The Role of Actin Binding Proteins in Cell Motility

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University Scholar Thesis

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Abstract

The actin cytoskeleton is responsible for many crucial cellular processes that rely on cell motility, including the immune response, wound healing, morphogenesis, and tumor cell metastasis. Actin binding proteins (ABPs) play key roles in the dynamic organization of the actin cytoskeleton during cell movement. The general localization of ABPs during dynamic cellular processes has been characterized. However, their specific functions and mechanisms of regulating the cytoskeleton remain unclear. ABPs can be classified by their presumed interaction with actin, such as cross-linkers and bundling proteins. We have chosen instead to classify them by their localization to particular actin structures. For instance, fimbrin, α-actinin, ABP-34, and fimbrin are known to localize to macropinocytic and phagocytic cups. Furthermore, they are found in leading edges of polarized moving cells, while filamin localizes to the rear of polarized cells during chemotaxis.

Two approaches have been utilized to ask how these proteins contribute to cytoskeleton functions. Using homologous recombination, double mutants lacking α-actinin and fimbrin (95-/fim-), and triple mutants lacking ABP-34 in addition to α-actinin and fimbrin (34-/95-/fim-) have been generated in *Dictyostelium discoideum*. By analyzing mutants devoid of proteins that localize to similar cell structures, we hope to disrupt processes that require a functional cytoskeleton such as phagocytosis, random motility, development, chemotaxis, and bacterial growth. Both 95-/fim- and 34-/95-/fim- cells exhibit a reduction in random cell motility, decreased persistence and polarized movement during chemotaxis, and increased rates of macropinocytosis. 95-/fim- cells develop normally when starved, while 34-/95-/fim- mutant cells fail to form fruiting bodies. However, both double and triple mutants were able to grow and develop into fruiting bodies on bacterial growth plates.

In addition, we have asked whether the presence of a particular ABP in excess influences cell function. *Dictyostelium* cell lines over-expressing several different ABPs have been created and analyzed for the effect of over-expression on random motility and chemotaxis. *Dictyostelium* cell lines over-expressing GFP-Enlazin and 34-GFP show reduced motility during random movement in the absence of a chemotactic signal. Additionally, GFP-Enlazin over-expressers exhibit enhanced cortical F-actin, and normal speed and persistence during chemotaxis. We have shown that over-expression of some ABPs, such as GFP-Enlazin, reduces random cell motility. We have also demonstrated that random motility, chemotaxis, macropinocytosis, and developmental patterns are affected in 95-/fim- and 34-/95-/fim-null mutants. The initial results provide motivation for further experimentation necessary to resolve the mechanistic cause for the observed alterations in cell behavior.
**Introduction**

I. **Overview of Cell Motility**

Most animal cell types possess the ability to move, and depend on the complex coordination of their cytoskeleton, membrane, and adhesion systems. A single cell migrating over a two-dimensional substrate requires protrusion, or the outward movement of the cellular membrane in the front of the cell. Following cell protrusion, adhesion of the cell body to substrate surface allows for cell movement along the substrate. Lastly, the cycle of cell migration concludes with de-adhesion of the rear of the cell and retraction. This general process of cell motility, however, varies among different cell types. For example, weakly bound cell bodies of neuronal growth cones do not retract, allowing for the production of axons as the cell extends forward (Bray, 1979). In contrast, cultured fibroblasts produce strongly adhered extended tails, and leave behind cell fragments during migration. Fast moving and weakly adhesive cells, such as amoeba and white blood cells, have a rounded tail, due to more efficient de-adhesion and cell retraction (Mitchison and Cramer, 1996).

The ability of cells to move and change shape is vital for cellular processes needed to carry out specific functions. An example is the need to feed, whereby individual cells in the environment crawl in search for food. Individual cells also migrate in response to signals from their surroundings; immune cells are recruited to diseased sites, and tissue cells are activated during wound healing. Cell motility is also vital for development of tissues and organs of entire organisms, as in the case of embryogenesis. Just as motility is employed for normal functioning of individual cells and whole organisms, several diseases have resulted from abnormalities in motile processes, such as cancer metastasis, and autoimmune syndromes like rheumatoid arthritis.
II. *Dictostelium Discoideum* as a Model Organism

*Dictostelium discoideum*, the soil-living amoeba commonly known as a slime mold, has served as a model organism since its discovery in 1935 by Dr. Kenneth Raper. *Dictostelium* reproduces by mitotic division, producing daughter cells with a cellular composition that most resembles animal cells, rather than yeast and plant cells. For example, *Dictostelium* cells have a flexible plasma membrane, which allows it feed on bacteria and axenic liquid medium (Williams, 2010). *Dictostelium’s* 35-Mb genome has been sequenced, and encodes for many proteins that are closely related to mammalian orthologs, and human disease-related proteins. All known genetic information and mutant phenotypes of *Dictostelium* can be found on the comprehensive database, Dictybase: [http://dictybase.org](http://dictybase.org) (Eichinger et al. 2005; Kreppel et al. 2004).

Rapid phenotype analysis is facilitated by the organism’s haploid genotype, which further supports the use of molecular genetics techniques. For example, gene function can be inhibited using an antisense or dominant negative construct under the control of a regulatory promoter. “Knock-ins” and REMI mutagenesis have been routinely used to modify or mutate genes respectively. Furthermore, gene disruptions are achieved via homologous recombination or a targeting construct, with an efficiency of up to 90% (Williams, 2010). The ease with which *Dictostelium* can be genetically manipulated is one of several reasons it serves as a designated NIH model organism and an attractive system for cell biological studies.

The lifestyle of this eukaryotic amoeba heavily depends on actin regulated processes. In culture, *Dictostelium* feeds on liquid nutrient media via macropinocytosis. During macropinocytosis, non-specific fluid uptake is accomplished through the formation of a clathrin-independent form of endocytosis (reviewed by Cardelli, 2001). Cells form macropinocytic cups, which are U-shaped pseudopod extensions, driven by actin polymerization. The cups then close to form large vesicles, or macropinosomes. *Dictostelium* cells are obligatory phagocytes in nature, internalizing bacteria as a
food source. Phagocytosis is similar to macropinocytosis, as it relies on actin driven pseudopod extension to engulf particles (Cardelli, 2001). However, the molecular mechanism of phagocytosis is more clearly defined than that of macropinocytosis; it involves receptor binding and particle recognition, followed by activation of internal signaling pathways leading to temporal and spatial regulation of actin filament formation. Subsequent generation of a phagocytic cup surrounding the particle, internalization of the particle within a phagosome, and removal of the actin coat from the phagosome, concludes particle internalization. While several studies attempt to elucidate the molecular signaling pathways that differentiate macropinocytosis and phagocytosis, both processes are proven to be regulated by actin cross linking proteins, such as coronin, α-actinin, and fimbrin (Cardelli, 2001).

Dictyostelium are constitutively motile in the absence of a chemotactic signal; they are in active search for chemical gradient signals, producing psuedopods while “randomly walking” in several directions (Li et. al, 2008). In response to a chemical gradient, polarized Dictyostelium cells move directionally up the gradient via chemotaxis. Dictyostelium use heterotrimeric G-protein coupled receptors to detect cAMP gradients. External detection of signal is translated to a number of internal responses through signal transduction pathways, which ultimately induce directional migration. In response, cells polarize and generate actin protrusions at the front of the cell, moving toward a higher or lower chemical concentration if performing positive chemotaxis, or negative chemotaxis respectively (Parent, et. al, 1998).

During starvation, chemotaxis is a central step in development, whereby up to 100,000 undifferentiated single Dictyostelium cells undergo programmed changes in gene expression and morphology. Development initiates with the aggregation of cells via chemotaxis and concludes with the formation of a spore containing fruiting body. During chemotaxis, individual cells respond to extracellular cAMP signals generated by forming multicellular aggregates, allowing cells to migrate towards the growing aggregates. After aggregation, the cells collectively migrate as a multicellular slug
towards favorable conditions with heat and light. During slug migration, cells further differentiate into pre-stalk and pre-spore cell types, which respectively form into the stalk and spore in the fruiting body. The spores formed wait for the return of favorable conditions for individual amoeboid growth before dispersal and germination. The developmental mechanisms of *Dictyostelium* are reviewed by Strmecki *et. al* 2005. The differentiation and migratory movement of *Dictyostelium* have made it an attractive system for scientific research in development, morphogenesis, chemotaxis, and cell motility (Parent, 2004).

Studies of *Dictyostelium* have given insight into cellular components and mechanisms of mammalian cells. For instance, the chemotactic abilities of leukocytes and the phagocytosis capability of macrophages and neutrophils are carried out by equivalent actin powered pathways in *Dictyostelium* (Noegel and Schleicher 2000; Rupper and Cardelli, 2001). Furthermore, the major structural and regulatory cytoskeletal elements of *Dictyostelium* have been conserved in humans (Rivero, 2008; Vlahou and Rivero, 2006). Basic cell physiology that would otherwise be very difficult to study in complex organisms is relatively easy to address in *Dictyostelium*. For these reasons, it is clear that *Dictyostelium* serves as a suitable model for a number of studies involving the actin cytoskeleton.

### III. The Role of the Actin Cytoskeleton During Cell Migration

Cytoskeleton filaments provide the structural support needed by cells as they undergo dynamic cellular processes. While intermediate filaments play key roles in cell structure, and microtubules are necessary for cell division, the actin microfilaments are central to changes in cell morphology and cell crawling. Actin filaments, also referred to as F-actin, are composed of highly conserved globular protein molecules found in most eukaryotes. Individual g-actin proteins polymerize into a helical arrangement to form polarized filaments, in which the “barbed” end of the filament undergoes a greater rate of polymerization compared to the opposite “minus” end of the filament. The barbed ends of filaments
located near the membrane directs the polymerization of actin in response to local signals, and is widely accepted as the source of the force needed to drive membrane protrusions (Small, 1988).

Actin filaments are proactively assembling, disassembling, and organizing to give rise to several cell structures. Lamellipodia are thin active cellular protrusions located at the front of cells, such as fibroblasts, where the actin filaments are organized in orthogonal arrays with the plus ends oriented toward the protruding membrane (Small, 1988). Pseudopodia are generally thicker than lamellipodia, as seen in the front of amoeboid cells, and also contain orthogonally arranged actin filaments (Mitchison, 1996). Filopodia are hair-like protrusive structures that contain a bundle of actin filaments organized in a parallel fashion, as extensively studied in neuronal growth cones (Matsudaria, 1994; Mitchison, 1996). Additionally, the cellular cortex contains a dense assembly of filaments oriented in a less polarized fashion (Cox et al. 1995).

IV. Actin Binding Proteins Regulate Actin Dynamics

Several actin binding proteins (ABPs) function by modifying actin filament structures and regulating filament turnover. For example, some ABPs participate by breaking old filaments, while others regulate the addition of actin subunits to filament ends. Assembled actin filaments found in cellular structures like pseudopods, lamellipodia, and filopodia are organized into stable networks by ABPs (Eichinger et al., 1999). Bundles of parallel actin filaments found in filopodia are linked together by the ABP fimbrin (Matsudaira, 1994). During polarized movement, ABPs α-actinin and coronin localize to the leading edge to organize actin filaments, while the ABP filamin localizes to the peripheral cortex and rear of the cell (Mishima and Nishida, 1999; Washington and Knecht 2008). Additionally, preliminary data from the Knecht Laboratory shows that the ABP enlazin localizes to the rear of polarized cells as well. Below, a general overview of the structure, function, and localization of various ABPs analyzed in this manuscript are described.
**α-Actinin**

α-Actinin is a calcium regulated ABP initially identified as one of the most abundant actin cross-linkers in amoebas of *Dictyostelium discoideum*. α-Actinin is also referred to as the 95,000-Dalton (95KDa) protein, because of its dimeric subunits that measure 95,000 Daltons each. A member of the calponin homology (CH) super family of ABPs, it organizes filaments in antiparallel arrays (Condeelis and Vahey, 1982). α-Actinin is known to localize to pseudopods of rapidly moving cells, phagosomes, and contractile vacuoles (Brier et al., 1983; Furukawa and Fechheimer, 1994). *Dictyostelium* cells lacking α-actinin show growth impairments under osmotic conditions, motility defects, and orientation defects (Eichinger, et. al 1996; Witke et. al, 1992; Rivero et. al. 1996).

**Fimbrin**

Fimbrin is a calcium regulated ABP that contains actin binding domains similar to those of α-actinin. Fimbrin is a monomer consisting of two actin binding domains of calponin homology (Korenbaum and Rivero, 2002). Fimbrin was initially identified as a part of the intestinal brush border, known as microvilli, in chicken. However, they are highly conserved from plants to humans. Three tissue specific isoforms of fimbrin have been identified in plants and vertebrates (Bretscher and Weber, 1980). Iso-forms of fimbrin found among vertebrates have been named plastins, with L-plastin in leukocytes, I-plastin in intestinal and renal brush borders, and T-plastin on all other tissues (Lin et al. 1993). Fimbrin localizes to the cell cortex, leading edge, phagosomes, and macropinosomes of *Dictyostelium* (Prassler 1997; Pikzack et. al 2005). *Dictyostelium* null mutants of fimbrin show decreased cell size (Pikzack et. al. 2005).

**Filamin**
Filamin is a homodimer ABP, with two 120,000 Dalton subunits arranged in a parallel fashion (Condeelis et. al, 1984; Brink et. al. 1990). Filamin contains two CH domains, which are characteristic of the α-actinin and spectrin superfamily of actin binding domains (Hartwig, 1995). The polypeptide organizes actin filaments into arrays at right angles to each other, forming rigid orthogonal networks seen in situ (Hartwig et. al. 1980; Hartwig and Shevlin, 1986). In addition to localizing to the cell cortex, lamellipods, phagocytic cups, and pseudopods of motile cells, filamins are involved in signal transduction pathways and GTPase interactions (Stendahl et al., 1980; Condeelis et al., 1981; Carboni and Condeelis, 1985; Stossel et. al., 2001; Feng and Walsh, 2004). Dictyostelium mutants lacking filamin exhibit reduced frequency and size in pseudopod formation, decreased motility, chemotaxis, and phagocytosis, possibly as a result of reduced cross-linking of actin filaments (Brink et. al., 1990; Cox et. al., 1992; Cox et. al., 1995).

**ABP-34**

The 34-kDa actin-bundling protein, termed as ABP-34, is a calcium-regulated actin cross-linking protein located at the leading and trailing edges of locomoting cells (Fechheimer, 1987), the phagocytic cup (Furukawa and Fechheimer, 1994), cleavage furrow (Furukawa and Fechheimer, 1994), and cell-to-cell contact sites (Fechheimer et al., 1994). The protein has three actin binding sites, one calcium binding EF hand, and intramolecular interaction zones that are important for regulation of actin binding via an amino terminal inhibitory domain (Lim et al., 1999). Dictyostelium cells lacking the 34-kDa actin binding protein can grow and develop normally, but show increased persistence during motility (Rivero et. al., 1996).

**Enlazin**
Enlazin is a large actin associated protein that contains amino-terminal talin-like domain, a α-helical domain, and a fimbrin domain. In contrast to the simple proteins of the fimbrin family of ABPs, the multiple domains of enlazin are thought to represent a unique class of proteins. Homologues of enlazin have yet to be found in other organisms. Three calponin homology domains have been identified in the fimbrin domain, which is 45% similar to mammalian T-plastin. Enlazin is known to be enriched in the cell cortex of Dictyostelium discoideum. During cytokinesis, enlazin is distributed in the cytoplasm and membrane ruffles. Silencing of enlazin in Dictyostelium demonstrates a decreased growth rate and reduced cell-surface adhesion (Octtaviani et. al., 2006).

V. My Project

Up to twenty different ABPs have been recognized, however their specific functions and the mechanisms by which their interaction with the actin cytoskeleton is regulated remain unclear (Laevsky and Knecht, 2003). In an effort to characterize the specific roles of individual ABPs, mutants lacking certain ABPs have been created and analyzed in a number of biological processes involved in cell movement. Extensive studies of the filamin ABP have revealed its significant role in cell migration during embryonic development; mutations of filamin in Drosophila melanogaster, the fruit fly, affect oogenesis (Sokol and Cooley, 2003), cause congenital defects among several organ systems in humans (Robertson et. al. 2003), and are commonly found among human breast and colon cancers (Tobias Sjöblom1 et. al., 2006). With anti-metastatic therapies turning to cell migration as a target of inhibition of tumor development, there is a significant need to understand the function and regulation of the motile machinery, like actin binding proteins, at the molecular level (King and Insall, 2009).

While some studies provide insight to a clear function of some ABPs, several other studies suggest that there is a considerable amount of redundancy in ABP function due to very subtle
phenotypic differences in null mutants, like those devoid of α-actinin or filamin (Rivero et al., 1999). Several studies also provide insight to the general localization of ABPs. For instance, coronin and filamin are known to localize to phagocytic cups (Maniak et al. 1995; Cox et al., 1996). α-Actinin and filamin have been shown to bind to actin structures in the same region, but also differentially localize to other regions; α-actinin localizes to leading edges of actively moving polarized cells, while filamin localizes to the peripheral cortex and rear of polarized cells (Washington and Knecht, 2008). Furthermore, the fact that α-actinin, fimbrin and filamin localize to some but not all of the same actin filament containing structures raises the question of how combinations of these ABPs compete or cooperate to organize the actin cytoskeleton. It is from this knowledge of general function and localization of ABPs that more sophisticated questions about ABP interactions arise.

All in all, the literature is bereft of extensive studies that involve mutants lacking more than one ABP. Chapter 1 of this manuscript will explore how the individually well characterized ABPs fimbrin, filamin, α-actinin, ABP-34, and enlazin regulate cytoskeletal dynamics in the model organism Dictyostelium discoideum. The first hypothesis is that if the function of these proteins is important, then the presence of too much of a particular ABP may have a distinct effect on cell motility. These proteins are fluorescently labeled by fusion to a fluorescent protein to allow for visualization using fluorescence microscopy. DNA expressing each of these fluorescent fusion proteins was transfected into wild-type Dictyostelium cells causing over-expression of the ABP. Chemotaxis and random motility assays with these cells were performed. In the random motility assays, movement of cells was recorded as they were allowed to move in nutrient rich media without any directional signals. During chemotaxis assays, starved cells were observed as they moved directionally along a chemical gradient. A computer software program (Image J) was utilized to quantify the average speed and directionality of cell movement.
Chapter 2 describes the second aspect of this project, involving the creation of a double mutant that lacks the ABPs α-actinin and fimbrin (95-/fim-), and a triple mutant cell line that lacks ABP-34, α-actinin, and fimbrin (34-/95-/fim-). Fimbrin, α-actinin, and ABP-34 have been targeted for combinatorial gene disruption because they are known to localize to macropinosomes, phagosomes, and leading edges in Dictyostelium. The morphological and behavioral characteristics of the mutant cell lines were investigated in bacterial growth, chemotaxis, macropinocytosis, development, and random motility assays. A Dictyostelium mutant that lacks α-actinin, as well as a mutant that lacks both ABP-34 and α-actinin have already been isolated (Rivero et al., 1999). The fimbrin gene of each cell line was replaced with a drug resistance gene by homologous gene targeting. Cells that successfully replaced their endogenous fimbrin gene with the drug resistant gene were selected and used for phenotypic analysis.
Chapter 1. The Effect of Actin Binding Protein Over-Expression on Cell Motility

I. Materials and Methods

Cell Culture:

Parental Ax2 Dictyostelium cells were maintained at 21°C in axenic liquid HL-5 medium (containing 10 g BBL Thione E Peptone, 10 g Glucose, 5 g Yeast Extract, 0.35 g Na₂HPO₄, 0.35 g KH₂PO₄, 0.1 mg/ml ampicillin pH 6.5-6.7 in 1 L tap H₂O). Cells were grown in sterile 100 mm (p100) or 60 mm (p60) polystyrene petri dishes, and passaged every 3-4 days upon growing to confluency.

DNA Isolation by Alkaline Miniprep:

Alkaline mini-prep was used to isolate vector constructs from bacterial cultures. Constructs and their corresponding drug resistance are summarized in Table 1. Constructs of proteins fused to green fluorescent protein and red fluorescent protein are denoted by GFP and RFP respectively. Briefly, 2 mL of bacterial cultures were grown between 9-12 hours in sterile capped test tubes, shaking at 37°C. About 1.5 mL from culture was then transferred to sterile 1.5 mL Eppendorf tubes, and bacteria were pelleted by micro-centrifugation at 13,000 rotations per minute (rpm) for 5 minutes. Supernatants were removed by aspiration, and the bacterial pellet was re-suspended in 200 μl of Buffer I (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). 100 μL of Buffer II (1% SDS, 0.2 M NaOH) was added to cell suspensions, and tubes were mixed by inverting, and then incubated at room temperature for 2 minutes. 150 μL of Buffer III (3.0 M Potassium Acetate, pH 5.5 with Glacial Acetic Acid) was subsequently added, and the tubes were mixed by inverting. Following incubation at room temperature for 5 minutes, the tubes were then micro-centrifuged for 10 minutes at 13,000 rpm. The resulting supernatants were transferred to new sterile Eppendorf tubes. 1 mL of 100% ethanol was added to the tubes, mixed by inverting, and then
centrifuged at 13,000 rpm for 15 minutes. Supernatants were removed by aspiration, then 500 μl of 70% ethanol was added, and tubes were centrifuged again for 5 minutes. The resulting DNA precipitate was re-suspended in 30 μl of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) containing 20 μg/ml RNase A. 1 μl of DNA samples were run on 1% agarose gels for UV detection and quantification.

**DNA Gel Electrophoresis:**

Routine DNA detection and quantification was performed via the following gel electrophoresis procedure. 1% agarose was prepared in 1x TAE buffer solution (40mM Tris acetate, 1mM EDTA, pH 8.0). 0.3 g of agarose was microwave heated in 30 ml of 1x TAE buffer in an Erlenmeyer flask. Once agarose was melted, 3 μl of 10 mg/ml Ethidium Bromide was carefully added to the agarose. The solution was poured into a gel apparatus, and allowed to cool for approximately 30 minutes. Upon solidification, prepared DNA samples were loaded into gel wells and resolved at approximated 70 – 90 volts. The DNA was subsequently imaged under UV light, using Image J computer software.

**Transformation:**

*Dictyostelium discoideum* AX2 cells (referred to as wildtype) were transformed with the DNA constructs outlined in Table 1. Each construct was maintained in *Escherichia coli*, and isolated by alkaline mini-preparation. Transformation was performed using a modification of the procedure previously described by Pang, Lynes, and Knecht 1998. Briefly, about 1 x10⁶ cells were harvested and washed twice with H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO₄, 5 mM NaHCO₃ and 1 mM NaH₂PO₄), at 1,500 rpm in a Sorvall Centrifuge at 4°C. Cells were re-suspended in 100 μl of H-50 buffer and transferred to an ice cold 0.1 cm electroporation cuvette. About 4 μg of plasmid constructs were added to cell suspension and cells were electroporated twice with a BTX electroporator at 0.6 kV/50 μF/25 Ohms with a 0.6 msec time constant. The interval between electroporation was between one to
five minutes. The electroporated cells were then removed from the cuvette and transferred to a 100 mm polystyrene dish containing 10 ml of HL-5. Following 24 hours of incubation at 21˚C, the media was changed and corresponding drugs were added (10μg/ml G418, blasticidin 4μg/ml final concentration). Three days after drug addition, the media was changed, and fluorescent cells were identified and isolated by cloning using a Zeiss epifluorescence widefield microscope. To obtain at least 80% fluorescence level of each population, some cell lines were subsequently sorted on the FACs Aria II flow cytometer to isolate fluorescent populations. The created cell lines were maintained in HL-5 medium at 21˚C as described previously, and subsequently analyzed in random motility assays.

Table 1. Summary of Over-Expressing Cell Lines Created

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Parental Line</th>
<th>Construct</th>
<th>Drug Resistance</th>
<th>Source of Cell line/Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-Enlzain</td>
<td>Ax2</td>
<td>GFP-Enlazin</td>
<td>G418</td>
<td>Octaviani et. al, 2006</td>
</tr>
<tr>
<td>GFP-Filamin</td>
<td>Ax2</td>
<td>GFP-Filamin</td>
<td>Blasticidin</td>
<td>Raymond Washington (former graduate student from Knecht Lab)</td>
</tr>
<tr>
<td>GFP-α-Actinin</td>
<td>Ax2</td>
<td>GFP-αActinin</td>
<td>G418</td>
<td>Raymond Washington (former graduate student from Knecht Lab)</td>
</tr>
<tr>
<td>marsRFP-Fimbrin</td>
<td>Ax2</td>
<td>GFP-Coronin</td>
<td>G418</td>
<td>Rander and Chin Chi Chen (former graduate students from Knecht Lab)</td>
</tr>
<tr>
<td>34-GFP</td>
<td>Ax2</td>
<td>ABP-34 GFP</td>
<td>G418</td>
<td>Reyes et. al, 2009</td>
</tr>
</tbody>
</table>

**Random Motility Assays:**

Transformants expressing fluorescent ABPs were confirmed by visual inspection with a fluorescence microscope. 2.5 x 10^5 cells were plated in 5 ml of fresh HL-5 media in a p60 dish. After overnight incubation of at least 12 hours at 21˚C, the media was aspirated and the cells were re-suspended in 3 ml of fresh HL-5 media and transferred to a new p60 dish. The cells were allowed to
adhere to the bottom of the plate one hour prior to imaging. Live cell imaging was performed on Nikon fluorescence microscope with a 20x dry objective using Micro-Manager computer software. Phase contrast images were captured every 30 seconds, while fluorescence images were captured every 5 minutes. Motility was analyzed by observationally choosing 10 cells with the greatest speed within the field of view, and cell movement was tracked using the mtrackJ plugin of ImageJ. Distances of migration were calibrated for the 20x objective, and average speed (total distance divided by total time) was calculated for each cell population. Statistical significance denoted by p values was calculated using unpaired two-tailed t-tests on GraphPad Prism Software.

Under Agarose Chemotaxis Assays:

Agar plates containing 0.02 mM folate were prepared as follows. 5.6 mL of warm 2% Seakem agarose was prepared in SM medium solution (10 g Difco Bacto-Peptone, 10 glucose, 1 g yeast extract, 1.9 g KH$_2$PO$_4$, 0.6 g K$_2$HPO$_4$, 0.43 g MgSO$_4$, pH 6.5-6.7, in 1L dH$_2$O), and poured in p60 dishes containing 1.4 mL of 0.1 mM folic acid (50x stock folic acid: 50 mM in NaOH). Once agar cooled, a single 2-5 mm wide rectangular tough was cut into the agar with a single edged razor blade. An equal concentration of Ax2 and GFP-Enlazin cells cultured in HL-5 were mixed in a 15 ml Falcon Conical Tube, and pelleted at 1000 rpm centrifugation at 4°C for 5 minutes. HL-5 supernatant was removed, and cells re-suspended in about 100 μL SM media to a final concentration of about 1 x 10$^7$ cells/ml. Suspended cells were then added to the trough, and imaged at 10x objective under wide-field fluorescent microscopy for about 8 hours. Phase contrast images were captured every 30 seconds, while fluorescent images were captured every 5 minutes. Motility was analyzed by choosing cells in the front of the migrating population, using the mtrackJ plugin of ImageJ. Average speed (total distance divided by total time) and persistence (displacement of cell divided by the distance traveled by cell) were calculated for each population.
Statistical significance denoted by p values was calculated using unpaired two-tailed t-tests on GraphPad Prism Software.

Phalloidin Staining and Ratiometric Analysis:

Four 18 x 18 mm glass coverslips were placed within p60 dishes. 5 ml of a 1:1 ratio of Ax2 to GFP-Enlazin (about 8 x 10^5 cells/mL) were allowed to adhere on glass coverslips overnight at 21°C. Glass coverslips were removed, and placed in a p60 dish with 1%/0.1% formaldehyde/glutaldehyde HL-5 fixing solution. Cells were permeablized in 0.1% triton X-100 for five minutes. Coverslips with fixed cells were then inserted into 50 mM solution of NH₄Cl. Cells on each coverslip were subsequently stained with 80 μl of 0.1 μl/ml rhodamine phalloidin for at least 30 minutes. Coverslips were washed three times with TBST (20 mM Tris/HCl, pH 7.5, 150 mM Na Cl, 0.005% Tween 20), and five times with distilled water. Wet cover-slips were blotted on kimwipe, and inverted on glass slides containing 15 μl Prolong Gold mounting media. Clear nail polish was used to seal the coverslip to the slide. Cells were imaged at 60x oil objective on the Andor Confocal microscope, under green and red fluorescent light exposure.

Ratiometric analysis was performed using ImageJ software to calculate the cortex: cytoplasm pixel intensity ratio of a fluorescent GFP-Enlazin expressing cell and a non-expressing cell. For each cell, the line tool was set to a pixel with of five, and was drawn along the cell cortex. A list of pixel values along the line was generated using the plot profile tool. The rounded rectangle tool was used to draw a circular region of interest (ROI) within the cytoplasmic region of the cell body, and the mean pixel intensity value of the ROI was measured. The cortex and cytoplasmic signal data collected was imported into Excel, and the cortex: cytoplasm ratio was calculated.

II. Results

Generation of Over-Expressing Cell Lines:
Ax2 cell lines over-expressing marsRFP-fimbrin, GFP-filamin, GFP-α-actinin, GFP-Enlazin, and 34-GFP were created as described in Materials and Methods. After drug selection, fluorescent colonies were successfully selected to obtain a fluorescent population. Subsequent sorting of select fluorescent cells lines was employed as needed to obtain populations that were at least 80% fluorescent.

Random Cell Motility:

Random motility assays were performed to investigate the effect of over-expression of ABPs on vegetative motility in comparison to the wild-type. Using ImageJ software, fluorescent cells were analyzed. All cell lines over-expressing a selected ABP show a statistically significant reduction in motility in comparison to the wild-type. However, GFP-Enlazin and 34-GFP show the most significantly reduced motility compared to the wild-type (p<0.0001) (Figure 1). The mean speeds of the GFP-Enlazin and 34-GFP population are 2.693 μm/min and 1.847 μm/min respectively, while the mean speed of the wild-type is 8.863 μm/min. Relative significance among all cell lines analyzed is show in Table 3.

To analyze whether GFP had an effect on motility of cells, Ax2 GFP (Ax2 expressing GFP) were imaged during random motility. Cells of the GFP Ax2 population were categorized as Expressers if fluorescent, and Non-Expressers if non-fluorescent under blue fluorescent light exposure. As shown in Figure 2, both GFP Ax2 expressers and non-expressers show a significantly reduced motility compared to Ax2 alone (p<0.0001). However, there is no significant difference between the mean speeds of the GFP-Ax2 Expressers and Non-Expressers (p= 0.891). This unexpected result suggests that GFP alone may affect cell motility, which prompts a concern for aberrations in cell behavior due to the GFP fluorescent tag in subsequent results gathered.

Within each assay performed, cells that were visibly fluorescent were labeled as “Expressers,” while cells that were not fluorescent were labeled as “Non-Expressers.” This is a subjective definition because detection of fluorescence above background signal is a function of the sensitivity of the camera
and imaging system used. However, it can be confidently stated that the “Non-Expressers” are expressing less fluorescent protein than the Expressers, even though the Non-Expressers may be expressing a greater amount protein than wild-type. With this rationale, we suspected that the non-expressing cells would exhibit mean speeds similar to wild-type during random motility. As shown in Figure 3, the difference in mean speed between the GFP-Enlazin non-expressers and the wild-type are statistically insignificant (p= 0.05404). Furthermore, GFP-Enlazin Expressers exhibit a significantly reduced motility compared to the non-expressing cells (p<0.0001). Rose plots depicting the reduced range of movement of GFP-Enlazin Expressers compared to Non-expressers are shown in Figures 5-7. Both 34-GFP Expressers and Non-Expressers show significantly reduced motility compared to that of wild-type (p<0.0001). Additionally, 34-GFP Expressers show a statistically significant reduction in mean speed in comparison to 34-GFP Non-expressers (p<0.0001). The measured average speeds and corresponding standard deviations over-expressing cell lines analyzed during random motility assays have been summarized in Table 8. The random motility movies collected of all over-expressing cell lines analyzed are referenced in Table 11. There were no clear differences in cell morphology that could further elucidate the cause of reduced motility. However, cell tracks of the Ax2, GFP-Enlazin, and 34-GFP population demonstrate the reduced paths of cell movement in the Expressers compared to the Non-Expressers (Figure 4).

Under Agarose Chemotaxis:

The cell movement GFP-Enlazin population was further analyzed during agarose chemotaxis assays, whereby polarized cells migrated up a folic acid gradient. Cells of the Ax2 and GFP-Enlazin population were mixed at a 1:1 ratio. Cells that were fluorescent and non-fluorescent under blue light exposure were designated as “Expressers” and “Non-Expressers” respectively. As shown in Figure 8, the mean speeds of Expressers and Non-expressers are 2.96 μm/min and 2.8 μm/min respectively.
Ojukwu

(p=0.498). Their persistence values are 0.367 and 0.327, respectively (p=0.111). The difference between their mean speeds and persistence are statistically insignificant. Images of cells suggest that the proportion of expressing and non-expressing cells at the front of the migrating population was similar to the ratio of fluorescent and non-fluorescent cells present in the over-all population. All in all, GFP-Enlazin seems to migrate normally in comparison to the wild-type. The measured average speeds, persistence, and corresponding standard deviations of the over-expressing cell lines analyzed during chemotaxis assays have been summarized in Table 8. The movies of chemotaxis assays performed are listed for reference in Table 11.

**Phalloidin Staining and Ratiometric Analysis:**

Phalloidin staining was employed to investigate if over-expression of GFP-Enlazin affected the distribution of F-actin within cells. Phalloidin stained Ax2 and GFP-Enlazin cells were imaged using the Andor Confocal microscope, as described in the Materials and Methods. Preliminary data show that the pixel intensity ratio of cortex: cytoplasm of GFP-Enlazin Expresser is greater than that of the Non-Non-expressor, which are 1.86 and 1.46 respectively (Figure 9). The increased cortex: cytoplasm ratio of phalloidin stained in GFP-Enlazin Expressers suggests that over-expression of GFP-Enlazin increases the presence of cortical F-actin network of cells.

**III. Discussion and Future Directions:**

Ax2 cell lines over-expressing fluorescent fusion proteins of α-actinin, fimbrin, enlazin, filamin, and ABP-34 were successfully constructed as described in the Materials and Methods. However, after cloning or sorting via flow cytometry, there were some cells that were not fluorescent, and thus not significantly over-expressing the fused actin binding protein within a cell line population. To visualize fluorescent cells of each cell line, random motility assays were performed using wide-field fluorescence
microscopy. Fluorescent cells were manually tracked using the mTrackJ ImageJ plugin, and mean cell speeds were measured from each population. Of the cell lines analyzed, 34-GFP and GFP-Enlazin demonstrated the most statistically significant reduction in motility compared to the wild-type. From this initial observation, we further categorized GFP-Enlazin and 34-GFP cells that were visibly fluorescent under blue light exposure as “Expressers” and cells that were not fluorescent as “Non-Expressers,” with the expectation that the non-expressing cells would be as motile as the wild-type population. This was indeed the case for the GFP-Enlazin population. The difference in mean speeds between the wild-type and the GFP-Enlazin Non-Expressers were statistically insignificant. In particular, there was a clearly reduced range of motion of the GFP-Enlazin Expressers compared to the Non-Expressers. In the 34-GFP cell line, both the Expressers and Non-Expressers of the 34-GFP population displayed a statistically significant reduction in cell speed compared to the wild-type. There was no clear change in cell morphology of cells of the GFP-Enlazin and the 34-GFP population during random motility. It is currently unclear why GFP-Enlazin expressers and both the 34- GFP Expressers and Non-Expressers exhibit reduced motility, and further experimentation is necessary to elucidate the cause of this phenotype.

As a control, GFP Ax2 cells were analyzed during random motility to determine if GFP alone has an effect on cell motility. As was seen for the 34-GFP population, both the GFP Ax2 Expressers and Non-expressers demonstrated a significantly reduced mean speed compared to the wild-type population. One possible explanation for the observation seen in the 34-GFP populations is that a relatively low level of over-expression is sufficient to cause a reduction in cell motility, and the fluorescent detection power used during the random motility assay was not strong enough to detect very low levels of fluorescence. Another plausible explanation is that cells in the majority of the 34-GFP and GFP Ax2 population had a reduced motility, independent of whether the cells were expressing GFP. This phenotype in both the GFP Ax2 and 34-GFP populations could have arisen from methodological errors due to the following explanation. A very few number of fluorescent colonies were picked for cloning following transfection.
Given that each colony arises from a single cell, any anomaly that the single cell possesses may be propagated to daughter cells during each round of mitotic cell division. If colonies were picked from cells that were abnormally slow compared to the majority of the population, then the resulting cell population may be slow as well. To resolve this problem, multiple fluorescent cells should be selected using flow cytometry, which is capable of sorting thousands of cells at one time. This would reduce the high probability of hand picking and subsequently analyzing a colony that came from a single cell possessing aberrations in cell behavior. An additional factor that may have produced such results is changes in cell behavior over time. The phenotypes may have arisen during prolonged propagation of cells in culture after isolating the transformed cells. In the future, Ax2 cell lines should be transfected with GFP constructs, sorted by flow cytometry, and imaged immediately after selection, so that the parental cell line and the transfected populations are analyzed in parallel with no opportunity for clonal selection of variants.

GFP-Enlazin was chosen for subsequent studies in cell motility. In the under agarose chemotaxis assays, the range of agarose concentration can be varied from 0.75% to 2.5%. The chemotaxis assays were performed using the higher range of agarose concentration. With an agar concentration of 2%, we hoped to subject the cells to more stressful conditions, during which an alteration in chemotaxis may become more apparent. A 1:1 ratio of GFP-Enlazin and Ax2 were allowed to chemotax under the agar toward a folate gradient. However, GFP-Enlazin does not seem to affect chemotaxis, as there is no significant difference in cell speed and persistence between the non-fluorescent and fluorescent cells.

Because phalloidin binds to F-actin and prevents its depolymerization, phalloidin staining is a useful tool for visualizing the distribution of F-actin within cells. To determine if over-expression of GFP-Enlazin alters that actin cytoskeleton, a 1:1 ratio mixture of the GFP-Enlazin and Ax2 population were fixed and stained with rhodamine phalloidin. As shown in Figure 9, the increased cortex: cytoplasm ratio of phalloidin stained of GFP-Enlazin Expressers compared to Non-Expressers suggests that over-
expression of GFP-Enlazin enhances the cortical f-actin network of cells. In the future, performing an assay to measure the total amount of F-actin present within GFP-Enlazin and wild-type cells may confirm this result.

It is currently unclear why GFP-Enlazin causes reduced speed during random motility and increased F-actin in the cell cortex. Further assays must be performed to determine GFP-Enlazin’s mechanistic cause for observed phenotypes presented in Chapter 1. Confocal microscopy can be used to analyze the spatial and temporal dynamics of GFP-Enlazin during random motility, and elucidate how over-expression GFP-Enlazin reduces cell speed. Also, cells should be imaged under high magnification to investigate clear changes in morphology during random motility. Furthermore, whether GFP alone has an effect on motility of cells must be resolved, as it causes concern of how GFP and other frequently used fluorescent fusion proteins affect the behavior of cells. One way to resolve this question is to analyze knock-in cell lines of GFP-Enlazin and 34-GFP, so that the cells are not over-expressing the actin binding proteins, but is simply expressing natural levels of the fluorescent fusion protein.
Chapter 2. Generation and Analysis of Fimbrin Double and Triple Null Mutants

I. Materials and Methods

Generation of Double and Triple Mutants by Homologous Recombination:

The single mutant 95- strain (Eichinger et al., 1996, Rivero et al., 1996) and double mutant 34-/95- strain (Rivero et al. 1999) were used as the parental cell lines for disruption of the fimbrin gene (FimA) via homologous recombination. Briefly, the 95- strain was previously created by homologous recombination using the gene replacement vector pΔAA. The pΔAA vector contained the neomycin (G418) resistance gene interrupting the 0.8-kb 5’ coding sequence and the 0.3 kb 3’ coding sequence of α-actinin (Eichinger et al., 1996). The 34-/95- strain was also created by homologous recombination. The 34- strain was previously created using a gene disruption vector that contained a hydromycin resistance gene within the 4 kb genomic DNA of ABP-34 (Rivero et. al., 1996). The 34-/95- strain, referred to as 34-/αA- by Rivero et. al. (1999), was generated by disrupting the α-actinin gene of the 34- strain using vector pDabα 1.2 via homologous recombination. The pDabα 1.2 gene replacement vector contains the N-terminal 1.2 kb EcoRI fragment of the α-actinin cDNA (Witke et. al., 1987). The gene replacement vector for fimbrin, pbsrfim1, was previously created by a former graduate student, Irene Jarchum. pbsrfim1 contains the blasticidin resistance cassette, which was inserted into the Swal restriction site of the full length fimbrin gene by blunt ligation. The complete vector construct of pBSRfim1 is shown in Figure 10.

The gene replacement vector was obtained from dh10b E. coli cells by alkaline mini-preparation, and quantified by gel electrophoresis as previously described in Chapter 1. The pBSRfim1 vector was digested using PstI and BamHI restriction enzymes to liberate a linearized fragment containing the blasticidin resistance cassette flanked by the full length fimbrin gene. The digestion reactions were
performed in a 1.5 ml epitube, with a final reaction volume of about 40 ul containing 8.8 ul dH$_2$O, 10 μl of pbsrfim1 plasmid from miniprep, 4 μl of 10x NEB Buffer 3, 0.4 μl of 10x BSA, 0.8 μl of 50x RNase (1 mg/ml), 2 μl of PstI (20,0000 units/ml) and 1 μl of BamHI (25,000 units/ml). The reaction was incubated at 37°C for approximately three hours.

The resulting digest was concentrated by phenol-chloroform extraction and ethanol precipitation. Briefly, an equal volume of phenol/chlorophorm (1:1 ratio) was added to the digestion reaction and vigorously mixed by vortexing. Organic and inorganic phases were separated by micro-centrifugation for 1 minute. The organic phase was removed from the Eppendorf tube, and an equal volume of chloroform was added. The solution was mixed by vortexing, and the phases were separated by centrifugation for 1 minute. Then the lower organic phase was moved. To precipitate the DNA, 1/10th volume of sodium acetate was added, followed by the addition of two times volume of 100% EtOH. 15 min. centrifugation was then performed, followed by the aspiration of 100% EtOH, addition of 70% EtOH, and centrifugation for 5 minutes. The 70% was then aspirated, and 20 μl TE buffer was added to re-suspend the DNA. Gel electrophoresis was performed to inspect DNA for release of the expected 3.3 kb fragment (Figures 10 and 11).

The resulting linearized pbsrfim1 vector was introduced to the 95- and 34-/95- cell lines by electroporation as previously described in Chapter 1. After cells were allowed to recover in HL-5 media at 21°C for 24 hours following electroporation, the media was changed and the cells were pre-treated with 4 μg/ml blasticidin. The following day, cells were harvested, and mixed with concentrated suspension of *klebsiella aerogenes* (*Ka*) in 1x KK2 medium (10x KK2 stock: 11 g KH$_2$PO$_4$, 3.5 g K$_2$HPO$_4$ pH-6.5-6.7). The suspended cells and bacterial mixture was placed on KK2 agar plates containing 40 μg/ml blasticidin. After 3-4 days of incubation at 21°C, cells were picked from multiple plaques that formed, and were plated in HL-5 media containing 4 ug/ml of blasticidin. Cells were incubated at 21°C under HL-
5 medium BSR selection for three days. Media was then changed, and putative mutants were subsequently analyzed by Western blot, and polymerase chain reaction.

**SDS Page and Western Blot Analysis:**

Western blotting was employed to confirm the absence of fimbrin in putative mutants. Briefly, cells were grown and harvested at mid-log phase, at an approximate density of $2 \times 10^6$ cells/ml. About $2 \times 10^7$ cells were pelleted by centrifugation, and re-suspended in 100 μl of cold PEE and protease inhibitors (0.02 M NaPO$_4$ pH 6.8, 2 mM EDTA, 2 mM EDGA, 20 ug/ml Leupeptin, 0.08 mg/ml Aprotinin, 20 ug/ml Pepstatin). 100 μL of 2x SDS Lysis buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) were added to putative mutant sample, and boiled at 95°C for five minutes.

25 μL from each lysate sample were loaded in duplicate for Coomassie stain assessment of equal protein loading, in addition to immunoblotting. Protein samples were resolved at 90 volts on a 7.5% SDS gel for about 3 hours. Resolved protein was transferred to methanol soaked Millipore Immobilon membrane at 12 volts for 3 hours. In parallel, one set of resolved protein samples was stained with Sigma Coomassie brilliant blue solution (0.2% Coomassie blue R250, 10% acetic acid, 25% isopropanol) to visualize protein. After transfer, the membrane was incubated for 30 minutes, shaking in 5% non-fat milk-PBST blocking solution. For blocking solution, 5.0 g of dry non-fat powdered milk were added to 100 ml of PBST stock solution (1000 ml dH$_2$O solution of 8.0 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$, and 0.05% Tween-20). The membrane was subsequently incubated with 1:1000 dilution of Hydbridoma Bank monoclonal fimbrin specific antibody 210-183-1 in 50 ml PBST for 1 hour. The membrane was then washed three times for approximately 5 minutes each with PBST, and then incubated with 1:2500 dilution of conjugated alkaline phosphatase goat anti-mouse antibody in PBST. Development Staining Buffer was made fresh, containing 22.5 ml Development Buffer (12g Tris Base,
0.95g MgCl₂, 5.85g NaCl in 1000 ml, pH 9.5), 2.5 ml Nitroblue tetrazolium (1 mg/ml in Development buffer), and 25 μl 1000x BCIP [50 mg/ml in DMSO]), and membrane was developed in buffer for approximately 5 minutes. Coomassie stain of the gel after transfer was performed in parallel to assess sufficient protein transfer.

**Screening of Putative Mutants by Hot-Start Polymerase Chain Reaction (PCR)**

Putative mutants were confirmed by hot-start PCR analysis using primers dk396 and dk397. dk396 targets the flanking sequence to fimbrin (FimA), and dk397 targets the BSR cassette. The sequence of dk396 is 5’-ATT GAT ATA AAT ATT TGG AG- 3’, which has a melting temperature of 48.1˚C. The sequence of dk397 is 5’- TCT TAA TTT CGG GTA TA- 3’, with a melting temperature of 47.5˚C. The following PCR procedure was modified from Charette and Cosson (2004). 1 μL of 20μg/μl proteinase K was added to 20 μl of lysis buffer prior to use. The lysis buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.45% NP40, and 0.45% Tween 20. Cells were harvested at a density of 2x10⁶ cells/ml. For each reaction, 2 μl of lysis buffer (with Proteinase K) was added to 20 μl of cells in a capped PCR tube. After about five minutes of incubation at room temperature, the tubes were then incubated at 95˚C to inactivate the Proteinase K. 43 μl of PCR master mix (described in Table 2A) was added to 2 μl of cell lysates in new PCR tubes. 5 μl of diluted Phusion Polymerase (0.2 μL of Phusion + 5 μL of sterile dH₂O) was added during the first extension cycle. The reaction took place under the PCR program outlined in Table 2B below. The fim(-) cell line was previously created by Irene Jarchum, using the same gene disruption vector as described above. Fim- was employed as a positive control and Ax2 served as the negative control. PCR products were subsequently resolved by gel electrophoresis and DNA was detected under UV light exposure (Chapter 1 Materials and Methods). Mutants confirmed by PCR and Western Blot were selected for phenotypic analysis.
Table 2A. PCR Master Mix

<table>
<thead>
<tr>
<th>PCR Reaction Component</th>
<th>Stock Concentration</th>
<th>Volume for Single 50 μL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer dk396</td>
<td>100 pmol/μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Reverse primer dk397</td>
<td>100 pmol/μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>PCR buffer NH4 (10x)</td>
<td>10x concentrated</td>
<td>10 μl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50 mM</td>
<td>2 μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2.5 mM each</td>
<td>5 μl</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>N/A</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

Table 2B. PCR Reaction Program

<table>
<thead>
<tr>
<th>Cycle Amount</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x</td>
<td>30”, 95°C</td>
</tr>
<tr>
<td>30x</td>
<td>1. Denature 10”, 95°C</td>
</tr>
<tr>
<td></td>
<td>2. Anneal 10”, 45°C</td>
</tr>
<tr>
<td></td>
<td>3. *Extension 2 min 30 sec, 68°C</td>
</tr>
<tr>
<td>1x</td>
<td>7 min, 68°C</td>
</tr>
</tbody>
</table>

* Diluted Phusion polymerase was added during the extension step of the first cycle.

Random Motility Assays and Chemotaxis Assays:

Random motility and chemotaxis assays were performed as previously described in Chapter 1. However, only phase contrast images were captured every 30 seconds using a 10x or 20x objective.

Macropinocytosis Assays:

Cells in culture were harvested and pelleted by centrifugation at 1000 rpm. Cells were re-suspended to a concentration of about 2 x 10⁶ cells/ml in fresh HL-5 medium. The cell suspension was added to a small scintillation vial, and cells were allowed to recover on shaker for 15 minutes. After recovery, 0.5 ml aliquots were collected from each vial, and added to correspondingly labeled Falcon 15 ml conical tubes, containing 5 ml of ice-chilled HL-5. 70 kDa FITC-Dextran (50 mg/ml stock made up in 10% DMSO and 90% HL-5) was added the scintillation vials, to a final concentration of 2 mg/ml. 0.5 ml aliquots were obtained from shaking scintillation vials at 0 min, 15 min, 30 min, 60 min, 90 min, 120 min,
150 min, and 180 min time points, and transferred to labeled 15 ml conical tubes containing 5 ml cold HL-5 on ice. Cell aliquots in conical tubes were washed two times by centrifugation at 4°C at 1200 rpm for 5 minutes, using 5 ml of cold HL-5. After the second wash, the pellet was re-suspended in 1 ml of HL-5, and transferred to Falcon round bottom flow cytometry tubes, and kept on ice. The FACs Caliber Dual Laser Flow Cytometer and Cell Quest Computer Software were employed to measure the mean fluorescence intensity of 10,000 cells collected from the aliquots. Data was exported, and mean fluorescence over time was plotted on Excel or GraphPad Prism software.

**Development:**

1–2 ×10^7 cells were harvested and washed twice by centrifugation at 4°C at 1000 RM, using 5 ml MCPB starvation buffer (filter-sterilized 1 L stock solution of 1.42 g Na₂HPO₄, 1.36 g KH₂PO₄, 0.19 g MgCl₂, 0.03 g Cl₂, 0.5 g Streptomycin dihydrosulfate, ph 6.5-6.7). Cells were re-suspended in 3 ml MCPB, and suspension was added to 60 mm Petri dishes containing 1 ml of 1.5% MCPB agar (1.5 g agar/100 ml MCPB). Cells were allowed to settle and attach to the agarose for 20 to 30 minutes before the buffer was carefully aspirated. Live cell imaging was performed on Nikon fluorescence microscope under 4x dry objective using the Micro-Manager computer software. Phase contrast images were captured every 20 or 30 seconds.

**Growth and Development on Bacterial Agar Plates:**

Cell growth, phagocytosis, and development were assessed by placing mutant cells on a Ka bacterial lawn as a food source. 20 ml of SM agar (20 g bacto agar melted by autoclave per 1L SM media) were poured into 100mm x 15 mm polystyrene agar plates. 10 or 100 cells were mixed in Ka bacteria concentrated in SM media, and the mixture was then spread on solidified SM agar plates. Cells were
allowed to grow for 7 days at 21˚C. Images of plaques and fruiting bodies were taken with a macroscope.

II. Results

Generation of Fimbrin Mutants:

Fimbrin, α-actinin, and ABP-34 have been targeted for combinatorial gene disruption due to their similar localization to macropinosomes, phagosomes, and leading edges in Dictyostelium. Analysis of mutants lacking multiple ABPs that localize to similar structures may reveal significant defects in cell behavior during various cellular processes. As described in the Materials and Methods above, 95-/fim- double mutants and 34-/95-/fim- triple mutants were generated using the pBSRfim1 vector construct, which contained the fimbrin gene interrupted by the blasticidin resistance cassette. The expected 3.3 kb band containing the homologous targeting sequence was released after digestion, and subsequently transfected into 95- and 34-/95- cells. Blasticidin resistant clones were grown and selected as described above, and putative mutants were screened for successful recombination.

PCR analysis was performed to confirm recombination of the linearized pBSRfim1 vector into the 95-, and 34-/95- strain genomes. PCR products were produced using primers dk396 and dk397 under a hot-start PCR using Phusion polymerase. Dk396 targets the flanking sequence to fimA, and dk397 targets the BSR cassette. 12 of the 17 putative 34-/95-/fim- triple mutant clones produced a DNA product of about 1.5 kb, indicating successful recombination (Figure 13B). Seven putative mutants confirmed by PCR were chosen for Western Blot analysis, and six of them did not yield the fimbrin antibody signal. There is a faint band corresponding to the 67 kD fimbrin protein in putative triple mutant 43 (Figure 14). Eight of the 13 putative 95-/fim- double mutant clones lacked the 67 kD fimbrin antibody signal under Western Blot analysis (data not shown). Of the eight double mutant clones successfully confirmed by Western blot analysis, 6 putative clones yielded the 1.5 kb band under PCR
analysis (Figure 13A). Figures 14 shows a repeated analysis of the double putative mutants, D7 and D8, which both appear negative for the fimbrin protein. 34-/95-/fim- triple mutant 14, and 95-/fim- double mutant 7 (D7) were subsequently chosen for analysis during growth on bacterial plates, chemotaxis, macropinocytosis, development, and random motility assays.

**Random Motility and Chemotaxis Assays:**

Because fimbrin, α-actinin, and ABP-34 localize to pseudopodia of actively moving cells, we anticipated that the 95-/fim- double mutant and the 34-/95-/fim- triple mutant would demonstrate altered motility, particularly during chemotaxis. The 34-/95-/fim- triple mutant, 95-/fim- double mutant, and fim- and 95- single controls exhibit significantly reduced motility compared to wild-type during vegetative motility (p< 0.0001), as shown in Figure 15. The mean speeds of the Ax2, 34-/95-/fim-, 95-/fim-, 95-, and fim- mutants are 8.863 μm/min, 2.920 μm/min, 1.918 μm/min, 3.697 μm/min, and 3.420 μm/min respectively. The relative significance among all cell lines analyzed during random motility is shown in Table 7, which shows that the 95-/fim- mutant exhibited a significant reduction in motility compared to the 95-, fim-, and 34-/95-/fim- mutants. The measured average speeds and corresponding standard deviations of null mutants analyzed during random motility assays have been summarized in Table 10. Images of all null mutants during random motility assays reveal episodes of cell flattening in a circular shape during movement. The random motility movies collected during analysis are listed for reference in Table 11.

The 95-/fim- and 34-/95-/fim- mutants were further analyzed during under agarose chemotaxis assays to analyze behavior when moving directionally towards a folic acid gradient. Preliminary data shown in Figure 16 suggests that both 95-/fim- and 34-/95-/fim- have a significant reduction mean speed in comparison to the wild-type, with average speeds of 6.2 um/min, 5.2 um/min, and 5.1 um/min respectively. In comparison to the wild-type, the p-values of 95-/fim- and 34-/95-/fim- are 0.0154 and
0.0001 respectively. The persistence of Ax2, 95-/fim-, and 34-/95-/fim- are 0.410, 0.344, and 0.396 respectively. Both 95-/fim- and 34-/95-/fim-shows a reduction in persistence compared to wild-type. However the reduced persistence of 95-/fim- is statically significant (p= 0.042), while the reduced persistence of 34-/95-/fim- is not (p=0.105). The measured average speeds, persistence, and corresponding standard deviations of null mutant analyzed during chemotaxis assays of have been summarized in Table 10. The chemotaxis movies collected of all null mutants analyzed are referenced in Table 11.

**Macropinocytosis:**

The endoocytosis ability of the double and triple mutant was assessed by the uptake of FITC-Dextran. As shown in Figure 17, the double and triple mutants increased in fluorescence at a greater rate that than the wild-type during the first 150 minutes of uptake. However, the disparity of mean fluorescence seems to decrease over time between 150 and 180 minutes.

**Growth and Development:**

To analyze the ability for the fimbrin mutants to develop, cells were imaged for 24 hours during over agarose development on 1.5% MCBP agar. Figure 18 shows images of cell streaming between 8-11 hours of development, aggregate and slug formation between 15-16 hours of development, and fruiting bodies at 24 hour of development. 95-/fim- develops normally in comparison to Ax2, with streaming and aggregate formation occurring by 8 hours, and 16 hours respectively. However, robust streaming of the 34-/95-/fim- does not occur until about 11 hours of development, compared to 8 hours seen in the wild-type. Furthermore, 34-/95-/fim- does not develop into fruiting bodies after 24 hours. Movies of cell development for reference are listed in Table 11. To visualize the morphological states of mutants after development, macroscope images were taken of Ax2, 95-/fim-, and 34-/95-/fim- cells. As shown in
Figure 19, 95-/fim- fruiting bodies are present and are morphologically similar to Ax2 fruiting bodies. 34-/95-/fim- mutants do not develop into fruiting bodies, but look to remain in aggregate and slug formation. However, both of the 95-/fim- and 34-/95-/fim- mutants develop into fruiting bodies on Ka bacterial lawns, suggesting that they are able to feed by phagocytosis (Figure 20). Although we were unsure what to hypothesize about mutant development, we had suspected that the developmental patterns of the mutants on Ka bacteria and MCBP agar would be relatively similar. Furthermore, we had suspected that streaming into aggregates may be affected, because of impairments in chemotaxis. The interesting observation that the 34-/95-/fim- triple mutant is capable of full development into fruiting bodies on Ka bacterial lawns, but does not fully develop on MCBP agar, is unexpected.

III. Discussion and Future Directions:

Generation of 95-/fim- Double and 34-/95-/fim- Triple Mutants:

Several reports confirm the localization of fimbrin, α-actinin, and ABP-34 to leading edges of polarized cells, phagosomes, and macropinosomes. Several Dictyostelium single null mutants, including those devoid of fimbrin, ABP-34, and α-actinin, reveal very little about their individual contribution to actin regulation, as the observed phenotypes are not significantly different from the wild-type. Thus, multiple ABPs that localize to the same cellular structures were targeted for elimination, and the 95-/fim- and 34-/95-/fim- mutants were created with the hope of driving phenotypic change. Mutants lacking these actin binding proteins are commonly created by homologous recombination. In addition to inserting a drug resistant cassette, the vector constructs used for homologous gene targeting often have segments of the targeted gene removed, contain sequences that lack a start codon within the gene disruption sequence, or introduce an early termination sequence (Witke et. al. 1987; Pikzack et. al., 2005). These modifications in the gene disruption vector would ensure that the targeted genomic DNA does not revert back to its original sequence, or produce the 5' fragment of the targeted protein after
homologous recombination. However, the pBSRfim1 gene replacement vector used to target the fimbrin gene was composed of the full length fimbrin gene disrupted by the BSR cassette. Possible reversion of the targeted fimbrin gene or production of the 5’ fragment of fimbrin following homologous recombination could complicate the generation of fimbrin null mutants.

The 95-/fim- and 34-/95-/fim- mutants were generated, and the elimination of fimbrin was confirmed by PCR analysis and Western Blot. However, the gene disruption vector should have been constructed with additional modifications, such as targeted gene deletions, or insertion of premature termination sequences, which would prevent gene reversion or partial protein synthesis. Additionally, probing for ABP-34 and α-actinin by Western Blot analysis should be performed again in the newly created 95-/fim- and 34-/95-/fim- mutant strains, though the 95- and 34-/95- lines used to create the mutants were previously confirmed for lacking ABP-34 and α-actinin (Eichinger et al., 1996, Rivero et al., 1996; Rivero et. al 1999).

Random Motility and Chemotaxis:

Upon the creation of the 95-/fim- double and 34-/95-/fim- triple mutants, we hypothesized that motility would be reduced during chemotaxis. Indeed, our results confirmed our expectation. Both mutants show a small but statistically significant reduction in speed and persistence during folate chemotaxis. Interestingly, previous studies of 34- showed increased persistence on glass surfaces coated with bovine serum albumin, which makes the glass surface less adherent (Rivero et.al 1996). Furthermore, 34-/95- mutants also demonstrated increased persistence only in the absence of a chemotactic gradient (Rivero et.al 1999). While increased persistence in the 34- and 34-/95- suggests that there is increased stability of cross-linked F-actin (Rivero et. al, 1996), the decreased persistence observed in the 95-/fim- and 34-/95-/fim- mutants suggests that there is a lower stability of cross-linked actin networks. Removal of fimbrin, α-actinin, and ABP-34 suggests that their collective cooperation in
regulating the actin cytoskeleton is important for maintaining cell speed and persistence during chemotaxis. Cell speed was significantly reduced during random motility as well. Further experimentation is necessary to investigate the mechanistic cause of cell speed reduction and persistence. Chemotaxis assays of 95-/fim- and 34-/95-/fim- will be repeated under high magnification, and morphological differences in pseudopod extension or tail release can be analyzed. Furthermore, cell behavior such as persistence and number of turns during movement can also be analyzed.

**Macropinocytosis:**

It was hypothesized that the combinatorial elimination of ABP-34, fimbrin, and α-actinin would cause impairment in macropinocytosis. However, preliminary observations suggest that macropinocytosis of 95-/fim- and 34-/95-/fim- is increased during the first 150 minutes, in comparison to the wild-type. Although the disparity between mean fluorescence level of the mutants and wild-type decreases after 150 minutes, the results were not expected. It is clear that the combination of ABP-34, fimbrin, and α-actinin is dispensable for macropinocytosis. However, such a finding must be further analyzed. It is possible that compensation for the loss of ABP-34, fimbrin, and α-actinin may be occurring within the mutant cells, which may be further causing an increased rate of macropinocytosis. In addition to repeating the macropinocytosis assay performed, microscopy can be employed to examine the rate of macropinocytic cup formation, as well as cup size and morphology.

**Over Agarose Development**

Previous studies have shown that there are no phenotypic differences in development of fim-, 95-, and 34- single mutants, and increased number of sori in 34-/95- mutants (summarized in Rivero et al., 1999). Furthermore, there is a decreased spore yield in 34-/fim- double mutants (Pikzack et. al, 2005). There was no clear hypothesis for the development of 95-/fim- and 34-/95-/fim- mutants. 95-
/fim- mutants demonstrated normal development patterns of streaming, slug formation, and fruiting body formation in comparison to the wild-type. However, preliminary observation of the development of the 34-/95-/fim- mutant was characterized by a slight delay of chemotactic migration compared to the wild-type. Furthermore, fruiting bodies of 34-/95-/fim- mutants were not clearly apparent. Instead, it seems as though development terminated after aggregation, and where slugs were formed, they did not actively migrate. Future experimentation can be used to further characterize cell regulation and morphology during development. Developmentally regulated genes have been identified, and monitoring their timing and expression levels throughout development can further assess the progression of distinct developmental stages within the 95-/fim- and 34-/95-/fim- mutants. Furthermore, spore yield of the mutant cells can be quantified.

*Development and Growth on Bacterial Lawn*

Cell growth on *K. aerogenes* bacterial lawn requires the interplay between several cellular processes, including phagocytosis, mitosis, and cell motility. When cells are allowed to grow on bacterial plates, single cells engulf bacteria by phagocytosis and divide. Plaques become apparent on the bacterial lawn, where clearings of bacteria are filled with a colony of cells. Cells begin to starve in the center of the growing colony, where development initiates, and individual cells stream into mounds and eventually form into fruiting bodies. Fruiting bodies are found at the core of a colony followed by a circular zone of aggregating cells, a zone of pre-starving cells, and an outer zone of actively feeding amoebae (Soll, 1987). Given that the 95-/fim- double mutant developed normally during over-agarose development and the 34-/95-/fim- did not, it was hypothesized that similar results would be seen at the core of plaque colonies during growth on bacterial agar plates. However, the results did not support our hypothesis. There were clear fruiting bodies in both combinatorial mutants, in addition to the single mutant controls.
In is unclear why the 34-/95-/fim- mutant was able to develop during growth on *Ka* bacterial lawn. Perhaps, the presence of *Ka* as a food source facilitated subsequent development, as cells were not subjected to the same stressful conditions in a nutrient poor over-agarose environment. It is also clear the both mutant cells are able to phagocytose *Ka*, which contradicts our initial prediction for reduced phagocytosis as well. Phagocytosis and growth rate can be further analyzed by measuring the rate of plaque diameter increase over time. Furthermore, growth rate and phagocytosis ability of the mutants can be measured as the cells are cultured in a shaking yeast suspension. Additionally, the rate of phagocytic cup formation can be monitored as the mutants engulf fluorescent yeast using fluorescent microscopy. Fruiting body size and spore amount can further be analyzed as well.
Overall Conclusions:

By over-expressing selected ABPs, it is clear that over-expression of some fluorescent actin binding proteins have an effect on random motility. In particular, over-expression of GFP-Enlazin demonstrates decreased speed and range of motion during random cell movement, while causing no apparent effect during chemotaxis. Phalloidin staining reveals that GFP-Enlazin also enhances cortical F-actin. Current uncertainty of the affects of GFP alone on cell motility emphasizes the need for consideration of methodological techniques in assessing cell behavior. Additionally, obtaining cell lines from multiple mutant clones may be conducive in avoiding single cell aberrations in cell movement.

Results presented in Chapter 2 demonstrate that ABP-34, fimbrin, and α-actinin are dispensable for growth and phagocytosis on bacterial Ka lawns. Furthermore, both 95-/fim- and 34-/95-/fim-mutants can grow and develop into fruiting bodies on bacteria growth plates. While 95-/fim- mutants develop similarly in comparison to the wild-type during ovar-agarose development, the 34-/95-/fim-triple mutant exhibits a slight delay in streaming, and seems to arrest at aggregation and slug formation. Both mutants show an increased rate of macropinocytosis, supporting the idea of ABP functional redundancy allowing for compensation in null mutants. Random cell motility is significantly reduced in both 95-/fim-, 34-/95-/fim- mutants, and fim-, 95- single mutants. Reduction of chemotactic movement is apparent in both double and triple mutants. Furthermore, slight reduction in persistence is significant 95-/fim- double mutants, but statistically insignificant in 34-/95-/fim- triple mutants. While several studies must be performed to further understand the observations obtained, the results begin to further elucidate the intricacies of cytoskeleton roles of actin binding proteins.
Acknowledgments

First and foremost, I would like to extend my sincere thanks to Dr. David Knecht for welcoming me into his lab. His guidance and words of encouragement have greatly facilitated the progression of this project, and further helped me develop as an independent thinker and a persistent problem solver. The critical thinking, work ethic, and networking skills I have gained in his lab will carry me far in my future career as an aspiring physician. His efforts as my advisor have culminated in endless opportunities for academic, professional, and personal achievement.

I would also like to give my great appreciation to my graduate mentor Michael Lemieux. His kindness and mentorship have proven invaluable, as he guided me through new scientific concepts and scientific techniques. I would also like to extend thanks to current and past Knecht Lab members, Evan Byron, Allie Goetjen, Nicholas Minutolo, Alex Marrotte, Gaurav Joshi, and Dr. Renee Gilberti for their friendship and assistance within the laboratory. Additionally, I would like to forward great thanks to Dr. Carol Norris for her assistance with the Confocal and Flow Cytometry Facility, which was vital for the completion of this project. Lastly, I thank the Office of Undergraduate Research and the donors of the Summer Undergraduate Research Fund for financing my research endeavors.
Figure 1. Box and Whisker Plots of Ax2 Over-Expressers During Random Motility. Box and Whisker Plot of Ax2 wild-type and Ax2 cell lines over-expressing a fluorescently labeled actin binding protein. Ax2 (n=32), α-Actinin (n=32), 34- GFP (n=30), GFP-Enlazin (n=30), mRFP-Fimbrin (n=30), and GFP-Filamin (n=32) cells were analyzed using mTrackJ computer software. GFP-Enlazin and 34- GFP cell lines show the most reduced motility compared to Ax2.
Figure 2. Average Speed of GFP Ax2 during Random Motility. Ax2 cells expressing GFP were imaged during random motility. Cells of the GFP Ax2 population were categorized as Expressers if fluorescent, and Non-Expressers if non-fluorescent under blue fluorescent light exposure. Upper and lower ends of boxes represent 75th and 25th percentiles of cell speed respectively. Top and bottom whiskers depict maximum and minimum speeds. The median is represented by solid line within box. Both GFP Ax2 expressers and non-expressers show a significantly reduced motility compared to Ax2 alone (p<0.0001). There is no significant difference between the mean speeds of the GFP-Ax2 expressers and non-expressers (p=0.891).
Figure 3. Average Speed of GFP-Enlazin and 34 GFP Expressers and Non-Expressers During Random Motility. Box and Whisker Plot of Ax2 (n=32), GFP-Enlazin Expressers (n=30), and GFP-Enlazin Non-Expressers (n=28), 34- GFP Non-Expressers (n=27), and 34-GFP Expressers (n=30) are shown. Cells of the GFP-Enlazin and 34-GFP populations were categorized as Expressers if fluorescent, and Non-expressers if non-fluorescent under blue fluorescent light exposure. Upper and lower ends of boxes represent 75th and 25th percentiles of cell speed respectively. Top and bottom whiskers depict maximum and minimum speeds. The mean speed of GFP-Enlazin Non-expressers is similar to the mean speed of Ax2 (p=0.05404), while the mean speed of GFP-Enlazin Expressers is significantly reduced compared to GFP-Enlazin Expressers and Ax2 (p<0.0001). The a similar reduction in mean speed in shown for both 34-GFP Expressers and Non-Expressers (p<0.0001), while the difference in mean speeds between 34-GFP Expressers and Non-Expressers are significant as well (p<0.0001)
Figure 4. Manual Cell Tracks of Expressers and Non-Expressers. Cell tracks of Ax2, GFP-Enlazin, and 34-GFP during random motility assays are shown. Red tracks represent Non-expressers and yellow tracks represent expressers under blue fluorescent light exposure.
Figure 5. Trial 1 Rose Plot of GFP-Enlazin Non-Expressers and Expressers. Single cell Tracks of five different non-expressing GFP-Enlazin cells (A) and expressing GFP-Enlazin cells are shown (B). Each Track is plotted as a function of position over a period of 90 minutes. GFP-Enlazin Expressers show a lower range of motility along the x and y axis compared to GFP-Enlazin Non-Expressers.
Figure 6. Trial 2 Rose Plot of GFP-Enlazin Non-Expressers and Expressers. Single cell Tracks of five different non-expressing GFP-Enlazin cells (A) and expressing GFP-Enlazin cells are shown (B). Each Track is plotted as a function of position over a period of 46.5 minutes. GFP-Enlazin Expressers show a lower range of motility along the x and y axis compared to GFP-Enlazin Non-Expressers.
Figure 7. Trial 3 Rose Plot of GFP-Enlazin Non-Expressers and Expressers. Single cell Tracks of five different non-expressing GFP-Enlazin cells (A) and expressing GFP-Enlazin cells are shown (B). Each Track is plotted as a function of position over a period of 57 minutes. GFP-Enlazin Expressers show a lower range of motility along the x and y axis compared to GFP-Enlazin Non-Expressers.
Figure 8. Speed and Persistence of Ax2 and GFP-Enlazin Expressers during Folate Chemotaxis. Box and Whisker plots are shown. Images were taken as a 1:1 mixture of the GFP-Enlazin and Ax2 population were allowed to chemotax toward a 0.02 mM gradient of Folate in SM agar. Cells were categorized as Expressers if fluorescent, and Non-expressers if non-fluorescent under blue fluorescent light exposure. There was no significant difference in cell speed and persistence between expressers and non-expressers.
Figure 9. Phalloidin Stain off GFP-Enlazin Expressers and Non-Expressers. Phalloidin Stain of 1:1 ratio of Ax2 and GFP-Enlazin Cells were imaged on Andor Confocal microscope 60x oil objective. Arrows and stars designates a GFP-Enlazin Expresser and Non-Expresser respectively. The pixel intensity ratio of cortex : cytoplasm ratio of the GFP-Enlazin Expresser is greater than that of the Non-Expresser, (1.86 and 1.46 respectively).
Figure 10. pBSRfim1 Gene Disruption Vector. pBSRfim1 vector, created by former graduate student Irene Jarchum, contains the BSR cassette (1360 bp) integrated internal to the SwaI restriction site of the full length FimA gene (1921 bp) by blunt ligation. Digestion at the BamHI and PstI restriction sites prior to transfection would liberate a fragment of approximately 3311 bp, corresponding to disrupted fimA gene.
Figure 11. Creation of Mutants using pBSRfim1 Vector. A) The full length fimbrin sequence (fimA) containing the BSR cassette from pBSRfim1 disruption vector was introduced into the genome of 95- and 34-/95- *Dictyostelium* mutants via double homologous recombination. B) Screening strategies with multiple primer pairs and expected band sizes are shown.* Indicates primer pair used for positive mutant screen.

<table>
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<th>Primer Pair</th>
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</thead>
<tbody>
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<td>*Dk396/dk397</td>
<td>No band</td>
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</tr>
<tr>
<td>Dk 364/367</td>
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<td>3.3 kb</td>
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<td>Fbsr/Rbsr</td>
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Figure 12. pBSRfim1 Gene Disruption Vector Resolved by Gel Electrophoresis. Alkaline Mini-prep sample of circular pBSRfim1 vector and BamHI and PstI digested vector are shown. The digest yielded fragments of approximately 3311 bp, containing the full length fimA gene disrupted by the BSR cassette. The products following digestion were used for transfection of α-actinin- and 34/-α-actinin- cell lines.
Figure 13. PCR Screen of Putative Mutants. PCR products of α-95-/fim- (A) and 34-/95-/fim- putative mutants resolved by gel electrophoresis are shown. PCR products were produced using primers dk396 and dk397 (Figure 11) under a Hot-start PCR using Phusion polymerase. Mutants confirmed by PCR produced a PCR product of about 1.5 kb, and were selected for Western Blot analysis.
Figure 14. Western Blot Analysis of 95-/fim- and 34-/95-/fim- Mutant Strains. A) Protein samples from Ax2 and mutant cells were resolved by SDS page, and transferred to Immobilon filter membrane. Fimbrin specific monoclonal antibody 210-183-1 was used to detect fimbrin. Two putative 95-/fim-double mutants samples confirmed are designated as D7 and D8. Triple mutant 34-/95-/fim- samples clones 10, 14, 22, 26, 34, and 43 were analyzed. There is a faint band corresponding to the 67 kD fimbrin protein in putative triple mutant 43. All other putative mutant clones are devoid of the 67 kD band signal. B) Parallel Coomassie Stain of protein lysate samples show presence of protein for all putative mutants analyzed.
Figure 15. Box and Whisker Plots of Ax2 Null Mutants During Random Motility. Box and Whisker Plot of Ax2 wild-type and Null Mutant Cells. Upper and lower ends of boxes represent 75th and 25th percentiles of cell speed respectively. Top and bottom whiskers depict maximum and minimum speeds. The median is represented by solid line within box. All null mutants show significantly reduced motility compared to Ax2 wild-type (p <0.0001).
Figure 16. Average Speed and Persistence of Fimbrin Mutants during Folate Chemotaxis. Cells of the Ax2, 95-/fim- and 34-/95-/fim- were imaged during chemotaxis toward a 0.02 mM Folate gradient in SM agar. Box and Whisker plots of mutant mean speed (A) and persistence (B) are shown. Upper and lower ends of boxes represent 75th and 25th percentiles of cell speed respectively. Top and bottom whiskers depict maximum and minimum speeds. The median is represented by solid line within box. Both mutants show a significant reduction in mean speed compared to wild-type (p= 0.0154 and p= 0.0001 respectively). 95-/fim- shows a significant reduction in persistence compared to wild-type (p= 0.042), while 34-/95-/fim- does not (p=0.105)
Figure 17. Percent Increase of FITC-Dextran Fluorescence Over Time during Macropinocytosis. About 1×10^7 Cells were allowed to uptake HL-5 medium containing 2 mg/mL of 70 kDa FITC-Dextran. 10,000 cells were collected by FACsCalibur flow cytometer at each time point, and mean fluorescence intensity was measured using Cell Quest Computer Software. Percent increase of mean fluorescence at each time point was calculated. Both 95-/-fim- and 34-/-95-/-fim- show a greater increase in fluorescence more rapidly than wild-type during the first 150 minutes.
Figure 18. Over Agarose Development of Mutants. Cells were imaged for 24 hours during development on 1.5% MCBP agar. Images of cell streaming (8-11 hours), aggregate and slug formation (15-16 hours), and fruiting bodies (24 hours) are shown. 95-/fim- develops normally in comparison to Ax2, with streaming and aggregate formation occurring by 8 hours, and 16 hours respectively. 34-/95-/fim- demonstrates a slight delay in time to visible streams (11 hours) compared to Ax2, and does not develop into fruiting bodies.
Figure 19. Fruiting Bodies of Fimbrin Double and Triple Mutants on Agar. Macroscopic images of Ax2, 95-/fim-, and 34-/95-/fim- fruiting bodies after 24 hours of over agar development are shown in low magnification (A) and high magnification (B). 95-/fim- fruiting bodies are present and are morphologically similar to Ax2 fruiting bodies. 34-/95-/fim- do not develop into fruiting bodies, remaining in aggregate mound and slug formation.
Figure 20. Fruiting Bodies of Fimbrin Double and Triple Mutants on Ka Bacterial Lawn. Images of 95- /fim- and 34-/95-/fim- mutants and single mutant controls were taken after 6 days of growth on SM plates containing Ka bacteria at 21°C. Fruiting bodies formed in the center of plaques in all mutants.
Table 3. Two-Tailed P-values of Random Motility Mean Speeds for Over-Expressing Populations. P-values of over-expressers from Figure 1 are shown. Unpaired two–tailed t-tests were performed by GraphPad Prism Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

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<th>Ax2</th>
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<th>34- GFP</th>
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<th>mRFP-Fimbrin</th>
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Table 4. Two-Tailed P-values of Random Motility Mean Speeds for GFP Ax2 Expressers and Non-Expressers. P-values of over-expressers from Figure 2 are shown. Unpaired two–tailed t-tests were performed by GraphPad Prism Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

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<tr>
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<th>Ax2</th>
<th>GFP Ax2 Non-Expressers</th>
<th>GFP Ax2 Expressers</th>
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Table 5A. Two-Tailed P-values of Random Motility Mean Speeds for GFP-Enlazin Expressers and Non-Expressers. P-values of over-expressers from Figure 3A are shown. Unpaired two–tailed t-tests were performed by GraphPad Prism Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

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Table 5B. Two-Tailed P-values of Random Motility Mean Speeds for 34-GFP Expressers and Non-Expressers. P-values of over-expressers from Figure 3B are shown. Unpaired two–tailed t-tests were performed by GraphPad Prism Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.
Table 6. Two-Tailed P-values of Mean Speeds for GFP-Enlazin Expressers and Non-Expressers during Chemotaxis. P-values of over-expressers from Figure 8 are shown. Unpaired two–tailed t-tests were performed by GraphPad Prism Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

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<td>GFP-Enlazin Expressers</td>
<td>0.4982</td>
<td>N/A</td>
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</table>

Table 7. Two-Tailed P-values of Random Motility Mean Speeds for 95-/fim- and 34-/95-/fim-Mutants. P-values of over-expressers from Figure 15 are shown. Unpaired two–tailed t-tests were performed on Prism GraphPad Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

<table>
<thead>
<tr>
<th></th>
<th>Fim-</th>
<th>95-</th>
<th>95-/Fim-</th>
<th>30-/Fim-/95-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ax2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fim-</td>
<td>&lt; 0.0001</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>95-</td>
<td>&lt; 0.0001</td>
<td>0.3331</td>
<td>N/A</td>
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<tr>
<td>95-/Fim-</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td>30-/Fim-/95-</td>
<td>&lt; 0.0001</td>
<td>0.0793</td>
<td>0.0036</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

Table 8. Summary Table of Over-Expression Chemotaxis and Random Motility Data. Summary of random motility and chemotaxis average speeds, chemotactic persistence, and corresponding standard deviation values are provided from GraphPad Prism Software.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Random Motility N</th>
<th>Random Motility Mean Speed (µm/min)</th>
<th>Random Motility Mean Speed Standard Deviation</th>
<th>Chemotaxis N</th>
<th>Chemotaxis is Mean Speed (µm/min)</th>
<th>Chemotaxis is Mean Speed Standard Deviation (µm/min)</th>
<th>Chemotactic Mean Persistence</th>
<th>Chemotactic Mean Persistence-Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ax2</td>
<td>32</td>
<td>8.863</td>
<td>3.772</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>α-Actinin</td>
<td>32</td>
<td>5.953</td>
<td>3.461</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>marsRFP-</td>
<td>30</td>
<td>6.356</td>
<td>2.529</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fimbrin</td>
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<td></td>
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<td>GFP-</td>
<td>32</td>
<td>6.668</td>
<td>2.585</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Filamin</td>
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</tbody>
</table>
Table 9. Two-Tailed P-values of Chemotaxis Mean Speeds for 95-/fim- and 34-/95-/fim- Mutants. P-values of over-expressers from Figure 16 are shown. Unpaired two-tailed t-tests were performed on Prism GraphPad Software. Significant difference in means (p<0.05) is indicated by red bold type in the chart below.

<table>
<thead>
<tr>
<th></th>
<th>Ax2</th>
<th>95-/fim-</th>
<th>34-/95-/fim-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ax2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>95-/fim-</td>
<td>0.0154</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>34-/95-/fim-</td>
<td>0.0001</td>
<td>0.3703</td>
<td>N/A</td>
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</table>

Table 10. Summary Table of Null Mutant Chemotaxis and Random Motility Data. Summary of random motility and chemotaxis average speeds, chemotactic persistence, and corresponding standard deviation values are provided from GraphPad Prism Software.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Random Motility N</th>
<th>Random Motility Mean Speed (μm/min)</th>
<th>Random Motility Mean Speed Standard Deviation (μm/min)</th>
<th>Chemotaxis N</th>
<th>Chemotaxis Mean Speed (μm/min)</th>
<th>Chemotaxis Mean Speed Standard Deviation (μm/min)</th>
<th>Chemotactic Persistence</th>
<th>Chemotactic Persistence Standard Deviation</th>
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<tbody>
<tr>
<td>Ax2</td>
<td>32</td>
<td>8.863</td>
<td>3.772</td>
<td>10</td>
<td>6.260</td>
<td>0.3340</td>
<td>0.4075</td>
<td>0.04868</td>
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<tr>
<td>34-/95-/fim-</td>
<td>30</td>
<td>2.920</td>
<td>0.9757</td>
<td>10</td>
<td>5.050</td>
<td>0.7028</td>
<td>0.3956</td>
<td>0.05553</td>
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<tr>
<td>95-/fim-</td>
<td>30</td>
<td>1.918</td>
<td>0.8274</td>
<td>9</td>
<td>5.400</td>
<td>0.9487</td>
<td>0.3440</td>
<td>0.07545</td>
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<tr>
<td>95- Fim-</td>
<td>30</td>
<td>3.697</td>
<td>1.006</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>34- Fim-</td>
<td>30</td>
<td>3.420</td>
<td>1.183</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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Table 11. Referenced Movies. Movies can be located in Knecht Laboratory data files.

<table>
<thead>
<tr>
<th>Movie Type</th>
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<tr>
<td>Ax2 Random Motility Movies:</td>
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<tr>
<td></td>
<td>AX2 Movie 2</td>
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<td>Ax2 Movie 3</td>
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<tr>
<td>GFP α-Actinin Random Motility Movies:</td>
<td>GFP a-actinin RM1 Movie</td>
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<td>GFP a-actinin RM2 Movie</td>
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<tr>
<td></td>
<td>GFP a-actinin RM3 Movie</td>
</tr>
<tr>
<td>34- GFP Random Motility Movies:</td>
<td>34-GFP RM Movie 1</td>
</tr>
<tr>
<td></td>
<td>34-GFP RM Movie 2</td>
</tr>
<tr>
<td></td>
<td>34-GFP RM Movie 3</td>
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<tr>
<td>marsRFP-Fimbrin Random Motility Movies:</td>
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<tr>
<td></td>
<td>mars-RFP Fimbrin Movie 2</td>
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<tr>
<td></td>
<td>mars-RFP Fimbrin Movie 3</td>
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<td>GFP-Filamin Random Motility Movies:</td>
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<td>GFP Filamin Movie 2</td>
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<td>GFP Filamin Movie 3</td>
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<td>GFP-Enlazin Random Motility Movies:</td>
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<td></td>
<td>GFP Enlazin Movie 2</td>
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<td>GFP Enlazin Movie 3</td>
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<tr>
<td>GFP-Enlazin Chemotaxis Movie:</td>
<td>GFP Enlazin Chemotaxis Movie</td>
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<td>DKO RM Movie 2</td>
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<td></td>
<td>DKO RM Movie 3</td>
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<td>34-/95-/fim- Random Motility Movies:</td>
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<tr>
<td>95-/fim-</td>
<td>TKO14 Development Movie Part 1 and Part 2</td>
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References


Octtaviani E, Effler JC, Robinson DN. Enlazin, a natural fusion of two classes of canonical cytoskeletal proteins, contributes to cytokinesis dynamics. Mol Biol Cell. 2006 Dec; 17 (12) :5275-86. PubMed PMID:17050732; PubMed Central PMCID: PMC1679690.


