mechanism of coordinated activities, resulting in a precise system of actin-based motility. Carefully we have begun to tease apart the activities of these protein components and the mechanism of their ordered interactions.
The interplay between actin and ADF/cofilins are one of the best-understood systems in actin-based motility. Their evolved coordination allows for precise timing and rate control in actin depolymerization. In motile cells and pathogens, the rapid actin polymerization utilized for motility is not sustainable without its corresponding rapid depolymerization, allowing for continuous recycling of actin monomers. Proteins in the ADF/cofilin family fulfill this role; they are the main actin depolymerizing factors in eukaryotic cells. The mechanism by which ADF/cofilins maintain rapid depolymerization with precise timing and balance is multi-dimensional (Bamburg et al. 1999; Pollard and Borisy 2003).

The Actin-Filament Biological Clock

Actin filaments themselves act as a timer. The actin monomers “age,” in that while ATP hydrolysis is fast with a half-time of 2 seconds, phosphate release is slow with a half-time of 350s (Carlier and Pantaloni 1986; Blanchoin and Pollard 2002). The result is an actin filament where the barbed end is composed of ATP-actin monomers while the majority of the filament is composed of the intermediate ADP-Pi-actin, and the pointed end is composed of ADP-actin. Among the different nucleotide states for actin, ATP-actin and ADP-Pi-actin are characteristically identical. The rate of ATP-actin monomer association at the barbed end is greater than the rates of association and disassociation at the barbed and pointed ends for both ATP and ADP-actin monomers (Pollard 1986; Pollard and Borisy 2003; Kuhn and Pollard 2005). This creates a property for actin filament kinetics, even in the absence of actin binding proteins, where ATP-actin monomers preferentially bind at the barbed end, and ADP-actin subunits are populated
and disassociate at the pointed end, resulting in treadmilling of actin subunits from the barbed end to the pointed end. Thus, the internal timer inherent in actin filaments are characterized by both the nucleotide state of actin monomers and their progression from the barbed end to the pointed end (Pollard 1986; Carlier and Pantaloni 1997).

ADF/cofilins have evolved to exploit the internal timer inherent in actin filaments resulting in rapid depolymerization. ADF/cofilin’s high binding affinity for ADP-actin filaments ensures targeting of ADF/cofilin severing activity towards the pointed end of actin filaments. To further facilitate this targeting, ADF/cofilins bind weakly to ATP and ADP-Pi-actin filaments (Maciver et al. 1991). Once ADF/cofilins bind to ADP-actin filaments, ADF/cofilins catalyze actin depolymerization through 4 stages. First, even with ADF/cofilin’s low affinity for ADP-Pi-actin filaments, ADF/cofilins accelerate the actin depolymerization timer by promoting inorganic phosphate release (Blanchoin and Pollard 1999). Inorganic phosphate release then weakens the attachment of the actin filaments to the Arp2/3 complex, resulting in debranching and exposure of the pointed end (Blanchoin et al. 2000). Next, upon binding to ADP-actin filaments ADF/cofilins change the twist of these filaments such that the crossover length is decreased by 10nm. This change in twist is retained in the region bound by the ADF/cofilins and is not propagated to the rest of the actin filament, ensuring that the effects of ADF/cofilins are targeted (McGough et al. 1997; Bamburg et al. 1999). Finally, the combination of ADF/cofilin binding and the resulting twist may weaken the lateral contacts between the actin subunits resulting in actin filament severing and increased ADP-monomer disassociation from the additional actin filament ends (Maciver et al. 1991; Carlier et al.)
Recent evidence suggest that these “stages” of cofilin function may be separated through differences in cofilin concentrations, where low cofilin concentrations sever actin filaments and higher cofilin concentrations debranch and promote inorganic phosphate release. If and how differences in cofilin concentration are regulated in the actin filament array of motile elements remains to be determined (Andrianantoandro and Pollard 2006).

**Recycling Actin**

The depolymerization activity of ADF/cofilin results in a pool of ADP-actin monomers. In order to “recycle” these ADP-actin monomers into ATP-actin monomers primed for rapid polymerization, profilin has evolved to modulate the activities of ADF/cofilin, ADP-actin monomers, and ATP-actin (Blanchoin and Pollard 1998; Didry et al. 1998; Mattila et al. 2004; Bertling et al. 2007). ADP-actin monomers bind tightly to ADF/cofilin at a $K_d=0.1\mu M$ about 5-fold tighter than profilin. Physiological concentrations of profilin can overcome this inhibition of ADP-actin release by promoting ADP disassociation. This reduces the strength of ADF/cofilin’s affinity for actin and due to ATP’s higher cellular concentrations, as well as its higher affinity for actin, the nucleotide free actin binds ATP. Profilin then binds ATP-actin monomers with a binding strength almost 50-fold greater than ADF/cofilin. Thus, through the above mechanisms profilin drives the pool of actin monomers towards the ATP bound state (Blanchoin and Pollard 1998; Didry et al. 1998; Pollard and Borisy 2003).
Formation of the profilin bound ATP-actin monomer state itself provides two functions. First, sequestration of ATP-actin monomers into profilin-ATP-actin monomer complexes prevents spontaneous nucleation. Second, profilin functions as a molecular shuttle in which profilin picks up ATP-actin monomers and delivers it to a specific location. In accordance to this function, the profilin-ATP-actin monomer complex accelerates polymerization at the barbed end of the actin filament while preventing ATP-actin monomer addition at the pointed end. (Pring et al. 1992; Didry et al. 1998; Vinson et al. 1998; Kaiser et al. 1999; Kang et al. 1999). Profilin shuttling targets to various proteins expressed in the cell and even from pathogens. These proteins contain poly-L-proline domains that act to attract profilin-ATP-actin monomer complexes. This strategy is implicated in vasodilator-stimulated phosphoprotein (VASP) in complex with ActA from Listeria and in the Shigella induced proteolytic product vinculin p90 (Kang et al. 1997; Laine et al. 1997; Zeile et al. 1998). Profilin shuttling can additionally function in gating formin proteins from a capped state to an open state allowing profilin to deposit its bound ATP-actin monomer onto the barbed end (Kovar et al. 2003; Romero et al. 2004). All these diverse functions of profilin work to accomplish the basic goal of controlled actin filament elongation at the barbed end.

Staying on the Treadmill

The treadmilling of actin filaments support the forward movement of the leading edge or the actin-based motility of Listeria. Profilin and ADF/cofilin work in concert to increase the efficiency of actin-monomer transfer from the pointed-end back to the barbed end thus catalyzing the “treadmilling reaction.” Alone, ADF/cofilin increases the
rate of treadmilling by 25-fold, but after addition of bovine profilin treadmilling rates increased up to 125-fold. Even though plant profilins have no effect on actin monomer nucleotide, adding plant profilin still increases treadmilling rates by 75-fold. Presenting further evidence that profilin’s synergistic role with ADF/cofilin in speeding up treadmilling includes binding actin monomers and allowing only barbed end addition (Perelroizen et al. 1996; Didry et al. 1998).

The increase in treadmilling rates observed in these experiments may not completely originate from an increase in the treadmilling rates of individual filaments. Instead profilin and ADF/cofilin may work synergistically to the increase in the treadmilling efficiency of the overall actin dendritic array in moving the load forward. Within this model, profilin and ADF/cofilin can accomplish this by supporting the inherent barbed to pointed-end treadmilling directionality of the actin filament and also ensuring actin monomers stay engaged with the treadmilling actin filament array during turnover. Moreover, recent work using total internal reflection microscopy (TIRF) presented evidence that the rate of subunit disassociation from an individual filament is not directly increased by ADF/cofilin binding but rather indirectly through ADF/cofilin’s ability to promote inorganic phosphate release. In fact, the rate of cofillin facilitated actin filament depolymerization equaled the rate of ADP-actin filament depolymerization. The evidence in this work further proposed that the increases in actin filament depolymerization rates seen in bulk assays are the result of ADF/cofilin’s contribution to the collective turnover of actin filaments through filament severing, Arp2/3 debranching as well as its promotion of inorganic phosphate release. Each activity serves to establish
an environment for maximal “natural” depolymerization rather than direct binding and removal of actin subunits (Carlier et al. 1997; Andrianantoandro and Pollard 2006). ADF/cofilin through three functions is able to increase the rate of turnover for the combined actin filament array since they work to harness rather than directly operate the barbed to pointed-end actin subunit association to disassociation treadmilling property already present among individual actin filaments.

In addition, to prevent exhaustion of the treadmilling machine, large reserves of ATP-actin monomers are maintained by several support mechanisms that work to keep subunits engaged with the treadmilling system. At the barbed end, ADF/cofilin is shown to inhibit depolymerization while profilin prevents its bound ATP-actin monomer from associating with the pointed-end (Andrianantoandro and Pollard 2006). Profilin available at high concentrations binds individual ATP-actin monomers and prevents spontaneous nucleation of superfluous filaments outside of the treadmilling network (Vinson et al. 1998). The synergy between profilin and ADF/cofilin serves a dual role of continuing to channel the intrinsic actin subunit association/disassociation property of actin filaments to increase treadmilling rates but also serves to retain actin monomers, maintaining an abundant supply of “raw materials.”

**Listeria and the Mystery of VASP Function**

One of the few proteins identified to physically bind to ActA on the surface of *Listeria* is the actin binding protein VASP (Niebuhr et al. 1997; Laurent et al. 1999). VASP was first recognized to assist in actin-based motility by binding profilin at its poly-
L-proline region (Reinhard et al. 1995; Gertler et al. 1996). In another experiment, *Listeria* motility is inhibited after addition of a peptide containing the poly-L-proline region of VASP, suggesting sequestration of profilin proteins (Kang et al. 1997). The inhibition of *Listeria* motility by a covalently crosslinked profilin-actin (PxA) compound was eliminated upon mutation of PxA’s poly-L-proline binding site, suggesting that VASP via its poly-L-proline region is necessary for recruitment of profilin to the barbed end – bacterial surface interface (Grenklo et al. 2003). These studies suggest that VASP functions in recruiting profilin to the site of activity, such as the barbed ends at the bacterial surface. In contrast to these findings, VASP has been shown to increase *Listeria* velocity in the absence of VASP in reconstitution assays (Loisel et al. 1999). In contrast to its activity in *Listeria* movement, a study found that VASP negatively regulates fibroblast motility at the leading edge instead of enhancing motility (Bear et al. 2000). The negative regulation observed from this experiment was determined to originate from VASP’s function in interfering with barbed end capping (Barzik et al. 2005). Recently it was demonstrated using TIRF microscopy that VASP alone does not enhance filament elongation but can accelerate elongation in the presence of profilin at rates faster then profilin alone (Pasic et al. 2008). This finding raises the question of how VASP improves motility in reconstitution assays in the absence of profilin and why this activity is particular to *Listeria* motility and not other pathogens.

**Cyclase Associated Protein: the Missing Link in Actin Monomer Recycling**

After pointed end disassociation of ADP-actin monomers a small pool of ADP-actin monomer/cofilin complexes accumulates. Cofilin, however, does not function in
sequestration of actin monomers and requires assistance in transferring its bound actin monomer to another protein, allowing cofilin to return to actin filaments and enhance disassembly. Profilin was identified to be the repository for actin monomers, particularly ATP-actin monomers. To efficiently carry out their particular functions cofilin must transfer its bound ADP-actin monomer to profilin. However, for ADP-actin monomers cofilin binding strength is 5-fold greater than profilin. This enormous difference in binding energies was never completely addressed (Blanchoin and Pollard 1998; Didry et al. 1998).

An elaborate explanation was made where profilin promotes ADP release from cofilin bound ADP-actin monomers which then causes cofilin release of actin monomers allowing for fast ATP exchange at which point profilin binds the ATP-actin monomers with binding energies greater than cofilin. Moreover, an assumption was made that the physiologic concentration of profilin in cells is large enough to compensate for this large unfavorable difference between profilin and cofilin for ADP-actin monomer binding and exchange. Even though actin filament rates are at least 50-fold faster than rates measured in vivo, indicating a missing cellular factor, this discrepancy was not explored further (Blanchoin and Pollard 1998; Didry et al. 1998; Balcer et al. 2003; Pollard and Borisy 2003).

An identified adenylyl cyclase activator, cyclase associated protein (CAP), was discovered to bind actin monomers (Freeman et al. 1996). Much later this protein was characterized biochemically and demonstrated to accelerate release of cofilin from
monomer binding (Moriyama and Yahara 2002). Further biochemical characterization of the yeast homolog, Srv2, demonstrated that CAP has a binding strength against ADP-actin monomers 5-fold greater than cofilin. These results suggest that CAP’s role may be to assist profilin in releasing ADP-actin monomers from cofilin. CAP was also identified to bind ATP-actin monomers 10-fold weaker than profilin. Moreover, addition of CAP enhances profilin’s nucleotide exchange rate lowering its half-maximal effect concentration from 1.5µM to 0.65µM. Thus, CAP strongly competes with cofilin for ADP-actin monomers releasing it for further rounds of depolymerization. Bound ADP-actin monomers are then strongly promoted to undergo nucleotide exchange in synergy with profilin and once ATP is exchanged CAP easily transfers binding of ATP-actin monomers to profilin for shuttling to the barbed-end for subunit association (Moriyama and Yahara 2002; Balcer et al. 2003; Mattila et al. 2004). As demonstrated by these studies CAP clearly addresses the discrepancy in the transfer of actin monomers from cofilin to profilin with a simple and direct mechanism more likely to be developed by evolution rather than a complex solution requiring many conditions.

**Fascin, Filaments and Filapodia**

Fascin is a 55 kD protein involved in crosslinking actin filaments in parallel bundles (Tseng et al. 2001). In cells, fascin localizes to long narrow cellular structures that need support such as filapodia, stress fibers, and microvilli (Bryan et al. 1993; Ishikawa et al. 1998; Svitkina et al. 2003; Vignjevic et al. 2006). As a crosslinker it is relatively considered to create more flexible actin networks. Preparations *in vitro* of fascin-actin networks give poor resistance to even small amounts of mechanical stress
(Xu et al. 2000). Even at high physiological concentrations fascin-actin networks exhibit very low elasticity (Palmer et al. 1999).

Recently, fascin has been characterized for its role in filapodia formation. \textit{In vitro} fascin is required for the formation of filapodia-like bundles, while \textit{in vivo} fascin depletion results in loss of filapodia and formation of abnormal long flimsy filament bundles (Vignjevic et al. 2003; Vignjevic et al. 2006). Fascin involvement in filapodia formation and its localization to long structures in the cell may indicate a role in \textit{Listeria} protrusion formation since many characteristics of filapodia actin filament structure match those seen in EM images of \textit{Listeria} protrusions through the cell membrane. In addition, involvement of VASP at the tips of filapodia structures further suggests possible involvement of fascin as a common element in \textit{Listeria} actin tails and filapodia bundles (Sechi et al. 1997; Lanier et al. 1999; Svitkina et al. 2003).

\textbf{Crosslinkers in actin-based motility}

Involvement of crosslinkers in actin-based motility has generally been ignored. A few studies have begun to explore the possibility of their involvement in \textit{Listeria} motility. Addition of $\alpha$-actinin to reconstitution assays revealed that $\alpha$-actin is not required for motility. Moreover, varying its concentration had no affect on velocity. Fascin, fimbrin and $\alpha$-actinin have all been localized to \textit{Listeria} actin tails (Dabiri et al. 1990; Kocks and Cossart 1993; Brieher et al. 2004). Their exact role and influence on the actin tail or the actin network in crawling cells is fairly unexplored. While there is no documentation of filamin localization in \textit{Listeria} actin tails, it has been implicated to be involved in actin-
based motility. Filamin null cells have reduced migratory speed and display high frequency of plasma membrane blebbing (Adelman and Taylor 1969; Cunningham 1995; Flanagan et al. 2001). Recent studies have demonstrated that addition of a high concentration of Filamin with a high concentration of gelsolin can produce faster migration speeds in beads coated with the Arp2/3 activator, verprolin/cofilin/acidic (VCA) domain from Wiskott-Aldrich Syndrome Protein (WASP). Recently, in another study fascin along with three other crosslinking proteins α-actinin, filamin, and fimbrin were necessary for the formation of a specialized tail that allowed a short burst of Arp2/3 independent motility (Brieher et al. 2004). The following study demonstrates that all four crosslinkers share a particular function in Listeria motility, we hope that by exploring crosslinking proteins we can help to determine what the unifying function of crosslinkers are in Listeria actin-based motility.

**Finding New Directions**

We used this vast body of work in the actin field to lead our work in new directions. Each study in the literature left us clues examine in more detail and in this goal we were fortunate to find an in vivo experimental system that gave us many of the freedoms employed in vitro but still enabled us to ask and answer certain questions that can only be revealed in an in vivo system. Even for proteins with an exhaustive body of work, such as profilin and cofilin, much of the data collected from studies in vitro remain untested for their role in vivo. Other proteins like CAP are only now being carefully examined. Our study provides one of the first sets of direct in vivo evidence that demonstrates CAP’s integral role in actin-based motility. Moreover, in our decision to
examine crosslinking proteins to discover their role in *Listeria* motility we expected to find a plain and direct result. Instead our studies reveal that each crosslinking protein plays a balanced role in both regulating speed and affecting actin dynamics.
Results
Materials and Methods

Bacterial strains and cells

Wild-type *Listeria monocytogenes* strain 10403S was grown in brain-heart infusion (BHI) medium (Difco) at 30°C without shaking (Bishop and Hinrichs 1987; Cheng and Portnoy 2003). *Drosophila* Schneider S2 cells were grown in Schneider’s *Drosophila* medium (SM; Invitrogen) supplemented with 10% heat-inactivated FCS (Invitrogen) at RT and incubated with dsRNA for 7 d as previously described (Clemens et al. 2000; Rogers et al. 2002; Rogers et al. 2003).

Infection of *Drosophila* S2 cells

Cultures of S2 cells in SM-FCS media are supplemented with 19µg ml⁻¹ of cholesterol (Sigma) and incubated at RT. After 30 min of incubation non-adherent cells and cholesterol supplemented SM-FCS media were removed. Remaining cells were resuspended, diluted in fresh SM-FCS media and infected with an addition of *Listeria monocytogenes* resuspended in PBS. After 1 h of infection, non-adherent cells and bacteria were removed, and fresh SM-FCS media containing 10 µg ml⁻¹ gentamicin (Sigma) was added (Cheng and Portnoy 2003). After 1 h of incubation cells were plated in 35 mm culture dishes with No. 1.5 coverslip bottoms (MatTek) that had been coated with a solution of 0.5 mg ml⁻¹ concanavalin A (ConA; Sigma-Aldrich).

Live cell imaging and analysis of *Listeria* motility and GFP-actin tails
S2 cells expressing GFP-actin were treated with dsRNA, infected with *L. monocytogenes* at d 7 and then added to culture dishes with coverslip bottoms coated with ConA as described above. Cells were observed 4-7 h after infection and images were collected at 5 s intervals by CCD camera (Coolsnap HQ) mounted on an inverted microscope (Olympus Ix70) with a 60x PH3 oil immersion objective at RT.

Prior to analysis, standard image processing steps were carried out using ImageJ. Individual Listeria were tracked using a set of algorithms written in the IDL language, obtained from Eric Weeks ([http://www.physics.emory.edu/faculty/weeks/](http://www.physics.emory.edu/faculty/weeks/)) or were tracked by hand using ImageJ. All subsequent numerical analysis was carried out using KaleidaGraph and custom-designed IDL routines. Image analyses of GFP-actin tails were performed with ImageJ.

**Results**

We previously developed a method for observing the *in vivo* roles of ~90 actin binding proteins in lamella formation and dynamics in *Drosophila* S2 cell lines, using RNA interference (RNAi) mediated inhibition of targeted proteins. Our results identified a key set of proteins required for lamella formation (Rogers et al. 2003). As a result we sought to investigate other aspects of the actin cytoskeleton, one of which is actin-based motility. However, it was difficult to induce cell migration in a cell line susceptible to RNAi. To overcome this difficulty we developed a method for analyzing actin-based motility in S2 cells using the pathogen *Listeria monocytogenes*. *Listeria* was ideal for our experimental goals for many of the same reasons it has been used in previous actin-based motility assays. First, *Listeria* is a discrete motile unit whose movement can be easily measured.
Second, in S2 cells expressing GFP-actin *Listeria* also robustly produces a discrete actin tail whose dynamics and dimensions, for similar reasons, can be easily measured. Third, *Drosophila* S2 cells, a cell culture system with a high sensitivity to RNA interference (RNAi), are susceptible to *Listeria* infection and display the same characteristics for *Listeria* actin-based motility as seen in mammalian cells used in previous studies.

In our previous work with S2 cells we sought to improve its cytology in order to better examine microtubule structure and dynamics. We used the substrate concanavalin A to promote cell attachment and formation of a flattened discoid morphology about 20µm in diameter (Rogers et al. 2002). We also used this strategy to examine actin-based lamella formation, where upon further examination using fluorescence microscopy on GFP-actin expressing S2 cells we were able to determine that about 90% of the cells adopt a radially symmetrical actin cytoskeleton organization. This actin cytoskeleton cellular structure can be divided into three zones; a dense actin network at the extreme periphery of the cells about ~1 µm wide, a second inner layer comprised of a lower density of actin about 4-6 µm wide and a core composed of a circular bundle of filaments that surround the nucleus. In our model the first two zones make up the lamella of the cell (Rogers et al. 2003). Most of the motile *Listeria* (90%) are visualized in the first two zones described above and are obscured when entering the area occupied by the nucleus. As a result, the dynamics of *Listeria* and its actin tail were difficult to analyze. When we examined the motility of *Listeria* using phase-contrast microscopy the trajectories were short and often were perturbed by interactions with the cell membrane. We encountered
many of the same problems when examining the actin tail under fluorescence microscopy.

In order to improve our visualization of *Listeria* motility and the dynamics of its GFP labeled actin tail we decided to use a S2 cell phenotype discovered from our earlier studies. Treatment of S2 cells with Pavarotti dsRNA results in large multi-nucleate cells. Pavarotti is a mitotic motor protein involved in central spindle formation and cytokinesis (Goshima and Vale 2003). We examined whether depletion of Pavarotti by RNAi caused any changes in *Listeria* motility; in comparison to WT we found no difference in the rate of *Listeria* movement (Figure 2C). The *Listeria* within the Pavarotti depleted S2 cells exhibited extended trajectories in its movement, remained outside of the visually obscuring central nucleus area longer, and encountered the cell membrane less often. Because of the significant improvement in the quality of our analysis this change imparted, we employed this strategy for subsequent examination of *Listeria* motility and actin-tail formation.

*Listeria* enters cells within one hour of inoculation where they lie in phagosomes (Cheng and Portnoy 2003). Using time-lapse phase microscopy the *Listeria* bacterial rods can occasionally be seen within the phagosome; they appear as dark rods ~2-3 µm in linear dimension. At 5 h after inoculation *Listeria* escape from phagosomes and enter the cytoplasm. Typically about 90% of the *Listeria* begin moving after exposure to the cytoplasm. Under phase microscopy, *Listeria* is shown to travel either in a linear trajectory directly into the cell membrane or with less directional persistence arching
either back towards the nucleus or along the second inner layer of the lamella. Motile *Listeria* display density at the tail end of the bacterial rod where it is strongest at the bacterial surface and tapers off. Under fluorescence microscopy the GFP labeled actin tail appears as a bright comet shaped structure that, again, has the highest intensity at the bacterial surface and tapers off. One notable difference is that the phase image of the actin tail is usually shorter than the fluorescent image of the actin tail by 50% or more.

In our experimental approach we approximated the instantaneous velocity of *Listeria* as the distance traveled in 5 s. The resulting instantaneous velocity profiles of a moving *Listeria* varied considerably. We treated our wild-type cells with the same conditions as our RNAi treated cells. In order to mimic conditions without causing any RNAi specific depletions we used dsRNA of a gene sequence that is not present in the Drosophila genome. The gene sequence we used was bluescript. Within the wild-type cells that were treated with bluescript dsRNA *Listeria* moved at a rate of 0.15 µm s\(^{-1}\) (Figure 2C) and Pavarotti dsRNA treated cells *Listeria* moved at a similar rate of 0.12 µm s\(^{-1}\) (Figure 2C).

**Depletion of Actin Turnover Proteins Profilin and ADF/Cofilin affects the velocity of *Listeria* motility**

As seen in experimental investigations *in vitro* profilin and ADF/cofilin are key proteins in *Listeria* actin-based motility, where the former protein was even found to be essential for movement (Theriot et al. 1994; Loisel et al. 1999). We tested this hypothesis by measuring rates of *Listeria* motility in live S2 cells depleted of either profilin or
ADF/cofilin by RNAi. To measure the rates of *Listeria* movement we analyzed infected cells using phase contrast microscopy. Under phase microscopy *Listeria* appear as dark dense rods with a very sharp visual contrast against the cellular environment. Using this characteristic we were able to track *Listeria* easily using either automated tracking methods or by hand.

Compared to rates of *Listeria* motility in control cells, velocity was severely inhibited in both Profilin and ADF/cofilin dsRNA treated cells. For both conditions there was about a 50% decrease in velocity (Figure 2C). A Mann-Whitney U-test showed a statistically significant difference in the distributions of instantaneous movement rates for *Listeria* in controls and in cells treated with either ADF/cofilin or profilin dsRNA (Figure 2C) (**p** = < 0.0001).

**Significance of Cyclase Associated Protein in actin-based motility**

Both biochemical and *in vivo* evidence have begun to demonstrate CAP involvement with actin polymerization and turnover (Freeman et al. 1996; Moriyama and Yahara 2002; Balcer et al. 2003). CAP has never been tested for activity in *Listeria* reconstitution assays and all of the *in vivo* data have mainly demonstrated its role in cell shape and actin structure (Benlali et al. 2000; Rogers et al. 2003). To determine whether CAP’s function in the actin cytoskeleton extends to actin-based motility we measured velocities of *Listeria* in CAP RNAi depleted S2 cells and compared them to our results with ADF/cofilin and profilin depleted cells.
Compared with control cells depletion of CAP from S2 cells resulted in reduced velocities of *Listeria* movement greater than 50% (Figure 2C). A Mann-Whitney U-test showed a statistically significant difference in the distributions of instantaneous movement rates for *Listeria* in controls and cells treated with CAP dsRNA (Figure 2C) (p = < 0.0001). Our results suggest that CAP does not serve a redundant role in actin polymerization and moreover, the similar degree to which CAP depletion inhibits *Listeria* velocities as compared to profilin and ADF/cofilin RNAi depletion posits the possibility of a more central role for CAP in actin-based motility.

**A Role for the Actin Crosslinker Fascin in *Listeria* Actin-Based Motility**

One of the crosslinking proteins, α-actinin, had no effect on the actin-based motility of *Listeria* in reconstitution assays (Loisel et al. 1999). In contrast to this, recently crosslinking proteins, in particular fascin, were shown to transiently mediate actin-based propulsion independent of the Arp2/3 nucleated dendritic array pathway. One model has been proposed in which crosslinking proteins contribute to the normal propulsive force of *Listeria* actin-based motility (Brieher et al. 2004). To test this hypothesis we analyzed the rate of *Listeria* motility in S2 cells depleted of fascin.

Depletion of fascin from S2 cells results in an increased rate of *Listeria* motility by at least 2-fold at a rate of 0.26 µm/sec compared against 0.12 µm/sec in control cells (Figure 2C). A Mann-Whitney U-test showed a statistically significant difference in the distributions of instantaneous movement rates for *Listeria* in controls and cells treated with fascin dsRNA (Figure 2C) (p = < 0.0001).
To test whether depletion of fascin from S2 cells also affected actin tail dynamics, we observed *Listeria* motility in S2 cells expressing GFP-actin under a fluorescent microscope. From these images we assessed the spectrum of actin tail lengths for each individual bacteria and recorded the maximum length. Fascin depleted cells were found on average to contain *Listeria* with actin tail lengths 2-fold greater than the length of actin tails found in control cells (Figure 3A). A two-tailed student t-test showed a statistically significant difference in the distributions of actin tail lengths for *Listeria* in controls and cells depleted of fascin by RNAi (Figure 2C) ($p = < 0.0001$). The extent of actin tail lengths found in fascin depleted cells can be dramatically large with the maximum length of *Listeria* actin tails at 47µm compared to a maximum length of 11µm in control cells (Figure 3B and 3C).

**Correlation Between *Listeria* Actin Tail Length and Velocity in Fascin Depleted Cells**

In previous studies *Listeria* actin tail length has been consistently shown to directly correlate with bacterial speed (Theriot et al. 1992; Soo and Theriot 2005). *Listeria* in fascin-depleted cells have longer actin tails, but also have higher velocities than in control cells. The property of longer actin tails may be directly due to the increase in bacterial velocity rather than due to any effect fascin depletion may have on actin tail dynamics. We tested this hypothesis by determining for each bacterium the averaged instantaneous velocities starting at the time point where the measured actin tail length (Figure 3A) first appears and ending at the time point when the actin tail decays.
In our control cells, actin tail length increased with increasing average velocity. The relationship between actin tail length and velocity approximates actin tail persistence time expressed by the slope of the linear fit as determined by the least squares method (Figure 4A) (Theriot et al. 1992; Soo and Theriot 2005). We divided fascin-depleted cells into two different cellular populations for our analysis based on an observed bimodal distribution of the individual linear relationships between actin tail length and bacterial speed. The first population of cells (type I) fit to a relationship that closely matches control cells (Figure 4B-4E). Indicating that in these population of cells the increased tail lengths is a direct affect of the increased speeds observed in fascin-depleted cells. The second population (type II) has a 50-75% positive offset from control cells (Figure 4F and 4G). For the linear relationship between actin tail lengths and bacterial speed a two-tailed student t-test showed a statistically significant difference between the fascin-depleted cell population type II versus control cells and versus cells depleted of fascin, population type I (p = < 0.0001 and p = 0.001 respectively).

For fascin depleted cells type II, the significant shift in the linear relationship between actin tail length and bacterial speed may indicate that fascin depletion at a certain level also causes changes in the actin tail dynamics. We tested this hypothesis with observations of the GFP-actin tail and its decay in fluorescence over time. As seen in previous studies on actin tail depolymerization, fluorescence decay was exponential (Theriot et al. 1992; Soo and Theriot 2005). To represent the rate of depolymerization we choose to calculate the half-life from the measured exponential decay constants. Based on
the global exponential fit (Figure 7) actin tails within control cells depolymerized with a half-life of about 36 s. This value closely approximates previous findings from *Listeria* actin tails in PtK2 cells (33 s) and in *Xenopus* extract (30 s and 42 s) (Theriot et al. 1992; Theriot et al. 1994; Soo and Theriot 2005).

As was seen in the tail length/velocity correlation analysis, a bimodal distribution is observed in a histogram of actin tail decay rates in fascin depleted cells, splitting the cells into two different pools. For the actin tail decay rates a two-tailed student t-test showed a statistically significant difference between the fascin-depleted cell population type II versus the fascin depleted cell population type I (p = < 0.0001). For the first set, the measured rate of actin tail filament depolymerization at 33 s closely matched values found in control cells and previous studies (Figure 7) (Theriot et al. 1992; Soo and Theriot 2005). In contrast, the second group of fascin-depleted cells contains *Listeria* with rates of actin tail decay 4-fold slower than rates in control cells (Figure 7). These results indicate that the long tails in fascin-depleted cells can be attributed to both faster velocities and slower rates of depolymerization.

**Depletion of other actin-bundling proteins and their effect on *Listeria* motility**

Fascin (Brieher et al. 2004), Fimbrin (Kocks and Cossart 1993), and α-actinin (Dabiri et al. 1990) all localize to *Listeria* actin tails as seen by immunofluorescence microscopy. We sought to determine if the effects of fascin depletion on *Listeria* motility is ubiquitous among actin crosslinking proteins or unique to fascin. We first examined velocity using the same techniques described above.
Within cells depleted of the bundling proteins $\alpha$-actinin and fimbrin, the rate of *Listeria* movement closely matched those seen in control cells, moving at a rate of 0.14 $\mu$m s$^{-1}$ and 0.12 $\mu$m s$^{-1}$ respectively (Figure 5). In contrast, *Listeria* in filamin depleted cells moved at almost twice the rate found in control cells at 0.20 $\mu$m s$^{-1}$ (Figure 5). A Mann-Whitney U-test showed a statistically significant difference in the distributions of instantaneous movement rates for *Listeria* in controls and cells treated with filamin dsRNA (Figure 5) ($p < 0.0001$).

**A Role for Crosslinking Proteins in Actin Dynamics**

To test whether depletion of $\alpha$-actinin, fimbrin or filamin affected actin tail dynamics we measured the tail length distribution, the correlation of actin tail length with bacterial speed, and the rates of actin tail depolymerization in cells depleted of one of the above bundling proteins. Cells depleted of anyone of the three bundling proteins resulted in the production of tail lengths slightly less than control cells (Figure 6). A two-tailed student t-test showed a statistically significant difference in the distributions of actin tail lengths for *Listeria* in controls and cells depleted of each one of the three above actin crosslinking proteins (Figure 6) ($p \leq 0.0001$).

In order to assess the significance of the observed small variation in tail lengths between controls and the three actin crosslinkers we plotted the actin tail/bacterial speed correlations of cells depleted of one of the three crosslinking proteins. Both $\alpha$-actinin and filamin depleted cells showed a significant shift from control cells. A two-tailed student t-
test showed a statistically significant difference in the linear relationship between *Listeria* tail length and velocity in cells depleted of either α-actinin or filamin when compared to controls (Figure 6) \( (p < 0.0001) \). In contrast from fascin-depleted cells the relationship between actin tail lengths and bacterial speed in α-actinin or filamin depleted cells became shallower when compared to controls. This suggests that for α-actinin and filamin, changes in tail length from varying speeds are much smaller in magnitude than in control cells and those seen in other wild type observations from previous studies (Theriot et al. 1992). Similar to fascin, we divided fimbrin depleted cells into two different cellular populations for our analysis based on an observed bimodal distribution of the individual linear relationships between actin tail length and bacterial speed. Fimbrin type I resulted in a linear relationship with a negative offset compared to controls, while fimbrin type II match results observed from fascin type II with a positive offset from the levels seen in controls (Figure 4B and 6).

The slower growth in actin tail lengths with increasing speed may be a direct result of an increase in the rate of actin filament depolymerization. To test this hypothesis we measured the fluorescence decay of actin tails in α-actinin and filamin depleted cells using the same technique and analysis as with fascin depleted cells above. Actin tails measured in both α-actinin and filamin depleted cells both resulted in a 2-fold increase in decay rates when compared to control cells (Figure 7). Again similar to fascin, a bimodal distribution is observed in a histogram of actin tail decay rates in fimbrin depleted cells, splitting the cells into two different pools. Fimbrin depleted cells type I rates of actin tail depolymerization matched results observed in α-actinin and filamin depleted cells while
fimbrin depleted cells type II match the slow rates of depolymerization seen in fascin depleted cells type II.
Conclusions
We dissected the mechanism of actin-based motility \textit{in vivo} and found a set of proteins whose depletion drastically changes the character and efficiency of actin-based movement. We accomplished this by using a form of molecular “fractionation”, a powerful technique only previously available for \textit{in vitro} studies. RNAi interference provided us this form of \textit{in vivo} “fractionation” by allowing selective depletion of any protein whose cellular function we wished to examine. Isolating characterization to specific proteins is especially important \textit{in vivo} because of the diverse and abundant protein make-up of the cell. We first examined two proteins, profilin and ADF/cofilin, whose functions have been well documented through biochemical assays and through \textit{in vitro} systems. We also sought to dissect actin-based motility further and determine if other actin-binding proteins are involved in its mechanism. There are over 90 actin-binding proteins implicated in actin-based motility. Our hypothesis is that with this diverse and abundant pool of proteins there are other key proteins that remain uncharacterized beyond the five in the current model. Our experiments identified that CAP is an essential protein for actin-based motility \textit{in vivo}. Through our investigation we also discovered that fascin and other crosslinking proteins, though not essential for motility, might serve a new unique role in regulating the speed and dynamics of \textit{Listeria} and its actin tail. Our observations have allowed us to identify and dissect the protein make-up of the actin-based motility machine.

\section*{Confirming Profilin and ADF/Cofilin’s Integral Role in Actin-Based Motility \textit{in vivo}}
Even though there is still a strong debate on the exact biochemical mechanism for both profilin and ADF/cofilin, there is very little doubt as to the importance of their role in actin-based motility and actin regulation in general. This was especially confirmed for actin-based motility in a reconstitution experiment that demonstrated profilin’s importance and ADF/cofilin’s essential role in maintaining motility (Loisel et al. 1999). Our results are consistent with their findings and show that profilin and ADF/cofilin is necessary for actin-based motility in vivo. In particular, we showed that they are both necessary proteins for actin-based motility function and that no other binding proteins expressed in S2 cells can replace the activities provided by these proteins.

With this study we also sought to directly quantify phenotypes in vivo; our main variable is velocity. For profilin and ADF/cofilin, their gravity of inhibition was fairly equal. This is in contrast to the findings from reconstitution assays that show that a lack of cofilin completely stops actin-based movement whereas in the absence of profilin *Listeria* motility is still viable though at a severely reduced rate (Loisel et al. 1999). This may be due to a basal level of protein that still remains after depletion with RNAi or that other proteins may fill in to substitute certain essential functions, albeit at a crippled level of activity. Nonetheless, the decrease of about 2- to 3-fold below the wild type rate of *Listeria* motility, as a consequence of depletion of either protein, is consistent with previous biochemical data that show enhancement of the treadmilling rate when both profilin and ADF/cofilin are present together (Didry et al. 1998).

**An Addition to the Essential Set of Proteins for Actin-Based Motility**

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Currently the main hypothesis for actin-based motility is that it can be fully reconstituted with the addition of five proteins: actin, Arp2/3, capping protein, profilin and ADF/cofilin (Loisel et al. 1999). Our results are inconsistent with this hypothesis in that we identified CAP whose depletion causes an equal level of *Listeria* motility inhibition as profilin and ADF/cofilin, indicating that CAP’s role in actin-based movement is both required and distinct. In the *in vitro* reconstitution assay, motility was initiated with the addition of five proteins. One of the main criteria for the reconstitution of motility consisted of a quantification of each protein’s contribution to the rate of *Listeria* motility and finding the optimal concentration for each protein that produces the maximal rate of movement. Our data suggests that with the set of proteins available and based on the criteria of velocity, actin-based motility wasn’t fully reconstituted. The highest rate achieved in the reconstitution assay equaled 0.05 μm a second, a rate that equals our lowest inhibited rate (Loisel et al. 1999). This caveat is also consistent with both *in vivo* and *in vitro* systems. Our wild-type rate of 0.12 μm/sec matches normal rates of motility seen in *Xenopus* extracts and cultured cells. Moreover, mutant rates in both these systems consistently fell within the range of 0.05 μm/sec as well (Geese et al. 2000; Geese et al. 2002; Grenklo et al. 2003; Soo and Theriot 2005). In order to determine the absolute reconstituted system a higher criteria needs to be set. If addition of CAP to the reconstituted system does not produce wild-type rates of motility there may be a need to possibly revisit *in vitro* fractionation experiments or attempt to screen the all actin-binding proteins using our system to determine the full complement of essential proteins involved in actin-based motility.
The Role of CAP in Actin-Based Motility

For S2 cells, CAP was first identified by our group as a possible essential and non-redundant component of the actin cytoskeleton in a focused screen of actin-binding proteins to find the minimum requirements of lamella formation. In this study we found that RNAi depletion of CAP caused a failure to spread and a high incidence of multiple nuclei. The morphology and phalloidin staining observed in CAP depleted cells matched closely to the same cytology observed in profilin depleted cells (Rogers et al. 2003). In our current studies we find that CAP depletion registers its inhibition of Listeria motility as strongly as profilin and cofilin. This indicates that CAP fills in a necessary and distinct niche in actin turnover and polymerization. This niche may originate from profilin’s weak competition against cofilin in binding ADP-actin monomers, since cofilin’s binding strength is 5-fold greater than profilin (Blanchoin and Pollard 1998; Vinson et al. 1998). CAP fills in this gap by catalyzing the release of cofilin molecules from sequestration with ADP-actin monomers to release cofilin for further rounds of actin turnover. In a previous study, CAP was shown to have a binding strength against ADP-actin monomers 5-fold greater than cofilin and a 10-fold weaker binding strength against ATP-actin monomers than profilin. CAP in addition enhances profilin’s nucleotide exchange rate lowering its half-maximal effect concentration from 1.5\(\mu\)M to 0.65\(\mu\)M. This combination of features enables CAP to compete for cofilin for ADP-actin monomers, promote nucleotide exchange and easily hand off ATP-actin monomers to profilin, strongly establishing a niche in enhancing the kinetics of actin turnover (Moriyama and Yahara 2002; Balcer et al. 2003; Mattila et al. 2004).
Contrary to a prior hypothesis, this synergy between cofilin, CAP and profilin may indicate that in the absence of CAP, the physiological concentration of profilin, either local or global, is inadequate to compete with cofilin for binding ADP-actin monomers, producing the severely reduced rates we observe in CAP depleted cells. Thus, in the absence of CAP the actin turnover system is unable to overcome this step in an efficient manner, illustrating that CAP serves a crucial and unique role in actin dynamics (Blanchoin and Pollard 1998; Didry et al. 1998; Vinson et al. 1998).

**Fascin Regulates the Rate of *Listeria* Motility**

The existence of an outer crosslinked actin shell in the tail was described as early as the first electron micrographs (EM) taken of *Listeria* and its actin tail (Tilney et al. 1992). Another study using EM imaged *Listeria’s* long protrusion into the cell membrane and discovered that the entire actin tail in the protrusion consisted of long bundled actin filaments (Sechi et al. 1997). Recently, high-resolution EM images of filapodia formation in cells revealed that actin filaments in filapodia resembled the structure of actin filaments in *Listeria* protrusions and that for filapodia this specific actin structure required fascin (Svitkina et al. 2003; Vignjevic et al. 2006). Based on these findings we sought to determine if fascin has a functional role in *Listeria* actin-based motility.

We have shown that depletion of fascin results in a 2-fold increase in velocity. Based on our results and findings from other studies, at the most basic level fascin may play a role in facilitating the connections between the actin tail and the bacterial surface. Experiments using optical traps demonstrate that as much as 10pN of force is needed to
separate a bacterial cell from its actin tail (Gerbal et al. 2000). EM images have also revealed attachments between the surface of ActA coated beads and individual actin fibers (Cameron et al. 2001). One model we can use to explain our observations and hypothesis of bacterial attachment is the modified tethered ratchet theory. However, this model describes the system at the level of individually attached actin fibers and lacks the breadth to adequately describe or model our findings, which require addressing the mechanics of a network of crosslinked filaments (Mogilner and Oster 2003).

Recently another theory has been proposed on the mechanism of *Listeria* actin based motility, the elastic gel model. This model looks at the problem on the mesoscopic level and seeks to describe the actin tail as a crosslinked elastic gel (Gerbal et al. 2000). The basic properties used to develop this model have already been demonstrated in symmetry breaking experiments involving beads coated with either ActA (Akin and Mullins, personal communication, 2004) or the VCA domain of WASP (van der Gucht et al. 2005). Several other studies have begun to test this model’s validity in actin-based motility (Giardini et al. 2003; Upadhyaya et al. 2003; Soo and Theriot 2005; Paluch et al. 2006).

In this model, we hypothesize that fascin functions to modify the actin filament network in the *Listeria* tail to exhibit specific properties that in turn regulates *Listeria* motility. Through previous studies, fascin and other crosslinking proteins have been identified as the main actin binding proteins with the ability to change a solution of actin filaments into a meshwork that has the properties of an elastic gel (Tseng et al. 2001). In
the model the actin tail is composed of two overlapping crosslinked elastic actin gels. The outer tail is polymerized from the side of the bacterium and forms a hollow cylindrical shell in the tail, while the inner tail fills in the hollow core of the outer shell and is polymerized from the polar end of the bacterium (Gerbal et al. 2000). Our model particularly involves the outer tail. This outer cylindrical tail has been observed in Listeria tails using EM and is described to appear as a crosslinked meshwork of actin filaments (Tilney et al. 1992). This outer tail has also been demonstrated to support brief burst of motility independent of Arp2/3 but requiring fascin (Brieher et al. 2004). Thus, it is in this outer tail that fascin may have the most influence in regulating actin motility.

In the model fascin has a role in regulating transition of Listeria into one of two states. In one state the bacteria is slowed and “locked in” with the actin tail with increasing connections between the outer tail and the side of the bacteria. In this state, the bacterium moves with the tail at the rate of actin polymerization (Theriot et al. 1992; Soo and Theriot 2005). In addition, during this period of confinement the elastic potential energy is stored as the polymerized actin gel accumulates on the bacterial surface. Listeria enters a second “fast” state if it is stochastically propelled forward fast enough to escape the retarding connections. Once in this state the bacteria propels forward through the energy released by the elastic recoil of the accumulated elastic actin gel on the surface of the bacterium. In particular, the force in the elastic recoil is determined by the magnitude of the stored elastic potential rather than directly from the rectifying forces of actin polymerization. Thus, through the accumulation of enough elastic potential energy the bacteria can be propelled at velocities faster than the rate of polymerization (Gerbal et
al. 2000; Soo and Theriot 2005). While fascin may contribute to the global elasticity of the actin tail (van der Gucht et al. 2005), our findings suggest that fascin’s major influence in *Listeria* motility is to prevent the bacteria from entering the state dominated by elastic recoil.

The largest physical factor that keeps the bacteria locked onto the tail is the frictional force on the bacterial surface caused by its physical connections with actin filaments in the tail. Fascin contributes to this frictional force since the strength of these physical connections is governed by the elastic modulus of bound filaments which is in turn inversely proportional to the average distance along the actin filament between the surface bound tip and the nearest crosslink. Thus, with depletion of fascin, the distance between crosslinks increases and the elastic modulus of the bound filaments decreases, giving the bacteria greater freedom of movement against the tangential frictional force. As a result of its increased freedom, *Listeria* is able to enter the elastic energy dominated regime at greater frequencies, increasing the average speed of motility (Gerbal et al. 2000). The conclusion of our data in relation to this model assigns all of the energy produced to the polymerization reaction but the use of this energy, both potential and kinetic, is specifically controlled by the connections of the outer actin tail with the sides of the bacterium which ultimately relates to the activity and density of actin crosslinkers, such as fascin, in *Listeria* actin based motility.

**An New Model for Arp2/3 Inhibited *Listeria* Motility**
In a recent experiment, after addition of the cofilin homology and acidic region of N-WASP (CA), Arp2/3 mediated nucleation of actin filaments are inhibited in an *in vitro* preparation of *Listeria* actin based motility. At the point of Arp2/3 inhibition, *Listeria* undergoes a short burst of movement caused solely by the extension of an outer sheath of parallel bundles. Through the use of fractionation experiments it was revealed that this phenomena required the presence of either fascin or other crosslinking proteins. The conclusions drawn from this work proposed that this bundled sheath of crosslinked filaments provide additional force through a Brownian ratchet model where the rectifying force is the bundling of actin filaments. The authors also rejected a model where the crosslinked outer bundled sheath propels the bacteria from forces originating from the elastic properties of crosslinked filaments, citing the lack of visual evidence of a collapsed tail during the Arp2/3 independent motility of *Listeria* (Brieher et al. 2004). The hypothesis of a rectifying bundled sheath using the energy of active crosslinking is incompatible with our data since depletion of fascin enhances the speed of motility rather than retard motility when fascin is depleted from our experiments. Our data suggests that the energy of movement seen in these experiments may still originate from Arp2/3 mediated polymerization, though indirectly. Otherwise, removal of fascin should have removed a source of energy for motility and the *Listeria* in our fascin depletion experiments would have moved at slower rates or at least remained at wild type speeds, instead of our actual result of faster motility.

Based on our data, our model can serve as an alternative hypothesis where the polymerization of actin is severely inhibited for both the inner actin tail and outer actin
shell via the loss of Arp2/3 actin filament nucleation and the only energy source remaining is the elastic potential energy of the outer actin shell from the accumulation of the actin gel matrix at the surface of the bacteria. Once Arp2/3 is inhibited the bacteria may lose its contact with both tails allowing it to enter the fast regime at which point the outer shell releases its remaining elastic energy producing the short burst of motility observed in these experiments followed by the formation of an actin tail in the form of a hollow cylinder of bundled actin filaments.

Moreover, the argument presented by the authors against a model similar to our own does not hold as a true caveat since the properties of our model do not require actual “squeezing” of the hollow actin cylinder (Brieher et al. 2004). Our model and data directs us to our main conclusion, that the energy driving the force of motility originates from the elastic potential energy stored within the gel measured by the actin accumulation at the bacterium surface. Thus, when this elastic energy is released, the force originates from the deformation of the actin gel as a result of the relaxation of the built up elastic potential energy. This can all occur at the bacterial surface and within the meshwork of the outer actin tail without any changes in the internal radius of its hollow core. One recognizable example of this type of deformation energy is the decrease in the thickness of a rubber band when stretched out and the energy released when it’s allowed to relax and return to its original thickness. Recent evidence supports our argument where photobleached marks are placed in the growing actin tail of a motile bead. The marks deform tangentially along the long axis of the bacterium and the actin tail but no deformation is seen orthogonally (Paluch et al. 2006). Moreover, the type of deformation
elastic energy described in our model has also been demonstrated in the symmetry breaking of actin gels around spherical beads. The movement of the bead during the process of symmetry breaking is due to the deformation of the actin gel while still connected at the bead surface and not due to any direct “squeezing” forces at one end of the bead (Aiken and Mullins, personal communication, 2004; (van der Gucht et al. 2005). Thus, again our model states that all the energy required for the movement Listeria originates from the polymerization of actin and crosslinkers such as fascin only serve to regulate the use of this energy.

The role of Other Crosslinkers in Regulating Actin-Based Motility

Based on our findings with fascin we sought to determine if other crosslinking proteins function with fascin in regulating the speed of Listeria velocity. There are only a few conclusive studies that have looked into the role of actin crosslinking proteins in Listeria actin-based motility. One study found that α-actinin was not required for actin-based motility and that changes in the concentration of α-actinin had no effect on the rate of movement (Loisel et al. 1999). This result generally agrees with our finding that depletion of α-actinin (and fimbrin) yielded no change in rate of Listeria motility. In contrast, depletion of filamin caused an almost two-fold increase in speed. This result is in slight conflict with recent findings that show that addition of high concentrations of filamin to a reconstituted medium with a high concentration of gelsolin increases the speed of VCA coated beads. However, at low concentrations of filamin the velocity of the beads first decreases with increasing concentrations before reaching a minimum and enhancing speed at higher concentrations (Paluch et al. 2006). Thus, our results suggest
that at the physiologic concentration, filamin serves to help retard the movement of 
*Listeria* actin-based motility, but based on previous studies maintains the potential to increase the velocity of movement at higher concentrations.

Based on the current data available on both the functions and effects of both fascin and filamin we can at most conclude that both these proteins may regulate the velocity of *Listeria* actin-based motility through a similar mechanism to the one described above in our model (Gerbal et al. 2000; Tseng et al. 2001; Tseng et al. 2004; Soo and Theriot 2005; van der Gucht et al. 2005; Paluch et al. 2006). For instance, filamin can crosslink filaments orthogonally and in addition they have been shown to crosslink actin filaments into very resilient and stiff networks when compared to filaments crosslinked by either α-actinin or fascin (Tseng et al. 2004). The relevance of this difference in the crosslinking activity of filamin and fascin for regulating actin-based motility in vivo is still undetermined.

**Cell-to-Cell Variation seen in RNAi Depletion Experiments**

In our analysis of tail length to bacterial speed correlation and actin tail decay rates we found that cells depleted of either fascin or fimbrin resulted in a bimodal distribution of phenotypes. However, for both fascin and fimbrin our findings in relation to their velocities remained consistent between all cells tested, indicating that this incongruity in phenotypes is not due to a complete failure in our RNAi depletion. One probable explanation is that with RNAi depletion there is some variability in the extent of protein depletion among different cells and possibly between experiments performed on
different days. There also may be high variability on the rate of protein turnover for different proteins and possibly from cell to cell. In addition, certain protein functions or roles may be more robust than others in that small changes may not effect one function such as modifying the rate of depolymerization, while other functions may be highly sensitive to small changes in concentration such as regulation of bacterial speed. This explanation is further supported by our observation that within cells the phenotypes among the bacteria analyzed remains consistent, so that the difference that we observe arise from the whole population of bacteria from different host cells and not from different individual bacterial cells.

**Properties of an Elastic Actin Meshwork Are Determined by Different Crosslinkers**

One of the first studies on *Listeria* actin-based motility investigated both the correlation between bacterial speed and actin tail length. They found that these two characteristics in *Listeria* motility were highly correlated and that this correlation stemmed from a constant rate of polymerization and depolymerization (Theriot et al. 1992). We find that depletion of each of the four crosslinkers we examined perturbed the relationship between tail length and bacterial speed by changing the rate of depolymerization. Our results show that depletion of fascin reduces the rate of depolymerization, while depletion of α-actinin or filamin increases the rate of depolymerization. The depletion of fimbrin yielded both, a population of cells with increased the rates of depolymerization and another population with decreased rates of depolymerization. Each crosslinking protein has the ability to modify actin networks to enable a variety of unique characteristics that are not fully understood, which makes it
difficult to isolate the mechanism behind that each crosslinking protein that explains their effect on actin depolymerization (Sato et al. 1987; Tseng et al. 2001; Svitkina et al. 2003; Tseng et al. 2004).

At a basic level each group of crosslinking proteins may modify the stiffness, density and availability of the actin network in particular ways that may help explain the results we observe in this work. Since depletion of fascin yields an actin tail that is more resilient and decays at a slower rate, the crosslinkers α-actinin and filamin may either individually or in coordination arrange the actin network into a structure that either stabilizes filament ends, prevents twisting/severing of actin filaments by cofilin, or inhibits the diffusion of proteins into the matrix reducing the frequency of cofilin-filament interactions (Tseng et al. 2004; Andrianantoandro and Pollard 2006; Paluch et al. 2006).

Among this set of crosslinking proteins fascin seems unique in that based on our data its presence in the actin tail regulates its structure in a particular way that allows for greater instability either through somehow arranging filaments in a particular configuration that promotes depolymerization or in a configuration that gives it greater access to actin binding proteins involved in depolymerization such as cofilin (Didry et al. 1998; Tseng et al. 2001; Svitkina et al. 2003; Andrianantoandro and Pollard 2006). One feature observed in fascin crosslinked filaments that may help in promoting depolymerization is the formation of non-Arp2/3 branchpoints usually consisting of short branches. These branch points may either provide greater freedom of motion that
promotes depolymerization, it may prevent tight bundling or simply enable free diffusion of proteins into the actin matrix (Tseng et al. 2001).

Our results suggest that depletion of fimbrin can either promote or hinder depolymerization. As stated above the effect of fimbrin’s depletion may be due to the sensitivity of fimbrin activity to concentration. The result seen with fimbrin depletion is more complex than the dual results seen with fascin-depleted cells since in fascin depleted cells the second population matches values seen in control cells. In fimbrin depleted cells one cell population matches the increase in depolymerization rates seen in α-actinin and filamin depleted cells, while the other population more closely matches depolymerization rate seen in fascin depleted cells. These results suggest that the effect of fimbrin on the actin network is not linear in nature. An example of this type of effect is seen in bead motility assays where a basic reconstitution mixture is supplemented with a high concentration of gelsolin, as filamin is added the velocity of movement decreases initially and once the concentration of filamin reaches higher values the velocity of bead movement increases (Paluch et al. 2006). Both the example presented and our case with fimbrin may be due to changes in crosslinker activity with higher concentrations. The change in response may be due either to changes in geometry with increasing concentrations or as more crosslinker is added to the system the network may start to develop major strain and as a result change the character of the crosslinkers in the tail. This shift in crosslinker activity with changes in strain has been observed in α-actinin. When a network of actin filaments and α-actinin is deformed rapidly, the network becomes 40-times more rigid than filaments alone, but when the α-actinin-actin filament
network is deformed slowly the elasticity of the network is fluid and indistinguishable from a mixture of filaments alone (Sato et al. 1987).

**Future Directions: Reconciling Findings With the Actin Field**

Our work discovered that our model for actin-based motility is not complete. Our most direct finding is that CAP is a key protein in actin-based motility. As stated in the discussion one important step would be to test out CAP’s role in a reconstituted system. We have already developed a construct and purified CAP protein. In collaboration with Dyche Mullins and students in the physiology course at Woods Hole we began a few qualitative experiments that resulted in a set of interesting motility patterns. However, the protein needs to be tested biochemically to ensure that there is proper activity and no gross changes in structure have occurred during the purification process. The most controlled experiment for addition of CAP to the reconstitution assay is to ensure both profilin and cofilin are present and measure the change in velocity versus concentration. One of the main hypotheses to be tested in this experiment is to determine if addition of CAP with a complete set of proteins increases the maximum velocity, such that maximum values are close to wild type velocities seen *in vivo*.

To determine the mechanistic reason for the shift in the depolymerization rate in our data with crosslinker depletion, fascin, α-actinin, filamin and fimbrin need to be examined using our experimental system with different combinations, since depletion of anyone of the four proteins resulted in a significant phenotype. However, based on our results we can divide the crosslinkers into two categories: sensitive to depolymerization
and resistant to depolymerization. Since one of the major components in this effect is the activity of coflin, a actin tail depolymerization assay with coflin using speckle microscopy either with TIRF or possibly use of a spinning disc confocal may help to determine in what way the actin tail is protected from or exposed to depolymerization. To determine accessibility of the actin tail core to actin binding proteins such as coflin, fluorescence resonance energy transfer (FRET) experiments may be designed to determine interactions between coflin and the actin tail.

The increase in velocity seen in fascin and filamin cells should first be examined more closely by analyzing changes in the tail morphology by EM or confocal microscopy. Theoretically if these fast tails are entering the non-linear regime of elastic recoil the tail may be mainly composed of the hollow outer tail. In addition, one of the main components of elastic recoil based motility is the accumulation of actin at the bacterial surface. This has already been analyzed carefully in wild type bacteria to a degree where instantaneous speed of the bacteria can be predicted from the measured actin density at the surface of the bacterium (Soo and Theriot 2005). Using our system or setting up a system in vitro to carefully analyze the tail densities versus velocity using higher resolution microscopy may help to elucidate if elastic recoil is occurring in these fast tails. An additional experiment to address this is through the use of speckle microscopy. This has already been used to examine elastic recoil in symmetry breaking experiments using ActA coated beads (Aiken and Mullins, personal communication, 2004). This will be borne out in future work.
**Figure 1. Tracking and Imaging of *Listeria* Actin-Based Motility in S2 Cells.**

S2 Cells were infected with *Listeria monocytogenes* and imaged as described in the Experimental Procedures by phase microscopy to observe *Listeria* actin-based motility within the cell.

(A) A whole-cell phase image of an infected S2 *Drosophila melanogaster* cell. *Listeria* appear as black rods. Scale bar, 10 µm.

(B) The whole-cell phase image of (A) is contrast enhanced and inverted resulting in *Listeria* that appear as white rods. Scale bar, 10 µm.

(C) Sub-region of (B) showing tracked *Listeria*. Objects elsewhere represent organelles and other phase dark cellular structures. Scale bar, 5 µm.

(D) Trajectories of 2 *Listeria* shown in (C), tracked at 12 frames/min. The total elapsed time for each trajectory is indicated in the figure. Scale bar, 5 µm.

(E) Time-lapse series showing motility of a single *Listeria*. Scale bar, 3 µm.
Figure 2. Velocity of Listeria Actin Based Motility in cells are inhibited by depletion of coflin, profilin and CAP, but is enhanced by depletion of fascin.

(A), (B) and (C) illustrate the experimental measurement of instantaneous velocities at the resolution of 5s. (A) Contrast inverted phase image of Listeria in the 4th frame of a 16-frame movie segment, with traced bacterial trajectory (yellow) superimposed with points at 5s intervals (blue). Each dot represents the bacterial centroid used to measure position. (B) A representative time-lapse series showing motility of a single Listeria and its trajectory in yellow. Instantaneous velocities are measured from the displacement of Listeria at each consecutive frame represented by the blue line. Scale bar 5µm.

(B) Histogram of the estimated instantaneous velocities for the trajectory shown in B.

(C) Histograms of the instantaneous velocities compiled from all the trajectories measured from control cells (treated with bluescript RNAi or Pavarotti RNAi) and cells treated with profilin RNAi, coflin RNAi, CAP RNAi and fascin RNAi respectively. The wild type average velocity determined from control cells are marked in each histogram (blue). The resulting average velocities from the depletion of the labeled protein are also marked and color-coded for an either a decrease in velocity (red) or an increase in velocity (green). (10 cells scored per condition from 2-4 separate experiments).
Figure 3. Listeria Actin Tail Length in Fascin Depleted Cells.

(A) Histogram of tail lengths measured from control cells and fascin depleted cells. Each event represents the maximum tail length for each unique *Listeria* within the cell. (5 cells scored from 3 separate experiments).

(B) Listeria actin tails visualized in S2 cells expressing GFP-actin in control cells (Pavarotti RNAi). Scale bar, 10µm.

(C) Listeria actin tails visualized in S2 cells expressing GFP-actin in fascin depleted cells. Scale bar, 10µm.
Control (Pav RNAi)
mean tail length (±std. dev)
5.1 μm (±2.1)

fascin RNAi
mean tail length (±std. dev)
10 μm (±7.4)
Figure 4. Fascin Depletion results in two populations of cells with different actin tail decay times.

(A). Correlation between bacterial speed and actin tail lengths in control cells. Line shown is best fit.

(B-G). Bacterial speed-tail length correlation plots for a representative set of bacteria from four fascin depleted cells (B) and plots for each of six fascin depleted cells separate from those examined in B (C-G). The upper right corner of each graph denotes cell population type (I or II) grouped based on a bimodal distribution of individual bacterial speed/tail length measurements. Each plot contains the regression line determined from control cells as a reference point (blue).
Figure 5. Fimbrin and $\alpha$-actinin depletion have no effect on *Listeria* velocities, but filamin depletion causes a 2-fold increase similar to fascin depletion.

Histograms of the instantaneous velocities compiled from all the trajectories measured from cells treated with $\alpha$-actinin RNAi, fimbrin RNAi and filamin RNAi respectively. The wild type average velocity determined from control cells are marked in each histogram (blue). The resulting average velocities from the depletion of the labeled protein are also marked and color-coded for an either a decrease in velocity (red), an increase in velocity (green) or unmarked if no significant change is present. (5 cells scored per condition from 2-3 separate experiments).
$\alpha$-actinin RNAi

mean instantaneous velocity ($\pm$ std. dev)
0.14 $\mu$m/sec ($\pm$0.070)

fimbrin RNAi

mean instantaneous velocity ($\pm$ std. dev)
0.12 $\mu$m/sec ($\pm$0.089)

filamin RNAi

mean instantaneous velocity ($\pm$ std. dev)
0.20 $\mu$m/sec ($\pm$0.084)
Figure 6. Shorter Average Tail Lengths in Cells Depleted of the Bundling Proteins α-actinin and Filamin Matches Faster Decay Times Determined From Actin Tail Length / Bacterial Speed Correlation Plots.

(A). Histogram of tail lengths measured from bundling proteins α-actinin, fimbrin, and filamin respectively. Each event represents the maximum tail length for each unique Listeria within the cell. (5 cells scored from 2 separate experiments).

(B). Correlation between bacterial speed and actin tail lengths in α-actinin, fimbrin, and filamin respectively. Line shown is best fit. Each plot also contains the regression line determined from control cells as a reference point (blue). For fimbrin depleted cells, each population type (I or II) is grouped based on a bimodal distribution of individual bacterial speed/tail length measurements.
A

- **α-actinin RNAi**
  - Mean tail length (±std. dev): 3.4 μm (±2.2)

- **Fimbrin RNAi**
  - Mean tail length (±std. dev): 4.1 μm (±2.2)

- **Filamin RNAi**
  - Mean tail length (±std. dev): 3.8 μm (±1.2)

B

- **α-actinin RNAi**
- **Fimbrin RNAi I**
- **Fimbrin RNAi II**
- **Filamin RNAi**

**Velocity (μm/sec)**

**Tail Length (μm)**
Figure 7. Measured Rates of Depolymerization Match Predicted Patterns of Actin Tail Persistence Determined From Actin Tail Length / Bacterial Speed Correlation Plots.

Rates of fluorescence decay were measured by averaging the 8-bit pixel intensities from an area of 7x7 pixels. Actin tails were chosen from the pool of samples used in tail length measurements (Figure 3A and 6A). Points were fit according to exponential decay with the equation $y = a(e^{-\lambda x})$ where $\lambda$ = decay constant.

Time course of normalized fluorescence intensity versus time where colored solid lines are the global fit rates and dark gray solid lines are the average individual actin tail decay rates and dashed light gray lines indicating standard deviations and solid blue line indicates global fit rate of control as a reference point. Fimbrin plot contain two separate population samples differentiated by spot and global fit rate color (type I; yellow and type II; black).
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