PaxB, an orthologue of mammalian paxillin, regulates adhesion, differentiation, and morphogenesis in *Dictyostelium discoideum*

by

MARIA-BERENICE DURAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2008

UMI Number: 3296981

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

This manuscript has been read and accepted for the Graduate Faculty in Biology
in satisfaction of the dissertation requirements for the degree of Doctoral of Philosophy.

12/18/2007
Date

Chair of Examining Committee
Dr. Derrick Brazill, Hunter College

01/15/2008
Date

Executive Officer
Dr. Richard L. Chappell

Dr. Patricia Rockwell, Hunter College

Dr. Thomas Schmidt-Glenewinkel, Hunter College

Dr. Joni Seeling, Queens College

Dr. Jeffrey E. Segall, Albert Einstein College of Medicine

Supervising Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

PaxB, an orthologue of mammalian paxillin, regulates adhesion, differentiation, and morphogenesis in *Dictyostelium discoideum*

by

Maria-Berenice Duran

Adviser: Derrick T. Brazill, Ph.D.

The adhesion of cells to other cells and to the surrounding extracellular matrix is essential for survival, proliferation, differentiation and migration. Focal adhesions, sites where cells are adhered to their substratum, are also important components for tissue formation and wound repair. As in animal cells, such adhesion is critical for the development of the eukaryote *Dictyostelium discoideum*. In fact, *D. discoideum* development displays many of the features of animal embryogenesis such as regulated cell-cell adhesion, cell motility and cell morphogenesis. *D. discoideum* live as individual cells under vegetative conditions, but develop into a multicellular organism under starvation. Cell-cell adhesion is a vital factor for *D. disciodeum* morphogenesis and is required for multicellular development past the aggregation stage. As in animals, *D. discoideum* cell adhesion molecules have a mechanical function and may interact with the signal-transduction processes regulating morphogenesis. One regulator protein, PaxB, is an orthologue of mammalian paxillin, a known focal adhesion molecule. Paxillin functions as a docking site on the

plasma membrane for signaling and structural proteins, and has been found to play a role in cell-cell cohesion as well as cell-substrate adhesion.

To gain a better understanding of the role and regulation of paxillin, we studied the role of *D. disciodeum* PaxB in differentiation and development. In particular we describe the effects of PaxB overexpression in adhesion and cell-type differentiation. PaxB overexpressing cells (PaxBOE) exhibit increased cell-cell cohesion in non-nutrient buffer, which is lost when Ca^{2+} is chelated by EDTA. This suggests adhesion mediated by PaxB is calcium dependent. Interestingly, cells overexpressing *paxB* are less adhesive to the substratum under equivalent conditions. We show PaxB undergoes serine phosphorylation. In addition, PaxB undergoes adhesion-dependent tyrosine and serine phosphorylation, which is also Ca^{2+} dependent. PaxBOE cells can aggregate and form mounds, but subsequent morphogenesis is blocked. This block can be rescued by addition of wild-type cells, indicating a non-cell autonomous role for PaxB. In these chimeras, wild-type cells predominantly localize to the middle section of the spore mass, while PaxBOE cells preferentially are excluded from the spore mass. Taken together, the data suggest calcium dependent cell-substrate adhesion correlates with changes in PaxB tyrosine and serine phosphorylation. The data also imply that proper PaxB protein expression is required for development past the mound stage and for appropriate cell sorting, cell-type differentiation, cell-cell cohesion, and cell-substrate adhesion.

ACKNOWLEDGMETNS

First and foremost I would like to acknowledge my husband Pedro Guzman for his support, understanding and encouragement.

I would like to thank my family, especially my sister Rosa. I am eternally grateful to my mother Eneida for her upbringing, dedication and sacrifices.

I have been honored to work with an outstanding mentor, Dr. Derrick Brazill. He has personally trained me in much of what I have learned as a graduate student. I will be infinitely grateful for his mentoring, encouragement, and support.

I have had the distinction to have Dr. Patricia Rockwell, Dr. Thomas Schmidt-Glenewinkel, Dr. Joni Seeling, and Dr. Jeffrey Segall as my dissertation advisors. I am very thankful for their insightful suggestions to my work.

I am extremely appreciative of the financial support throughout the program by the Diversity programs at the Graduate Center, the MBRS program at Hunter College, and Dr. Derrick Brazill.

To Gertrude Rivera, your words of wisdom will forever help me endure life's challenges.

Last but not least, I would like to extend my sincere and upmost gratitude to all the members of the Brazill laboratory for making it such a wonderful experience and a great place to work in.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Introduction

1.1 Adhesion

In eukaryotic organisms, developmental processes are often characterized by the expression of cell adhesion molecules (CAMs), which are involved in a complex series of cellular interactions during morphogenesis. CAMs are essential in the physiological processes of development, differentiation, and motility [1-3]. In particular, cell-substrate adhesion and motility are involved in wound healing, lymphocyte migration, metastasis, and phagocytosis [2, 4-6]. These complex events involve cell surface receptors, signaling molecules at focal adhesion sites (FAS), the actin cytoskeleton, and many associated proteins [7-10]. In essence, cell surface proteins bind to the extracellular matrix and connect it with the actin cytoskeleton and a complex network of cytosolic proteins controlling cell spreading, migration, proliferation, and survival [1]. The mammalian protein paxillin, a focal adhesion molecule which serves as an adapter and anchor protein at the plasma membrane for an array of signaling and structural proteins, is important for the integration and processing of adhesion and growth factor related signals [11-15].

1.2 Paxillin

The adhesion of cells to the surrounding extracellular matrix is a key component of the environmental cues necessary to control a cell's decision to survive,

proliferate, differentiate or migrate. Paxillin is a focal adhesion molecule involved in these events during the regulation of normal development as well as the recovery from injury or infection. Mammalian paxillin is a 68-kDa molecule that serves as an anchor protein for the integrating and processing of adhesion and growth factor related signals [12]. During the1990s paxillin was strongly believed to function mainly as a focal adhesion molecule [13-15]. Currently, paxillin is more commonly described as a multidomain protein that primarily functions as a molecular adapter or scaffold protein, providing multiple docking sites at the plasma membrane that facilitate its interaction with signaling and structural molecules [11, 12]. Today, paxillin is extensively investigated in a number of systems such as *Drosophila* [16], and mammalian cells [14] among others [10].

1.2.1 Structure

The multidomain structure of paxillin and the lack of identifiable enzymatic motifs first suggested an adaptor protein function [17]. Paxillin harbors five highly conserved LD domains, four extensively preserved LIM domains, two Y-phosphorylation motifs, and a proline rich region (Fig. 1A). The amino terminus of mammalian paxillin contains the five LD regions, consisting of leucine-rich motifs, that function in protein recognition [11, 15, 18], and regulate cell spreading and motility [12]. The carboxy terminus comprises the four cysteine rich, zinc-binding lin-11, isl-1, mec-3 (LIM) domains that also mediate protein-protein interaction [18]. In 1998 it was shown by Breen and colleagues, and by

Brown and colleagues that the LIM domains of paxillin regulate its focal adhesion activity [13, 15]. They are known to bind zinc and are found in many different proteins, some of which also contain kinase domains, homeodomains, and cytoskeleton interacting domains [15, 16]. The molecule has two highly phosphorylated tyrosine sites that form the core of SH2-binding motives [19]. These tyrosine residues are phosphorylated in response to growth factor signaling and adhesion to the extracellular matrix (ECM), and permits the formation of focal adhesions mediated by paxillin [12, 20, 21]. In addition, paxillin contains many serine/threonine and other tyrosine phosphorylation sites dispersed throughout its domains. Phosphorylation of specific serines localizes paxillin to focal adhesions [13]. Moreover, the proline rich domain provides multiple binding sites for proteins containing SH3 domains [12].

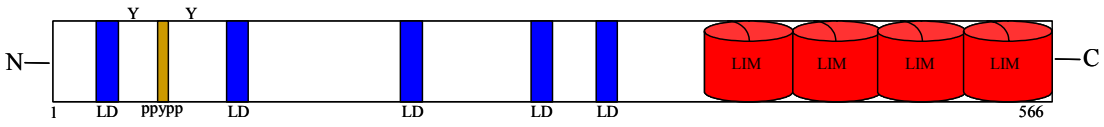


Figure 1. Paxillin contains a multidomain structure
Mammalian paxillin contains four LIM domains, five LD motifs, two highly phosphorylated tyrosine motifs, and a proline rich domain.

1.2.2 Regulation

As an adaptor protein, paxillin's activity is regulated by its localization and its ability to interact with its binding partners. Both of these properties are modulated by phosphorylation [11, 12, 22]. Paxillin phosphorylation, to a high

degree, occurs during various cellular events associated with cell adhesion, remodeling of the actin-based cytoskeleton, and growth control [13, 22-24]. Specifically, tyrosine phosphorylation has been shown to be involved in the formation of focal adhesions, the organization of the actin cytoskeleton, and morphology [17, 25]. Likewise, the phosphorylation of serine/threonine residues regulates the localization of paxillin to focal adhesion sites [13, 21], the stability of paxillin, membrane protrusion dynamics, cell migration, and cell spreading [26, 27]. In contrast, during cell mitosis, which is associated with an absence of adhesive complexes, paxillin serine phosphorylation is decreased [28]. These sites of serine/threonine phosphorylation are largely conserved in paxillin orthologues such as *Dictyostelium discoideum* PaxB.

1.2.3 Mammalian function and protein interaction

The proline rich domain and tyrosine phosphorylation (YXXP) motifs are believed to mediate paxillin's role in signal transduction. Paxillin has been shown to bind the SH3 domains of Src and Crk family members, most likely through the proline rich domain which contains three putative SH3 binding sites [17, 29]. In addition to its proline rich domain, paxillin can also bind Crk through its YXXP motifs [14]. The paxillin LD4 motif is responsible for regulating cell spreading and motility through an interaction with PKL, paxillin kinase linker [11]. West and colleagues showed that paxillin interacts with the ARF-GAP of paxillin kinase linker (PKL), a critical event in the regulation of Rac-dependent changes in the actin cytoskeleton that accompany cell spreading and motility [11].

Numerous studies suggest that paxillin is involved in the transduction of signals from growth factor receptors to focal adhesions [10, 29, 30]. Paxillin can be tyrosine phosphorylated during various cellular events associated with cell adhesion, remodeling of the actin based cytoskeleton, and growth control [17]. Paxillin can be tyrosine phosphorylated by growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) as well as in response to several small peptide growth factors [14, 30]. Paxillin is also tyrosine phosphorylated in response to integrin-mediated cell adhesion and during embryonic development [2, 10, 29]. The interaction of paxillin with numerous proteins lead to changes in the organization of the actin cytoskeleton that are necessary for cell motility events associated with embryonic development, wound repair, and tumor metastasis [10, 12]. It is speculated that these proteins probably use paxillin both as a substrate and as a scaffold protein to disrupt and even bypass the normal adhesion and growth factor signaling cascades necessary for controlled cell proliferation [10].

1.2.4 The role of paxillin in cancer

Paxillin is involved with numerous types of cancer. A critical importance for paxillin was confirmed upon the discovery that paxillin was a substrate for the hematopoietic oncogene *p210 BCR/ABL* [31]. Specifically, it was found that tyrosine and serine phosphorylation of paxillin is remarkably and constitutively increased in myeloid cell lines transformed by *BCR/ABL* [32, 33]. In addition, it's

prominently tyrosine phosphorylated in Rous sarcoma virus transformed fibroblasts [34]. Paxillin has also been shown to directly associate with the NF2 tumor suppressor (Merin/Schwannomin) and may mediate pathogenesis of neurofibromatosis type 2 [35]. A role for paxillin in cells transformed by papilloma virus associated with cervical cancer is proposed to be mediated in part through an association of paxillin with the E6 protein [21, 36]. Paxillin has also been proposed to play a role in tumorigenesis and invasion through its association with integrin and growth factor receptor ligation [37-39]. In addition, it has been proposed that paxillin is transcriptionally upregulated during breast cancer treatment with heregulin in aggressive breast cancer lines and grade III human breast tumors [40], while being decreased in metastatic breast cancer [41, 42]. Similarly, in lung cancer a decrease in paxillin expression and tyrosine phosphorylation has been correlated with the onset of metastatic cancer [43, 44]. More recently, the association of paxillin has been linked to various cancers such as prostate cancer [45], bladder cancer [46], leukaemia PLB-985 cells, and small cell lung cancer [47]. These findings indicate paxillin is intimately employed in various types of cancer, and depict its importance for human health.

1.2.5 Paxillin in the dark

Paxillin is an essential protein for normal development [48] and for the metastatic acquisition of numerous types of cancer [41-44]. However, its function and regulation have not been completely characterized. Being that paxillin is highly conserved between species, we studied its ortholog, PaxB in the simple model

organism *D. discoideum* to further elucidate the role and regulation of paxillin. *D. discoideum* contains 33% amino acid identity and 46% homology with mammalian paxillin [20, 49].

1.2.6 *Dictyostelium* paxillin (PaxB)

Many of the processes that paxillin is involved in, such as cell motility and morphogenesis, are important in the life cycle of *Dictyostelium discoideum*. The genome contains a single *D. discoideum paxB* gene that is highly homologous to mammalian paxillin. Unlike paxillin, PaxB contains only four of the five LD motifs found at the amino terminus (lacking paxillin LD4 region) (Fig. 2). Yet, all four LIM domains on the carboxy terminus are present, and a putative proline rich domain containing a possible SH3 binding site between the LD1 and LD2 motifs exist (Fig. 2) [20]. However, very little is known about its role in growth and development, or its binding partners. Due to their high homology, gaining an understanding of the function and regulation of PaxB, we will lend insight into the role of paxillin in mammalian development and disease.

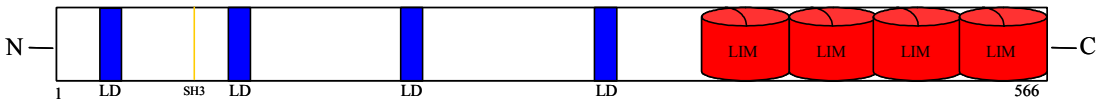


Figure 2. *Dyctiostelium* PaxB is highly homologous to paxillin
There is 33% amino acid identity and 46% homology between human paxillin and *Dictyostelium discoideum* paxillin, PaxB. *D. discoideum* PaxB contains four LIM domains, four LD motifs, and a putative proline rich domain.

1.3 *Dictyostelium discoideum*

*D*ictyostelium *discoideum* is a simple eukaryote with a well defined life cycle that displays many of the features of animal embryogenesis such as regulated cell-cell cohesion, cell substrate adhesion, cell motility, and morphogenesis [50-52]. It

feeds on bacteria and grows as single amoeboid cells, but multicellular development is triggered subsequently by starvation. The developmental process is divided into two phases. Through multiple stages of development, cells aggregate to form a multicellular fruiting body composed of differentiated cells. These processes involve regulated cell-cell and cell-substrate adhesions, motility, differentiation, and morphogenesis [8, 53]. Similar to paxillin, PaxB has been shown to play a role in cell-substrate adhesion, cell sorting and migration during *D*. *discoideum* development [49]. These characteristics and similarities to paxillin make D. discoideum a perfect candidate to study adhesion.

1.3.1 A model organism

The social amoeba *Dictyostelium discoideum* is one of the simplest eukaryotes able to form multicellular structures [54, 55]. It is a model organism extensively studied worldwide for the past 70 years [56]. Commonly, it's used as a model for developmental studies in cellular and developmental biology at a level of complexity greater than yeast, but simpler than plants and animals. *D. discoideum* cells possess signal transduction pathways employed by higher eukaryotes, and undergo a relatively simple program of multicellular development, which in many ways resembles animal development. Unlike embryos arising from a fertilized egg, where multicellularity is the outcome of repeated cell division, in *D*. *discoideum* the multicellular organism, called a slug and later on a fruiting body, arises from the gathering of thousands of individual cells into a multicellular agglomerate [56].

A major advantage of *D. discoideum* as an experimental system is that its molecular genetics are highly developed: the cells are easily transformed by integrating and non-integrating vectors, several resistance and auxotrophic markers exist, and gene disruption is favored by the small, haploid genome. Single or multiple knockout mutants generated by homologous recombination have been widely used to characterize the function and developmental role of gene products [57]. This approach has been complemented by the development of restriction enzyme mediated insertional (REMI) mutagenesis to generate new mutants and recover the tagged gene by plasmid rescue [58]. *D. discoideum* cells are easy to grow in liquid nutrients, with cell numbers duplicating every 8-10 hours. Cell development is initiated by removal of nutrients and is normally completed within 24 hours. Since *D. discoideum* is eminently suited for cell biology and biochemistry experiments, the introduction of these molecular genetic tools has made available a unique combination of approaches to investigate this system.

1.3.2 Life cycle

Growth and development are temporally separated and mutually exclusive in *Dictyostelium discoideum*. During growth, known as the vegetative stage, *D. discoideum* cells behave as free-living amoebae, which feed on bacteria by phagocytosis. The cells are barely cohesive, actively move on the substratum in search of food, and readily divide by binary fission [56]. When their food source is depleted, *D. discoideum* undergo a switch in behavior and begin a survival

mechanism that culminates in the formation of a multicellular organism (Fig. 3) [59].

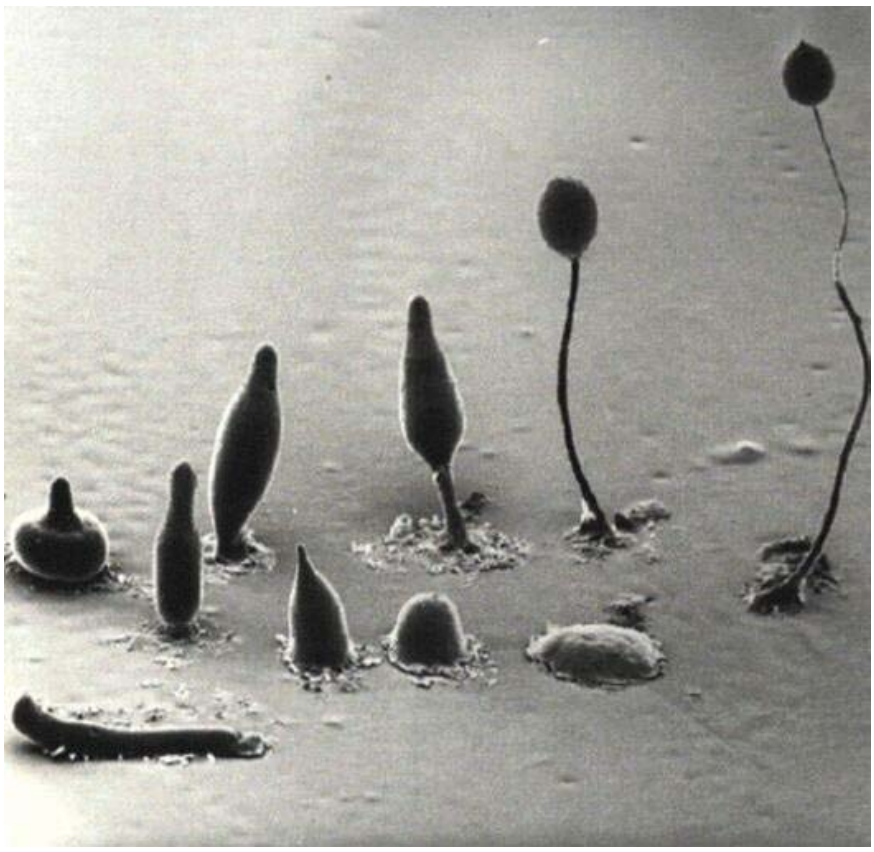


Figure 3. *Dictyostelium discoideum* developmental stages
In their vegetative stage, *D. discoideum* cells exist as single amoeba that feed on bacteria. When their food source is depleted, they undergo a switch in behavior to form a fruiting body. This is a differentiated multicellular structure composed of spores and stalk cells. Moving clockwise from lower right, the stages are loose aggregate, the tight aggregate (mound), the tipped mound, the finger, a slug (lower left), and the culmination stages from the Mexican hat stage to the final fruiting body. This scanning electron microscope is by R. Lawrence Blanton and Mark Grimson at Texas Tech. University.

The developmental process is divided into two phases, an aggregation phase and a post-aggregation phase, each lasting a period of approximately 12 hours. Four to five hours of starvation triggers the developmental process to produce and

respond to chemoattractants, and the synthesis of adhesion molecules for cell-cell cohesion and cell-substrate adhesion [60, 61]. In the pre-aggregation phase the concerted action of chemotactic cell motility and intercellular adhesion transform a monolayer of single cells into streams (Fig. 4) that give rise to multicellular three-dimensional aggregates.

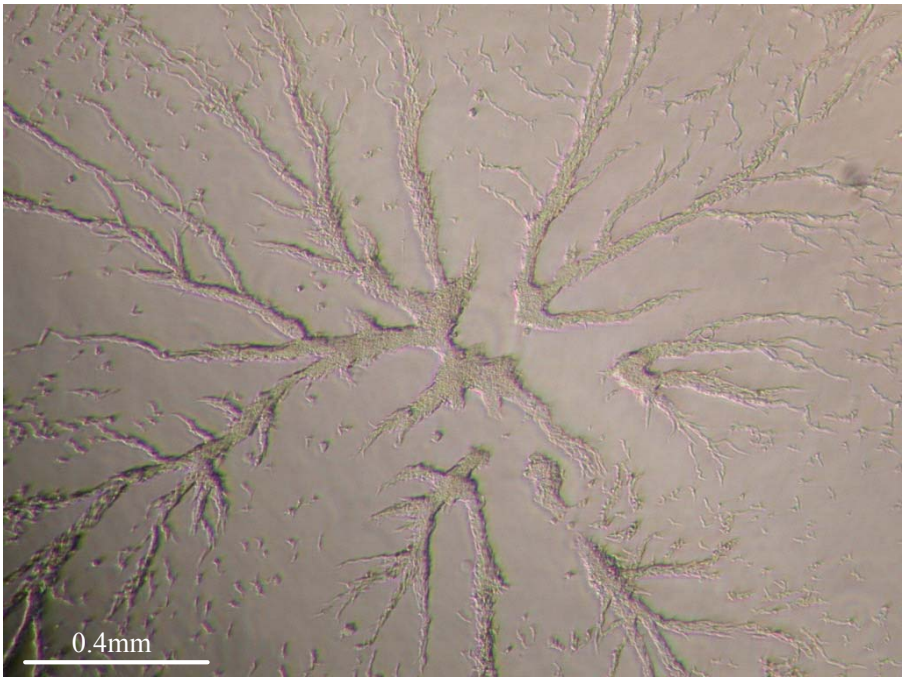


Figure 4. Streams of *Dictyostelium discoideum* starved cells
Representative photograph of wild-type cells undergoing streaming. After 5 hours of starvation, *D. discoideum* cells chemotax towards c-AMP and form streams composed of approximately 10^5 cells. These cells will eventually form aggregates.

This greatly simplifies the study of development and provides a great route to examine cell-cell adhesion through studies of aggregation and cell surface binding. In the post-aggregation phase, expression of cell-cell adhesion

molecules establishes cell-cell contacts and loose mound structures comprised of homogeneous cells [8, 53]. Cell differentiation then produces a prestalk and prespore population, after which, cell sorting takes place. It has been suggested that cell differentiation and sorting occur simultaneously, and that this is coupled to cell adhesion and motility [62-64]. During cell sorting, shortly after 12 hours of development, a dynamic movement of differentiated cells leads to the formation of tipped mounds, which extend into finger-like structures. Each finger gives rise to a slug, a sausage-shaped unitary organism capable of undergoing extended migration towards light and temperature gradients [65-68]. A slug possesses an anterior-posterior pattern of prestalk and prespore cells, as well as a dominant anterior tip which acts as an organizer for cell migration [60, 69]. Elaborate polarized cell movements occur inside the cell mass during the slug phase in order to reach the culmination phase, after which the fruiting body is generated [60, 70]. The fruiting body consists of a mass of spores (approximately 80% of the cells) supported by a stalk of vacuolated cells (approximately 20% of the cells), which are in turn anchored to the substratum by a basal disc [60, 71]. The spores can remain dormant for long periods of time but are able to germinate when food is available.

1.4 *Dictyostelium* adhesion

Dictyostelium growth phase cells are barely cohesive. When incubated on a solid substratum, they form transient contacts only, whereas in shaken suspension they form small, loose aggregates, which are easily dissociated with 10 mM EDTA or

EGTA [72, 73]. The class of adhesion molecule responsible for this form of adhesion was named contact site B (csB) [74]. A 24 kDa glycoprotein, with some homologies to cadherins, has been identified as csB [75, 76].

Aggregating cells differ from growth-phase cells by their stronger cohesion, which leads to formation of large EDTA-resistant aggregates when shaken [51, 77]. This is due to the expression of a class of adhesion molecules called contact site A (csA). csA is a glycoprotein of 80 kDa, which undergoes homophilic interactions [77]. The csA glycoprotein is expressed on the cell surface three hours after the onset of starvation and reaches maximal expression two hours later at the onset of aggregation [77]. Disruption of the single csA encoding gene generates null mutants which fail to form EDTA-resistant aggregates at the aggregation stage. The mutant cells are, however, able to undergo aggregation and complete development when incubated on agar [78]. If tipped aggregates or slugs formed by the mutant are dissociated, and the cells are incubated in shaking culture in the presence of EDTA, EDTA-resistant aggregates are formed, which suggests the existence of a third EDTA-resistant class of adhesion molecules, different from csA and active after the tip stage [67].

The molecular basis of the post-aggregative adhesion system is less well understood. The aggregates formed at the tipped mound or slug stage show a much more compact, tissue-like, structure than aggregates from the aggregation stage. In addition, prestalk and prespore cell populations within the slug display

different degrees of adhesiveness [52]. It is not known whether these differences between prestalk and prespore cells result from the expression of new cell type specific, adhesion molecules or from quantitative differences in adhesion molecules common to both cell types. A glycoprotein of 150 kDa is involved in post-aggregative cell-cell adhesion [79]. The glycoprotein could be identical with the product of the *lagC* gene, which encodes a 95 kDa polypeptide containing a putative transmembrane domain [80]. *lagC*-null cells are blocked at the loose mound stage and can be rescued when mixed with wild-type cells in a proportion that is compatible with LagC being a cell adhesion molecule [81]. If so, LagC could be a component of a heterophilic adhesion molecule, since constitutive expression of the protein in growth phase cells failed to induce cell clumping [81].

Modulation of cell adhesion by anti-adhesive molecules is also an important component of *D. discoideum* development. The anti-adhesive molecule AmpA modulates adhesions during development, and its inactivation causes increased cell-cell and cell-substrate adhesion that delays or arrests development [82]. Axenically grown *ampA* null cells progress normally through development to the mound stage, and then are delayed by four hours in the process of forming the tip on the mound. Bacterially grown *ampA* null cells are more adherent to the substratum, which delays their migration into mounds, and are completely arrested at the mound stage [82, 83]. The migration of cells is important at the mound stage for cell sorting of differentiated populations [56].

1.5 PaxB

Today, there are no reports indicating true focal adhesion sites in *D. discoideum*. It has been shown that PaxB is required for proper cell-substrate adhesion in *D. discoideum*, and PaxB-GFP localized in small stationary spots located at the interface of the cells with the substrate [49]. Interestingly, actin also localizes in stationary spots at the cell substrate interface and has been shown to colocalize with PaxB [49]. These structures were formed at the leading extending edge of the cells and stayed present as long as the cells were attached to the substrate [49]. These structures are suggested as possible focal adhesion sites. This observation is also indicative of possible focal adhesion sites being formed in *D. discoideum*.

As a model organism *D. discoideum* proves to be ideal for the study of adhesion. Here we add to our understanding of the paxillin orthologue in *D. discoideum*, PaxB, by showing changes in its phosphorylation in response to adhesion. In addition, we find that overexpression of *paxB* affects adhesion by increasing cell-cell cohesion, but decreasing cell-substrate adhesion. Finally, we demonstrate a role for PaxB in differentiation and morphogenesis by showing that overexpression of *paxB* blocks development past the mound stage and prevents differentiation of cells into spores.

1.6 Significance

Although its development differs in many respects from that of higher eukaryotes, *Dictyostelium discoideum* provides many opportunities to study cell processes which are common to mammalian development. Thus during the growth phase, the molecular basis of phagocytosis and cytokinesis can be analyzed [65, 84, 85]. Both morphologically and molecularly, these processes are basically similar in *D. discoideum* and in animal cells.

The aggregation stage offers the best possibility for dissecting chemotaxis and intercellular adhesion, two processes which play a key role in the development of higher organisms. Chemotaxis, which in *D. discoideum* development is mediated by cyclic AMP, involves both producing and sensing the chemoattractant as well as transducing the membrane signal to the actin cytoskeleton in order to stimulate oriented cell motility. Adhesion molecules also play a role in the propagation of motility. A wealth of biochemical and molecular genetic studies has defined the properties of motility and adhesion processes [65-67, 86]. Similarly, several actin-binding proteins have been characterized and their role in the organization of the actin cytoskeleton during chemotaxis, or other motility events, investigated [87].

The process of aggregation also requires the expression of new classes of cell adhesion molecules, which are responsible for the compaction of aggregates [67]. Both the cadherin-like glycoprotein csB [75, 88] and the IgG superfamily protein

csA have been shown to play a role in aggregation [77, 89]. After aggregation, a third adhesion system containing LagC, is involved in early structure formation [79, 81]. However, another adhesion system during final morphogenensis remains to be elucidated in *D. discoideum*. Post-aggregation stages, and late pattern formation, as well as cell type differentiation can be conveniently studied in this organism and give insight into animal embryogenesis.

Given its feasibility to switch from a unicellular amoeba into a multicellular organism, *D. discoideum* proves to be ideal for the study of adhesion. Differential exposure to gradients formed by extracellular diffusible substances, differential adhesiveness, and stochastic differences among cells, which depend on their past history from the end of growth, determine the cell fate [90, 91]. These events lead to the expression of cell-type specific genes to form the multicellular organism [90, 91]. The expression of adhesion molecules in *D. discoideum* greatly determines its development and morphology. We investigated the function and regulation of the adhesion protein PaxB in *D. discoideum*, to further elucidate its mammalian orthologue paxillin.

CHAPTER 2

Materials and Methods

2.1 Cell culture

Dictyostelium discoideum wild-type cells were grown in axenic HL5 medium (0.5% (w,v) yeast extract, 0.5% protese pedptone, 0.5% thiotone peptone, 1% dextrose, 4.7 mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH 6.5) on a 180 rpm shaking platform at 22^0C [92]. Strains used for the experiments, grown in axenic HL5 medium, consist of the wild-type Ax2 cells Ax2, HR30 (Ax2 expressing β-galactosidase) with 10 μg/ml blasticidin, Tet-7 (Ax2 containing the endogenous MB35 vector) with 20 μg/ml G418, and the mutant PaxBOE (*paxB* overexpressing cells) with10 μg/ml blasticidin and 20 μg/ml G418. The MB38 vector containing the *paxB* gene was transformed into Tet-7 cells by electroporation as described previously [93, 94]. Transformed cells were selected on GYP plates containing 20 μg/ml G418 and 10 μg/ml blasticidin in association with a G418 resistant strain of *Escherichia coli*. Clones were grown on HL5 medium containing 20 μg/ml G418 and 10 μg/ml blasticidin and analyzed for expression of the desired product. Wild-type cells were also grown on SM/5 agar plates with *Klebsiella aerogenes*. HR30, Tet-7 and PaxBOE cells were also grown on GYP agar plates with the blasticidin and G418 resistant strain of *Escherichia coli*.

For development, cells at mid-log phase (2-5 x 10^6 cells/ml) were washed with PBM (20 mM KH_2PO_4, 10 μM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1 with KOH), plated on filter pads at 1 x 10^7 cells/pad and incubated at 22^0C.

2.2 Construction of PaxBOE plasmid

The PCR amplification of *paxB* was prepared using a proof-reading enzyme (Roche Expand High Fidelity PCR System, Indianapolis, IN), using genomic DNA as a template and the annealing temperature following manufacture's protocol. The 5' PCR primer begins with the restriction enzyme SphI, followed by the in-frame *paxB* insert sequence (5'GCGCATGCATGGCAACAAAAGGATTAAATATG). The 3' primer begins with the restriction enzyme AatII, followed by the end of the triple codon, to maintain the reading frame, and a stop codon (3'GCGACGTCTTAAGCGAATAATTTATTATGACAA). The PCR product, as well as the MB38 vector, was digested with SphI and AatII. The digested products were separated by one percent agarose gel electrophoresis. The bands of interest were gel extracted and purified with UltraClean DNA Purification Kit (MO BIO Bedford, MA). Ligation of the *paxB* gene into the MB38 vector was done in a 1:4 molar ratio using T4 DNA Ligase (BioLabs, Beverly, MA), overnight at 15^0C. The ligated product was transformed into TOP 10F' *E. coli* cells. Positive transformants were tested by PCR using the primers for the *paxB* gene and further corroborated by restriction digest with SphI and AatII. The

MB38-*paxB* construct was transformed into Tet-7 cells and positive transformants confirmed by RT PCR.

2.3 Construction of PaxB RNAi plasmid

The RNAi construct was created by PCR amplification of two middle portions of the *paxB* gene, selected from the RNAi primer design program in Just-bio.com, using genomic DNA. The 5' PCR primer (PaxB L), begins with the restriction enzyme Bgl2, followed by the in-frame *paxB* insert sequence (5'GAAGATCTTCAAATTCACCACCACCATCAT). The first 3' primer (PaxB SR), to amplify a 600 bp fragment, begins with the restriction enzyme EcoRI, followed by the in-frame *paxB* insert sequence, and a stop codon (3'CGGAATTCCGACAACCACCACATGTACC). The second 3' primer (PaxB LR), to amplify a 900 bp fragment, begins with the restriction enzyme EcoRI, followed by the in-frame *paxB* insert sequence, and a stop codon (3'CGGAATTCCGTATGGACGACCATCACGTTC). Both of these fragments were amplified from the same starting location on the *paxB* gene with the same forward primer, but terminate at different locations through the use of different reverse primers. Therefore, the first 600 nucleotide of the 900 bp fragment are complementary to the 600bp fragment. The two fragments were ligated in an opposite orientation, and transformed into the RNR-P vector containing the BsR cassette (Blasticidin) as the selectable marker (RNR-PaxB). The construct was transformed into Ax2 cells, and upon transcription, the dsRNA and stem loop formation occur, followed by RNA interference.

2.4 PCR-Ready *Dictyostelium* genomic DNA: whole cell PCR

To test positive *paxB*-RNAi transformed cells we created the 5' PCR primer starting 1100 bp upstream of the paxB-RNAi constructs on the RNR-BsR vector (RNR-PaxB) 5'TCTAGAATCGGAGTGGTACC, and the 3' PCR primer utilizing the first twenty bp of the inversed PaxBLR primer sequence (PaxBLRinverse) 3'CTTGCACTACCAGCAGGTAT. Electroporated cells allowed to grow for 5 days were harvested from 24 well-plates to test for positive transformants. 200 µl of cells in HL5 media were centrifuged at 5000 rpm for 5 minutes, and the pellet resuspended in 25 µl of TETP (TE containing 0.3% Tween and one mg/ml Protease K(Qiagen protease Maryland, NJ)). The cells were incubated for 45 minutes at 56^0C, then 15 minutes at 95^0C. Two µl were used as template for PCR amplification.

2.5 Northern Blot and Western Blot analyses

For Northern blots, RNA was prepared using Trizol Reagent (GIBCO BRL, Carlsbad, CA), according to manufacturer's protocol. Samples containing 10 µg of RNA were separated on one percent agarose gel (0.6% formaldehyde, 10mM MOPS, pH 7.5), and blotted to Hybond-N+ (Amersham Bioscience, Piscataway, NJ). A probe specific for the endogenous *paxB* gene was labeled with ^{32}P-dATP (Perkin Elmer, Boston, MA), according to manufacturer's protocol (Invitrogen DNA Labeling Kit, Carlsbad, CA), and hybridization was performed as described by Engler-Blum et al., [95]. For Western blots, proteins were separated by 10 % SDS-PAGE, electrophoretically transferred to Hybond-P membranes (Amersham

Bioscience, Piscataway, NJ), immunoblotted with a peptide purified α–PaxB antibody, and visualized by enhanced chemiluminescent substrate for HRP detection (PIERCE, Rockford, IL).

2.6 Chimeras and β-galactosidase staining

Chimeras were created and stained as previously described by Jermy and Williams [96] with some adjustments. 1.0×10^7 cells were collected from HR30 strain and PaxBOE strain. Chimeras consisted of 20 percent HR30 cells and 80 percent PaxBOE cells. For β-galactosidase staining, cells were developed on white filter pads, 0.8um pore size (Millipore, Billerica, MA). Developed chimeras were stained for early culminant and fruiting body structures. Cells were fixed with glutaraldehyde solution (25% glutaraldehyde, 4% Triton X-100 in Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$)), followed by staining with X-gal solution (5 mM $K_3[Fe(CN)_6]$, 0.4 mg/ml X-gal, and 0.5% Tween 20 in Z Buffer), and incubated at 37^0C overnight. Pictures were taken with a dissecting microscope utilizing SPOT Advanced program with SPOT insight color camara, (Diagnostics Instruments, USA).

2.7 Cell-cell cohesion assay

The cell-cell cohesion assay was done as previously described by Secko et al., [72] with some adjustments. Exponentially grown cells were harvested and washed three times with PBM buffer (20 mM KH_2PO_4, 10 μM $CaCl_2$, 1 mM $MgCl_2$, [pH 6.1] with KOH) and resuspended to 2×10^7 cells/ml. Two milliliters

were transferred into a plastic 50 ml centrifuge tube (Corning 430828), and shaken horizontally (175 rpm) at 22^0C for four hours. 2.5×10^6 cells were collected, vigorously vortexed for 15 seconds to disperse aggregates, and placed on a platform shaker (160 rpm) at room temperature to allow aggregates to reform. Cells were collected at specific times and single and duplex cells were counted using a hemacytometer. To visualize aggregation, approximately 2.5×10^4 cells were incubated in PBM in tissue culture dishes under gentle agitation (60 rpm) for three hours. Cell agglomerates were viewed using a Nikon Eclipse TS 100 inverted microscope and photographed with a Nikon E 995 camera.

2.8 α-phospho-serine immunoprecipitation of PaxB

Logarithmic growing cells were collected, washed twice with PBM, and resuspended in PBM for cell-cell cohesion or HL-5 media for substrate adhesion. For cell-cell cohesion, 1.0×10^7 cells were resuspended in PBM and agitated at 175 rpm in a rotatory shaker for four hours. The cells were vortexed to disrupt cell clumping and allowed to readhere. Samples were collected at zero and 60 minutes and washed in PBM. For substrate adhesion, 2×10^6 cells were placed in suspension or tissue culture plates in the presence or absence of 10 mM EDTA for two hours. Samples were collected and resuspended in 500 µl of IP Buffer (20 mM HEPES, 150 mM $NaCl_2$, 1.5 mM $MgCl_2$, 1 mM EGTA in NaOH, 10% glycerol, 2 mM Na orthovanadate, 50 mM NaF, 10 mM Na pyrophosphate, 1% Triton X 100, 1X protease inhibitor complete tablets (Roche, Germany)). Cells were lysed via a 5 µm nylon filter (Sterlytech, WA) and incubated at 95^0C for 5

minutes. Lysates were incubated overnight at 4^0C with either 1 µg mouse α-phospho-tyrosine monoclonal antibody clone 4G10 (Upstate Cell Signaling Solutions, MA) or 1 µg mouse α-phosphoserine monoclonal antibody clone PSR-45 (Sigma, MO) and 50 µl protein A sepharose beads (GE Healthcare Bio-Sciences AB, Sweden). The immunoprecipitates were washed three times with IP Buffer and resuspended in 50 µl IP Buffer plus 10 µl 6X protein sample buffer. Protein samples were boiled for 3 minutes, separated by 7% SDS PAGE, and transferred onto Hybond-P membrane (Amersham Bioscience, Piscataway, NJ). Immunoblot analysis was done using an α-PaxB peptide purified antibody as previously described in the text.

2.9 Cell-substrate adhesion assay

The cell-substrate adhesion assay was done as previously described by Chen and Kat [97] with some adjustments. Exponentially grown cells were harvested and washed three times with PBM buffer and resuspended to 1.0×10^6 cells/ml in the same buffer. Four milliliters of this suspension was incubated in 50 ml glass cell culture flasks on a gyratory shaker at 120 rpm for 10 minutes at room temperature. The cells were incubated 2 hours further without shaking to allow them to adhere. The flasks were then agitated gently for 3 minutes at 60 rpm and the supernatants were transferred to a test tube. Non-adherent cells in each supernatant were determined using a hemacytometer.

2.10 Spore ratio and spore viability assays

The spore ratio was assayed with chimeras consisting of 20 percent HR30 cells and 80 percent PaxBOE cells or Ax2 cells as previously described, [98] with some adjustments. Chimeras were allowed to develop for 36 hours, followed by collection of spore mass using one milliliter PBM buffer. Spores were stained with X-Gal solution, vigorously vortexed, and incubated at 37^0C for 24 hours. Spores were washed three times using Z Buffer and the number of blue colored spores (HR30) and uncolored spores (PaxBOE or Ax2) were counted using a hemacytometer. The spore viability was assayed as previously described by Dynes et al. (1994) [81] and Bishop et al. (2002) [98]. The cells were plated on filter pads at 1.0×10^7 cells/ml and allowed to develop for 36 hours. The spores were collected with one milliliter PBM buffer and detergent (10 mM EDTA, 0.1 % Nonidet P-40), and serially diluted directly into *Klebsiella aerogenes* suspension and plated on SM/5 agar plates. The number of plaques formed was counted and scored as viable spores.

2.11 F-actin staining by Alexa Fluor 488-conjugated rhodamine phalloidin

For F-actin staining, vegetative and starved cells from axenic cultures were deposited onto glass slides for 30 minutes, fixed in 3.7 percent formaldehyde solution for 10 minutes, permeabilized in 0.2 percent Triton X-100 solution for 5 minutes, and incubated in 0.1 mM RNase A at 37^0C for one hour. Cells were then stained for F-actin with Alexa Fluor 488-conjuated Rhodamine phalloidin (Molecular Probes, Eugene, OR) solution for one hour and TO-PRO nuclei stain

642/661 (Molecular Probes, Eugene, OR) for 45 minutes. F-actin staining was viewed using a Leica microscope and software system.

2.12 Cell motility assay

Cell motility was assayed as previously described [99] with some adjustments. Vegetative and starved cells were seeded at low density, (~2 $\times 10^4$ cells/cm2), on plastic dishes in HL5 media or PBM buffer, and allowed to adhere for one hour. A time lapse movie was compiled by capturing an image of the cells every minute for 30 minutes using an inverted Nikon TE 200 Eclipse microscope using a Metafluor Image System viewed through a 40X objective. The individual amoeba tracks were traced and the distance traveled for 30 minutes was measured using the Image J software.

CHAPTER 3

Expression pattern and localization of PaxB

3.1 Expression of *paxB* during development

The pattern of expression of many genes in *Dictyostelium discoideum* is greatly correlated to its developmental function. Genes that are expressed at a specific developmental stage may directly play a role in the structural formation occurring at that point. In order to confirm the developmental regulation of PaxB, we performed Northern blot and Western blot analyses. A northern blot of total RNA from developing wild-type cells was probed with a fragment of *paxB*. *paxB* mRNA is expressed throughout all stages of development, but the strongest expression peaks at 8 and 12 hours (Fig. 5A). IG-7 is constitutively expressed in *D. discoideum* and its transcript levels were measured to ensure equal loading (Fig. 5B). Western blot analysis using a peptide purified α-PaxB antibody showed a similar pattern of protein production (Fig. 5C). PaxB is produced at lower levels in vegetative cells, but expression then rises dramatically by 8 to 12 hours, which correspond to the mound and tipped mound developmental stages. The protein starts to decrease by 16 hours, the slug stage, and declines gradually until 24 hours, the culmination and fruiting body formation. The expression pattern of *paxB* suggests the protein is highly produced during the formation of mound structures, which are developmental stages where cell-cell contact, cell substrate adhesion, and orchestrated cell motility for the formation of culminants occurs.

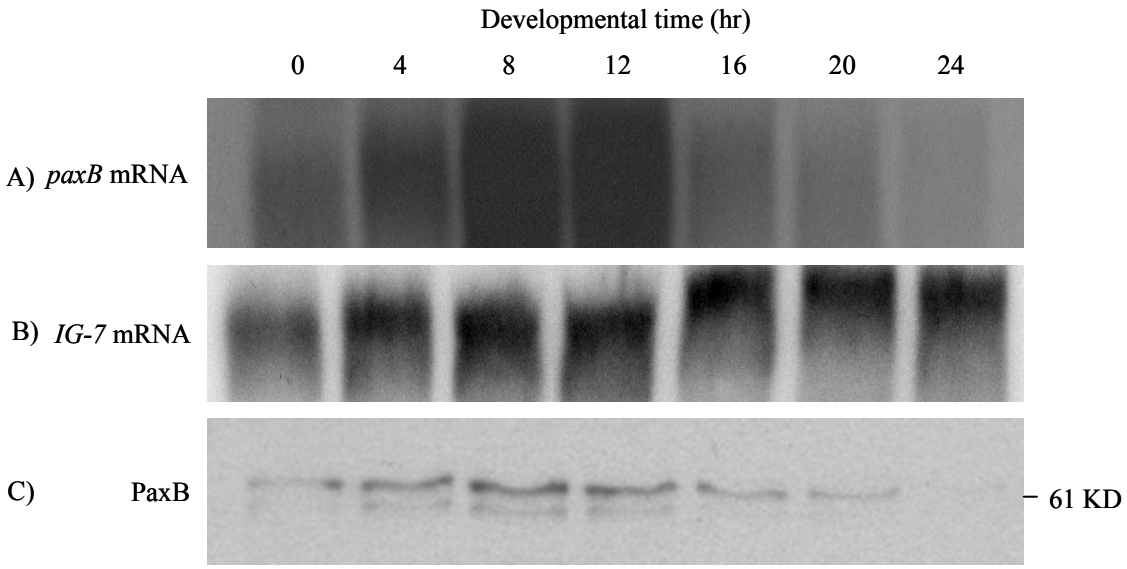


Figure 5. Northern blot and Western blot of lysates from developing wild-type cells. Hybond-N^+ and Hybond-P^+ membranes were probed with a ^{32}P-*paxB*-labeled probe and an α-PaxB peptide purified antibody, for Northern blot (A) and Western blot (C), respectively. To ensure 10 μg equal loading, *IG-7* mRNA (B) was measured and protein quantities were monitored by Coomassie Blue staining of another gel run simultaneously with the same samples. Protein size was measured by protein marker run in the same gel.

3.2 Overexpression of *paxB* using the Tet-off system

To better understand the function of *paxB* we created an overexpressor system using the tet-off system [100]. The tetracycline-controlled inducible expression system was first established in mammalian cells [101]. The system was then established in *D. discoideum* cells [100]. The overexpression is composed of a system that employs two vectors. The first vector consists of a genomically integrated, negatively regulated tetracycline-controlled transcriptional activator (tTA) under the actin15 promoter harbored in the MB35 vector, which contains a

G418 resistance cassette [100]. The second vector consists of an extrachromosomal response plasmid, MB38, containing a Blasticidin resistance cassette, and a minimal promoter (Pmin) governed by a tetracycline responsive element (TRE). The promoter and TRE regulate the expression of the gene of interest [100]. The *paxB* gene was inserted into the MB38 vector. When Tetracycline (Tet) is absent tTA binds to the TRE and activates the minimal promoter, initiating the overexpression of the *paxB* gene. In the presence of Tet, tTA binds it and undergoes a conformational change that prevents tTA from binding to the TRE, and thus the promoter is inactive [100].

To confirm that the Tet-off system works for the overexpression of *paxB,* we measured the transcript level in wild-type cells and three overexpression strains of *paxB*, PaxBOE 3, 5, and 10. There is no significant difference in *paxB* transcript levels of wild-type cells in the presence and absence of Tet (Fig. 6A). Therefore, addition of Tet does not affect transcript expression from the endogenous promoter. All three uninduced strains of PaxBOE cells show slightly higher transcript levels than the wild-type cells(Fig. 6A). However, when induced by the removal of Tet, the mRNA expression of overexpressors 3, 5, and10 is much higher than in the wild-type cells with an approximate increase of 5, 3, and 2 fold, respectively (Fig. 5A). To ensure equal loading, *IG-7* transcript levels were measured (Fig. 6B). Thus, we have successfully created three overexpressor constructs of the *paxB* gene that can be regulated with Tet using the Tet-off system.

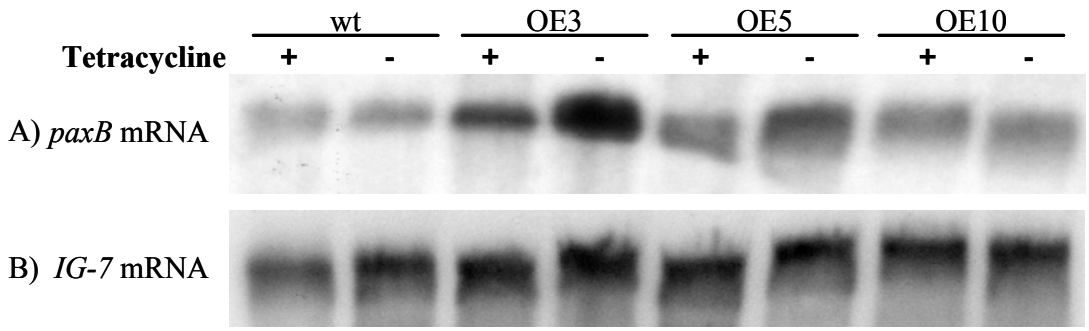


Figure 6. RNA overexpression of the *paxB* gene
Three strains of *paxB* overexpression were created using the Tet-off system. Total mRNA in the absence and presence of tetracycline, extracted from vegetative cells was probed with a 900 bp fragment of the endogenous *paxB* gene (A). *IG7* gene (B) probed with a ^{32}P-*IG-7*-labelled probe were measured as control for equal loading.

3.3 PaxB is overproduced in OE3 cells

The above results showed that the *paxB* overexpressing cells from strain number three (PaxBOE3) expressed the highest transcript levels. To confirm that the increased transcript levels parallels the overproduction of PaxB we measured the transcript level and protein production in wild-type and PaxBOE3 cells. There is no significant difference in the transcript levels of wild-type cells in the absence and presence of Tet as we had seen previously (Fig. 7A). As previously observed under uninduced conditons, PaxBOE3 cells show slightly higher transcript levels than in the wild-type cells, however, when Tet is removed and the gene is induced, the transcript level is increased (Fig. 7A). To ensure equal loading of RNA, *IG-7* transcript levels were measured (Fig. 7B). To equate the increased transcript level with increased production of the protein, we performed western blots using an α-PaxB peptide purified antibody. In the wild-type cells equal production of PaxB is observed in the absence and presence of Tet (Fig. 7C).

PaxBOE3 cells in the presence of Tet show slightly higher protein production than in the wild-type cells. However, in the absence of Tet this expression is 6 fold higher than in the wild-type cells (Fig. 7C). Thus the increased transcript level of PaxB correlates with an increased production of the protein. This confirms that we successfully created a Tetracycline regulated system for the overproduction of PaxB.

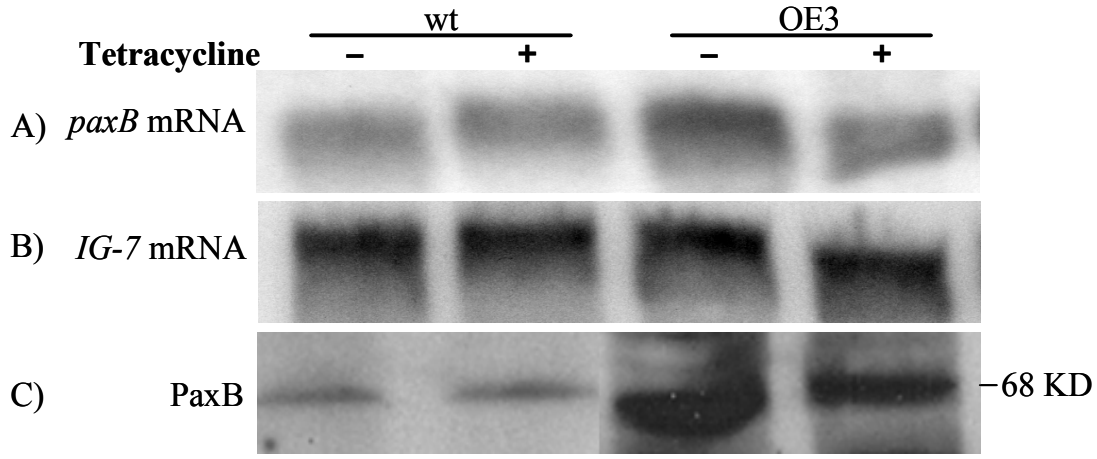


Figure 7. Overexpression of the *paxB* gene and protein overproduction
Northern blot and Western blot analysis of PaxB in wild-type and PaxBOE3 overexpressing cells. Total mRNA in the absence and presence of tetracycline, extracted from vegetative cells was probed with (A) a 900 bp fragment of the endogenous *paxB* gene, and with (B) *IG-7* gene as a control. (C) Western blot analysis of the PaxB protein extracted from vegetative cells, and resolved by SDS-PAGE was probed with α–PaxB, a purified peptide antibody.

3.4 *paxB* RNA interference

We wanted to create a system that would grant us the ability to regulate transcription at a specific developmental times. To accomplish this we created a

RNAi construct of PaxB. The *paxB*-RNAi construct was successfully created utilizing complementary arms of the PaxB gene (Fig. 8). The RNR-P vector containing a ribonucleotide reductase promoter (rnrp) that is activated through short exposures of ultra violet light [102] was used to create the RNA interference construct. A Blasticidin resistance cassette was inserted into the RNR-P vector through restriction digest (RNR-BsR). The *paxB*-RNAi construct was also inserted into the RNR-BsR vector through restriction digest. Ax2 wild-type cells were transformed with the RNR-BsR *paxB*-RNAi construct through electroporation. Upon activation of the rnr promoter, transcription of the *paxB*-RNAi construct will be activated, and down regulation of the protein occurs through RNA interference.

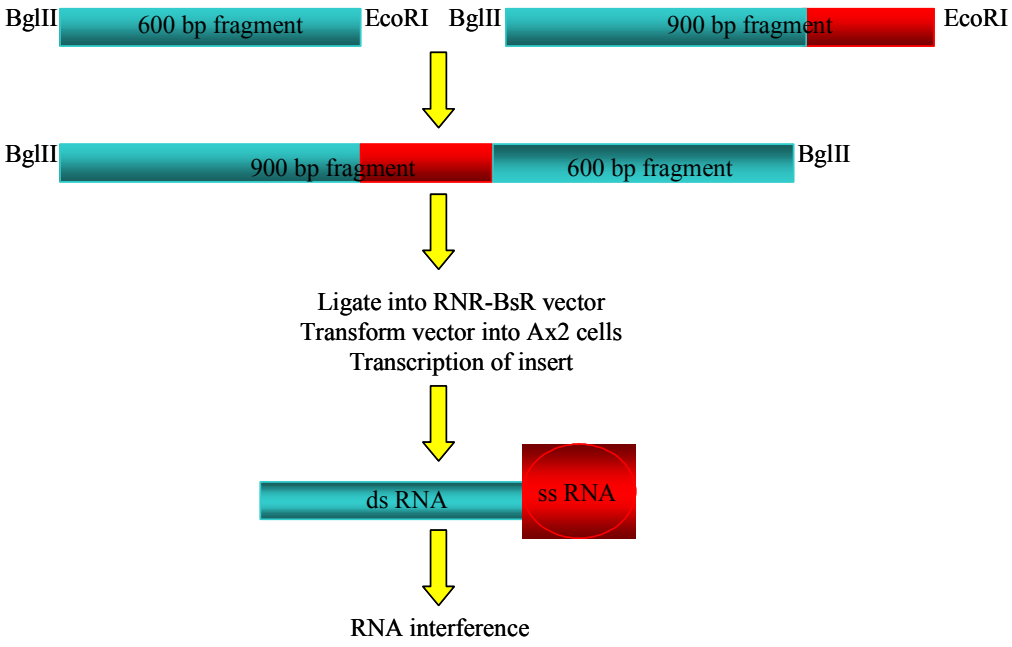


Figure 8. *paxB*- RNA interference construct
The *paxB*-RNAi construct was designed utilizing flanking arms of a 600 bp and a 900 bp fragments as described in materials and methods. The construct was inserted into the RNR-BsR vector containing a ribonucleotide reductase promoter.

Positive transformants were tested through whole cell PCR amplification as described in materials and methods. Denatured transformed cells and the RNR-BsR*paxB*-RNAi maxiprep were used as template for PCR amplification. The PCR products were separated by agarose gel electrophoresis, and a DNA marker was used as reference (Fig. 9). The positive transformants were plated on SM/5 agar plates containing a *Klebsiella aerogenes* (Ka) bacterial lawn, which resulted in no plaques. For unknown reasons, the transformants were not able to continue proliferation beyond two weeks after transformation, nor were they able to form plaques on bacterial lawn. When the cells on the 24 well plates were re-tested

through whole cell PCR, the positive transformants were no longer present. This suggests that something within the paxB-RNAi construct caused the cells to die.

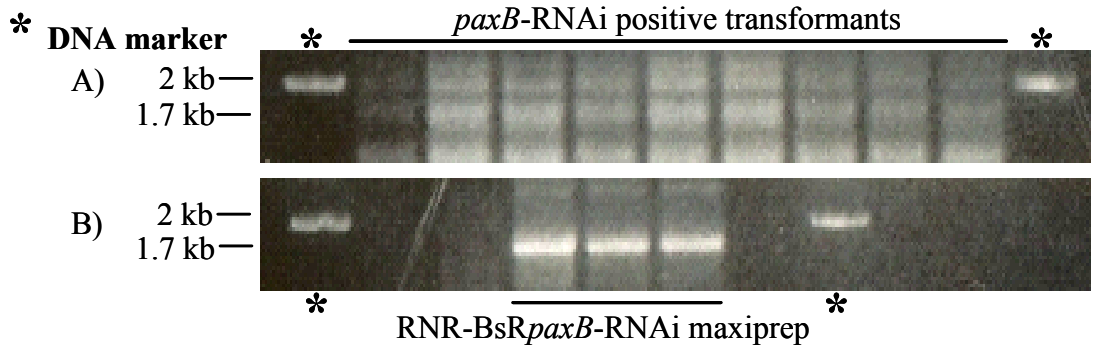


Figure 9. PCR amplification of positive *paxB*-RNAi transformants
The *paxB*-RNAi transformants were tested through PCR amplification of the 1.7 kb RNAi fragment. A 2 kb DNA marker is shown with an asterisk (*) on the agarose gel electrophoresis. (A) Amplification of the positive transformants, (B) amplification of the 1.7 kb RNR-BsR*paxB*-RNAi construct maxiprep utilizing different concentrations.

3.5 Discussion

The developmental time of expression of a protein in *Dictyostelium discoideum* can be directly correlated to its function. *paxB* RNA is expressed during all stages of *D. discoideum* development. The production of PaxB parallels its expression with the highest levels observed at 8 and 12 hours of development. These developmental times pertain to the mound and slug stages, respectively. Moreover, the localization of PaxB takes place at the interface of the cells with the substrate, and at sites of cell-cell cohesion [49]. The localizations of PaxB is indicative of its function, since the adhesion of cells to other cells and the underlying substrates are part of the developmental requirements for the formation of the mound and slug structures, as well as aid in motility during slug migration [3, 52, 67].

We have successfully created three overexpressor constructs of the *paxB* gene that can be regulated with Tetracycline using the Tet-off system. *paxB* mRNA overexpression was observed in all three overexpression strains (PaxBOE 3, 5 and 10). The above results showed that *paxB* overexpressing cells pertaining to strain number three (PaxBOE3) contained the highest mRNA expression. PaxBOE3 mRNA overexpression parallels the overproduction of the protein. We utilize this strain in consecutive experiments to investigate the role and regulation of PaxB. Also, we created an RNAi construct of the *paxB* gene that is regulated through small exposures of ultra violet light, with the intention of regulating expression of the gene at specific developmental stages. Unfortunately, the viability of

positively transformed cells appears to be hindered with the design of the RNAi construct, since cells failed to continue proliferation after two weeks of being transformed. Further attempt with this system, coupled with adjustments in growth environments of the transformed cells, might generate a viable population carrying the *paxB*-RNAi construct. Both of these systems are suitable to study the function and regulation of PaxB in *D. discoideum*.

CHAPTER 4

Developmental localization and activity of PaxBOE cells

The integration of single-celled amoebae into a multicellular organism is a major part of the developmental program of *D. discoideum*. Thus, a protein capable of modulating cell-cell adhesion is a candidate for influencing developmental progression and morphogenesis. Therefore, we examined the effect of overexpressing *paxB* on development.

4.1 Overexpression of the *paxB* gene arrests development at the mound stage

In order to observe the phenotypic effects of overexpression of *paxB*, we developed cells on filter pads and agar. We plated vegetative wild-type cells and PaxBOE3 cells to observe developmental progression. The initial stages of development are normal in the PaxBOE3 cells in that they reach the mound stage at the same time as wild-type cells, approximately 12 hours into development. However, there is a drastic difference after this point. Under uninduced and induced conditions, wild-type cells form fruiting bodies by 24 hours (Fig. 10A, 10B). In contrast, PaxBOE3 cells formed early culminants under uninduced conditions (Fig. 10C), while remaining arrested at the mound stage under induced conditions (Fig. 10D). These arrested phenotypes are able to be rescued under non-induced and induced conditions by addition of 20 percent wild-type cells, suggesting that the defect may be non-cell autonomous (Fig. 10E, 10F). Addition of 5% wild-type cells did not rescue the phenotype (data not shown), indicating a

stronger signal is required for development to proceed. This suggests normal production of PaxB is required for development past the mound stage and that wild-type cells can provide something which will allow PaxBOE3 cells to progress to full development.

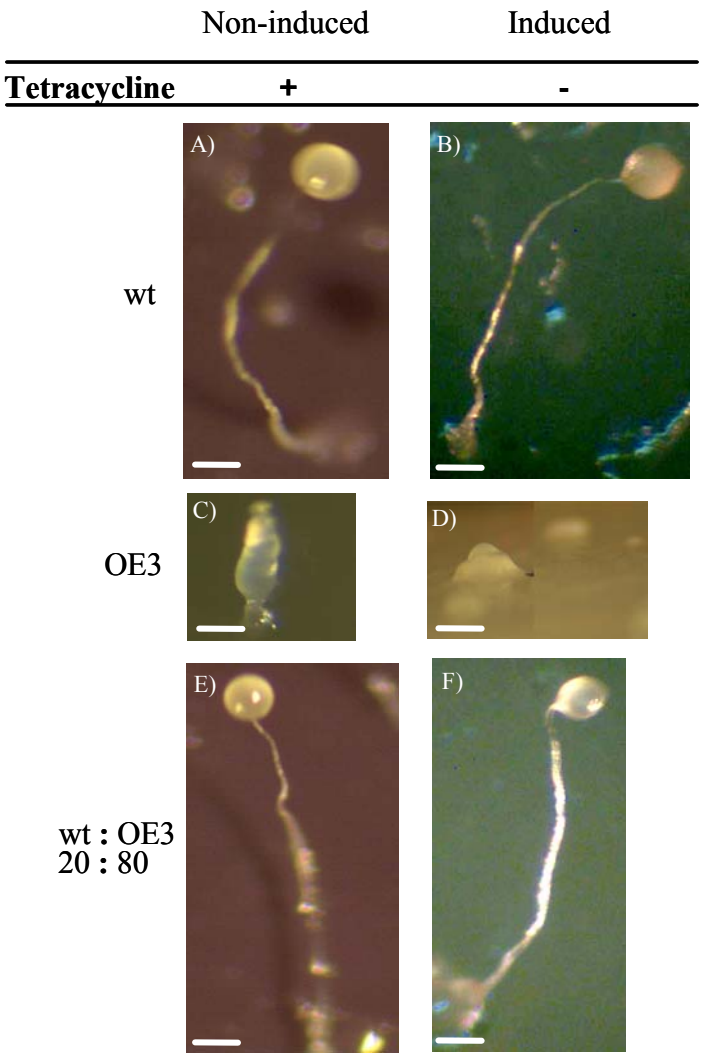


Figure 10. Development of wild-type and PaxBOE3 cells
Logarithmically growing cells were washed free of growth medium and plated for development on non-nutrient agar for 24 hours. Wild-type cells in the presence (A) and absence of Tet (B). PaxBOE3 cells in the presence (C) and absence (D) of Tet. Chimeras containing 20% wild-type and 80% PaxBOE3 cells in the presence (E) and absence (F) of Tet. Bars 0.5 mm.

4.2 Mound tip in chimeras is composed of wild-type cells

The initial homogenous cell population starts to differentiate in the mound generating prestalk cells and prespore cells [60, 69]. The prestalk cells sort out to form a tip that functions as an organizer, directing further development into slugs and fruiting bodies [64, 103]. After observing the rescued developmental phenotype of the PaxBOE3 cells with addition of wild-type cells, we investigated the localization of the wild-type cells in tipped mounds chimeras using a β-galactosidase expressing strain. As a control, we examined the localization of wild-type cells that express β-galactosidase in chimeras with the wild-type parental cell line. Chimeras with wild-type cells showed random distribution of the wild-type *β*-galactosidase expressing cells in the tipped mounds (Fig. 11A), thus expression of β-galactosidase have no effect on cell fate. However, in chimeras containing the *paxB* overexpressing cells, the wild-type *β*-galactosidase expressing cells sorted to make the tip of the mound (Fig. 11B). This suggests that PaxB plays a role in cell sorting or differentiation.

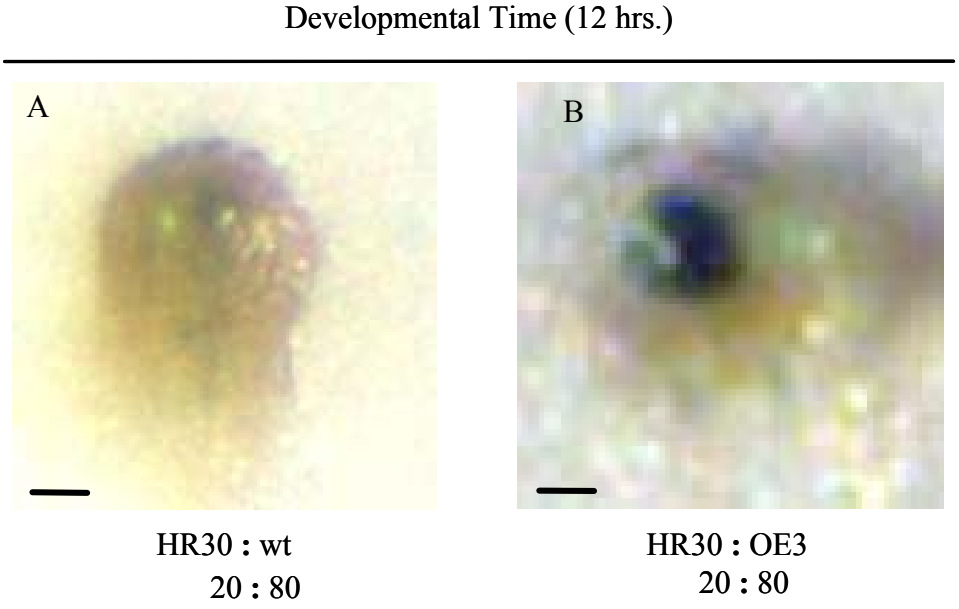


Figure 11. Localization of wild-type and PaxBOE3 cells in tipped mound chimeras
Wild-type cells carrying the actin15:lacZ reporter construct mixed in a 20:80 ratio with wild-type cells (A) or PaxBOE3 cells (B), were developed on filters. Structures were stained at 12 hours of development as described in materials and methods. Bars 1.5 mm

4.3 *paxB* overexpressing cells localize to the upper and lower cups of fruiting bodies in chimera structures

After observing that PaxBOE3 overexpressing cells were predominantly excluded from the tip of the mounds, we investigated the localization of the wild-type β-galactosidase expressing cells in chimeric culminants and fruiting bodies. As a control, we examined the localization of wild-type cells that express β-galactosidase in chimeras with the wild-type parental cell line. All chimeras were observed under non-induced and induced conditions. Chimeras with wild-type cells showed random distribution of the wild-type *β*-galactosidase expressing cells

in culminant and fruiting body under non-induced (Fig. 12A, 12C) and induced conditions (Fig. 12B, 12D), thus expression of β-galactosidase and the presence of Tet have no effect on cell fate. In chimeric culminants and fruiting bodies under non-induced conditions, some random distribution of both cell types is observed (Fig. 12E, 12G). However, in chimeric culminants and fruiting bodies under induced conditions, the wild-type *β*-galactosidase expressing cells were predominantly localized to the anterior of the culminant (Fig. 12F) and the upper and middle section of the spore mass (Fig. 12H). Therefore, the overexpression of the *paxB* gene appears to interfere with cell sorting or differentiation in the various developmental structures of *D. discoideum*. Specifically, PaxBOE3 cells preferentially sort out to the upper and lower cup of the spore mass.

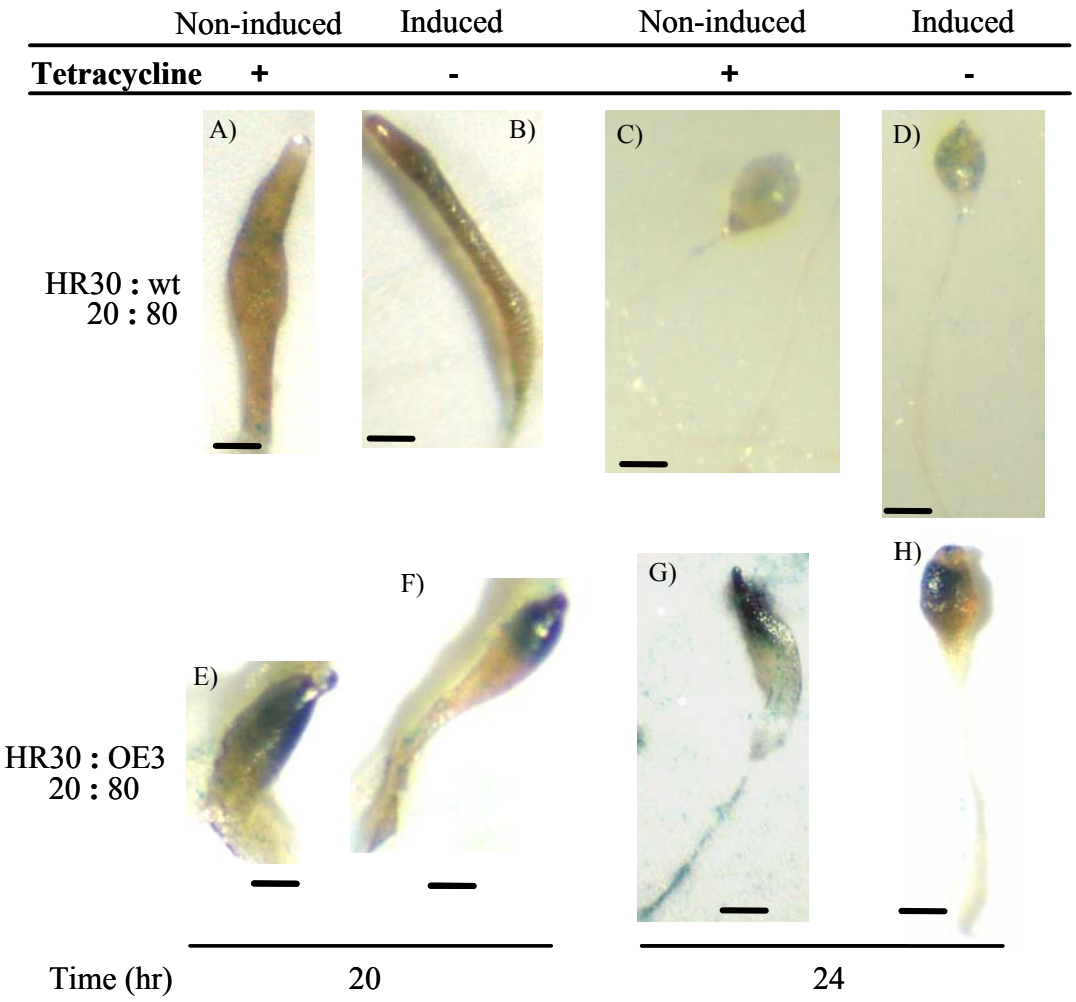


Figure 12. Localization of wild-type and PaxBOE3 cells in chimeras
Wild-type cells carrying the actin15:lacZ reporter construct mixed in a 20:80 ratio with wild-type cells or PaxBOE3 cells were developed on filters in the presence and absence of Tet. Culminants were stained at 20 hours of development for the wild-type and overexpressing cells in the presence (A), (E) and absence (B), (F) of Tet, respectively. Fruiting bodies were stained at 24 hours of development for the wild-type and overexpressing cells in the presence (C), (D) and absence (G), (H) of Tet, respectively as described in materials and methods. Bars 0.5 mm

4.4 Overexpression of the *paxB* gene inhibits differentiation into spores

After observing that PaxBOE3 cells predominantly sorted out from the spore mass of the fruiting body, we investigated whether PaxBOE3 cells were deficient

in spore formation. To test this, we created chimeras of wild-type *β*-galactosidase expressing cells with wild-type cells or PaxBOE3 cells. We then counted the number of blue and nude spores using a hemacytometer. In wild-type chimeras, approximately 40 percent of the spores came from non- *β*-galactosidase expressing wild-type cells (Fig. 13). However, in PaxBOE chimeras, the percentage of spores coming from PaxBOE cells was decreased to approximately 23 percent (Fig. 13). This suggests that *paxB* plays a role in cell-type differentiation. Specifically, overexpression of *paxB* interferes with the ability of cells to differentiate into spores.

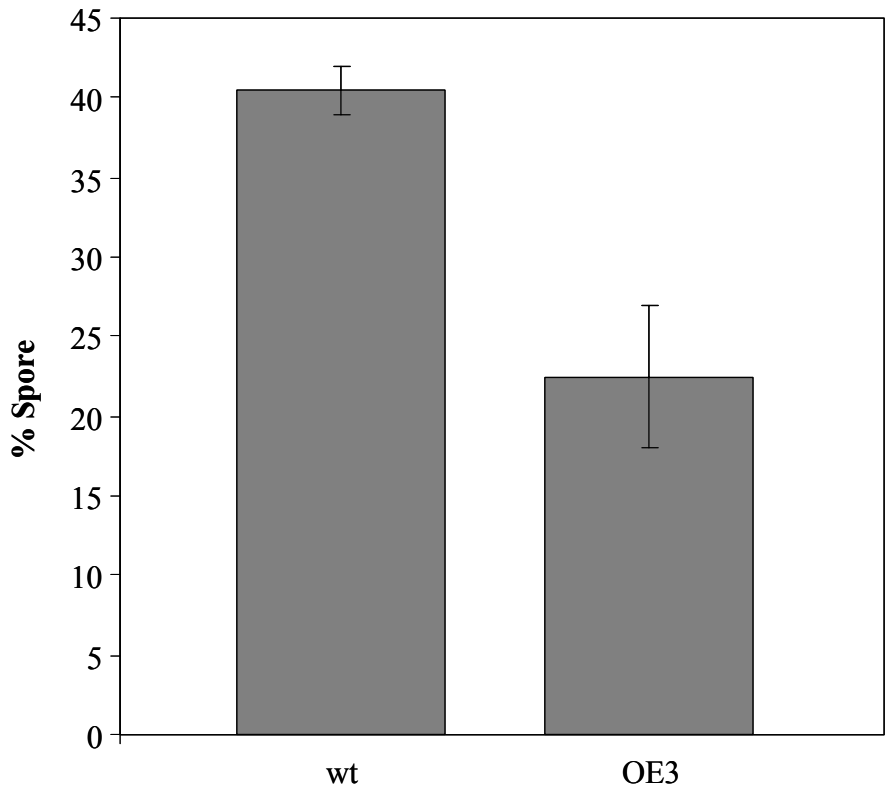


Figure 13. Spore ratio analysis of wild-type and PaxBOE3 cells in chimeras

Wild-type cells carrying the actin15:lacZ reporter construct mixed in a 50:50 ratio with wild-type cells or PaxBOE cells were developed for 36 hours on filters. Spores were collected and counted as described in materials and method. The percentage of spores developing from wild-type and PaxBOE cells was calculated and plotted. Values represent the mean of four independent experiments ± SEM.

4.5 Spore viability decreases in PaxBOE cells

Given that PaxB appears to be involved in spore differentiation, we wanted to test whether PaxBOE3 cells could produce viable spores. Wild-type and PaxBOE cells were developed for 36 hours. The cells were then heated in a detergent solution to kill non-spores and surviving spores were plated on bacteria and allowed to form plaques. Table 1 illustrates that while wild-type and PaxBOE3

cells produce equivalent number of viable cells, more than 10^6 viable spores germinated from the wild-type cells, versus only 10^2 viable spores from PaxBOE3 cells. This suggests that the *paxB* gene plays a role in the development of viable spores.

Cell line	Viable cells ($\times 10^4$)	Viable spores ($\times 10^4$)
wt	775	349
OE3	509	0.042

Table 1. Spore viability of wild-type and PaxBOE3 cells
1×10^7 wild-type and PaxBOE3 cells were starved on filters and allowed to developed for 36 hours. The developed structures were collected and assayed for cell and spore viability. Data shown are from one experiment with similar results obtained from three other experiments.

4.6 Discussion

The *paxB* gene is expressed throughout the different developmental stages of *D. discoideum*, but peaks during mound and slug stages [49]. This is consistent with its role in cell-cell cohesion, as cell-cell interactions are necessary for mound formation and subsequent slug formation. Overexpression of the *paxB* gene arrests development at the mound stage, indicating that normal expression of *paxB* is required for development past the mound stage. This is consistent with the findings that *paxB*$^-$ mutant cells also arrest at the mound stage [49]. According to Bukahrova (2005), this too could be due to adhesion defects, as PaxB may play a role in cell movement, particularly in the cells that form the tip of the mound (prestalk cells), a structure that controls the movement of all other cells in development [49, 64]. In addition, there is a greater expression of *paxB* in prestalk cells than prespore cells [49]. This problem is resolved in chimeric mounds. We found that the tips of the chimeric mounds are composed of wild-type cells. This suggests that the reason why chimeras can form fruiting bodies is because the wild-type cells are able to move to the tip of the mound and possibly create the signals for further development. This would explain the non-cell autonomous defect in the PaxBOE3 cells. Therefore, while PaxBOE3 cells cannot create a tip and signal further differentiation, they can still respond to those signals.

The results from the experiments with chimeras also lend insight into the role of PaxB in the correct sorting of cells in the fruiting body, particularly in the spore

mass. In chimeras of wild-type and overexpressing cells, PaxBOE3 cells predominantly sorted to the upper and lower cup of the spore mass, which are derived from prestalk cells. Bukahrova and colleagues (2005) found that PaxB is more strongly expressed in prestalk cells than in prespore cells [49], which can further explain the localization of the overexpressing cells to the cups of the fruiting bodies. Given that we are overexpressing *paxB* in these cells, they will be more inclined to localize to these regions. It is known that prestalk and prespore cell-types differentiate in a spatially independent manner throughout the mound [69, 70, 104], and their complete sorting is reached by the culmination of the tipped mound. Therefore, cell movement needs to occur in order for cells to sort. Given that cell movement depends on regulated cell-cell adhesion and cell-substrate adhesion, it is not surprising that PaxB influences differentiation as it is involved in these two processes. The overexpressing cells cannot sort out correctly, therefore, they cannot differentiate properly. In this light, the effect of PaxB on spore viability is most likely a result of the cells not differentiating into spores, rather than *paxB* playing a direct role in spore viability.

Given that the expression of *paxB* peaks during mound formation, it is not surprising that overexpression of the gene arrests development at this stage. Addition of wild-type cells rescued the phenotype, presumably by providing the signals necessary to continue development since in chimeric mounds the tip (organizer) was composed of predominantly wild-type cells. These results depict the importance of PaxB in *D. discoideum* development as a very important

adhesion molecule. It is important to mention that just like PaxB, mammalian paxillin knockout arrests development due to the inability of cell migration. These data enhance our notion that PaxB and paxillin behave similarly in their developmental function.

CHAPTER 5

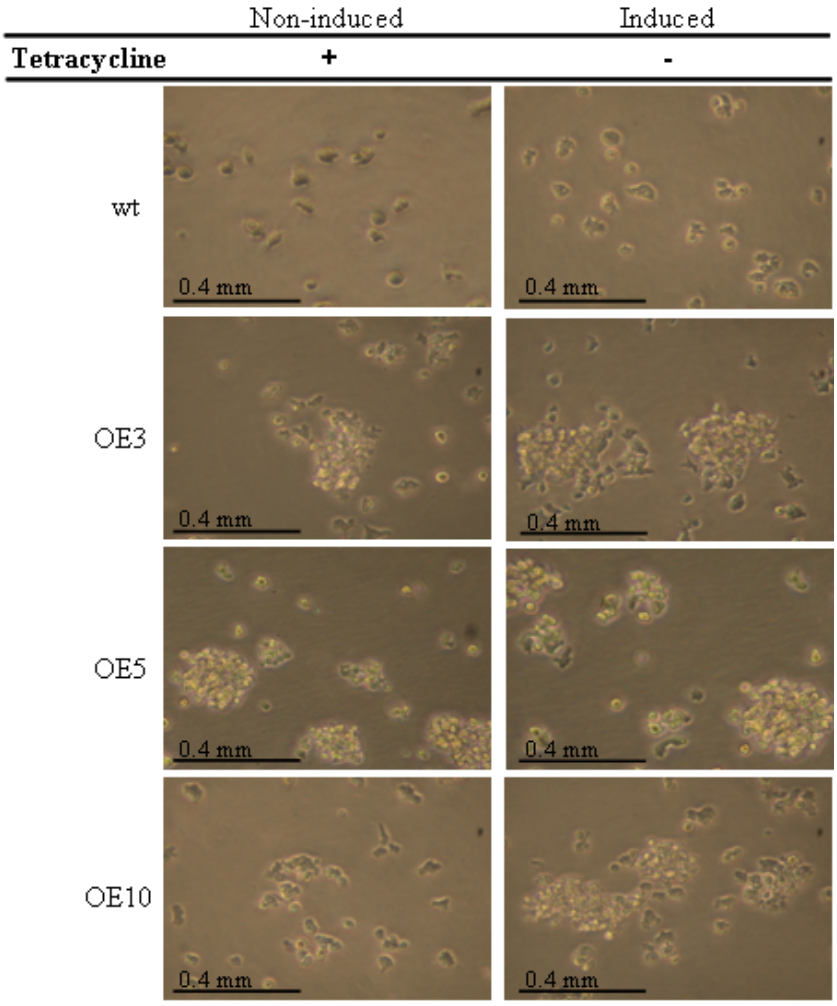
PaxB is a positive regulator of adhesion

5.1 Cell-cell cohesion increases in PaxBOE cells

Given that paxillin is an adhesion molecule, we examined whether overexpression of *paxB* affects cell-cell cohesion. It has been shown that when cells are washed free of growth medium, resuspended in non-nutrient buffer, and allowed to develop in suspension, cell agglomerates are formed [72]. Wild-type cells formed a few small agglomerates but, for the most part, remained as single cells after three hours of starvation, both in the absence and presence of Tet (Fig. 14A). This suggests that Tet does not interfere with the normal cell cohesion activity of the cell. In contrast, very few single cells of PaxBOE 3, 5, and 10 strains were present under induced conditions in the absence of Tet, with cell agglomerates of approximately 0.4mm (Fig. 14A). Under uninduced conditions with addition of Tet, only cell agglomerates from PaxBOE10 overexpressing cells were reduced in size, similar to that of the wild-type cells. Under the same conditions, the cell agglomerates of PaxBOE 3 and 5 strains remained relative the same. It is worth mentioning that PaxBOE10 cells had the lowest level of overexpression. This suggests that overexpression of *paxB* increased the cohesion of cells in all three overexpression strains, and addition of Tet can only decrease cohesion in PaxBOE10 overexpressing cells.

To quantify cell-cell cohesion, cell agglomerates were disrupted and allowed to readhere under gentle agitation. We counted the number of non-cohered single and duplex cells in the wild-type and the three overexpressor strains, at specific time points, and subtracted this from the total number of cells to calculate how fast cell-cell cohesion occurred. Quantification of cell-cell cohesion in presence of Tet shows parallel results to that observed in the cell agglomerates, with approximately 15 percent and 30 percent cohesion by five minutes and close to 45 percent cohesion by 60 minutes for the wild-type and PaxBOE10 cells, respectively (Fig. 14B). However, PaxBOE 3 and 5 strains agglomerated faster and to a greater extent than wild-type cells in the presence of Tet. Approximately 55 percent of PaxBOE 3 and 5 strains agglomerated by 5 minutes, while close to 85 percent by 60 minutes, versus 15 percent and 50 percent for the wild-type cells, respectively (Fig. 14B). Quantification of cell-cell cohesion in the absence of Tet shows that all three *paxB* overexpression stains, PaxBOE 3, 5, and 10, agglomerate faster and to a greater extent than the wild-type cells. Approximately 55 percent of PaxBOe 3 and 5 cells and 30 percent of PaxBOE10 cells agglomerated by 5 minutes, while close to 85 percent of PaxBOE 3 and 5 cells and 75 percent of PaxBOE10 cells by 60 minutes, versus 5 percent and 50 percent for the wild-type cells, respectively (Fig. 14C). This suggests that *paxB* plays a positive role in cell-cell cohesion.

A)



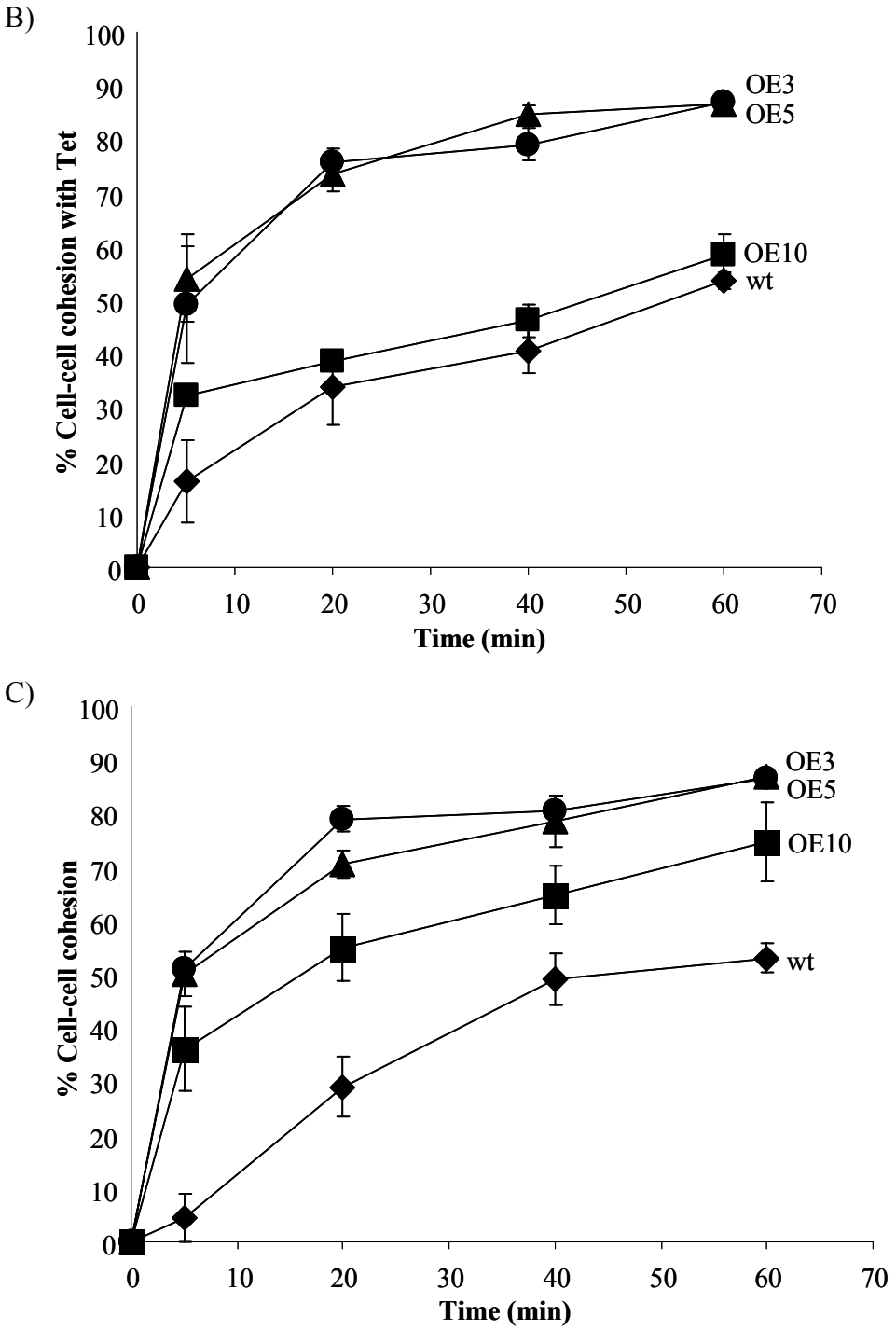


Figure 14. Cell-cell cohesion is increased in PaxBOE cells
(A) Representative photographs of wild-type and PaxBOE 3, 5, and 10 overexpressing cells agglutinants that formed after 3 hours of starvation in suspension. Scale bars, 0.4 mm. Wild-type and PaxBOE 3, 5, and 10 cells in the presence (B) and absence (C) of Tet, starved for four hours and vortexed, were allowed to readhere. Values represent the mean of three independent experiments ± SEM.

5.2 PaxB-mediated cell-cell cohesion is Ca^{2+} dependent

Given that *D. discoideum* cells display Ca^{2+} dependent and independent adhesion, we wanted to determine whether PaxB mediated cell-cell cohesion is calcium dependent. Therefore, we examined cohesion in the presence of 10 mM EDTA. As previously illustrated, PaxBOE3 cells agglomerate faster and to a greater extent than wild-type cells (Fig. 14). Under induced conditions in the absence of Tet, more than 50 percent of the overexpressing cells agglomerated by 5 minutes and over 80 percent by 60 minutes, versus 5 percent and 50 percent for the wild-type cells, respectively (Fig. 15). As expected, there is decreased cell-cell cohesion in the presence of 10 mM EDTA in wild-type cells (Fig. 15). Interestingly, there is decreased cell-cell cohesion in the PaxBOE3 cells in the presence of EDTA. It is important to note that the increase in cell-cell cohesion caused by overexpressing *paxB* is completely negated by EDTA. This suggests that the cohesion of cells mediated by PaxB is calcium dependent.

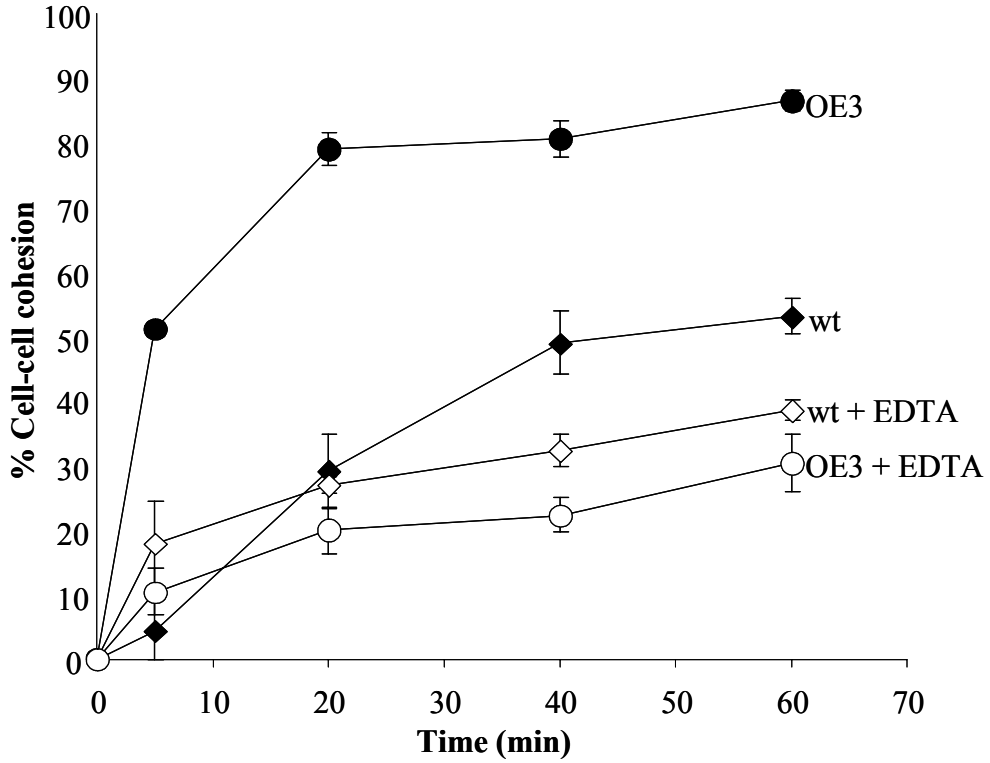


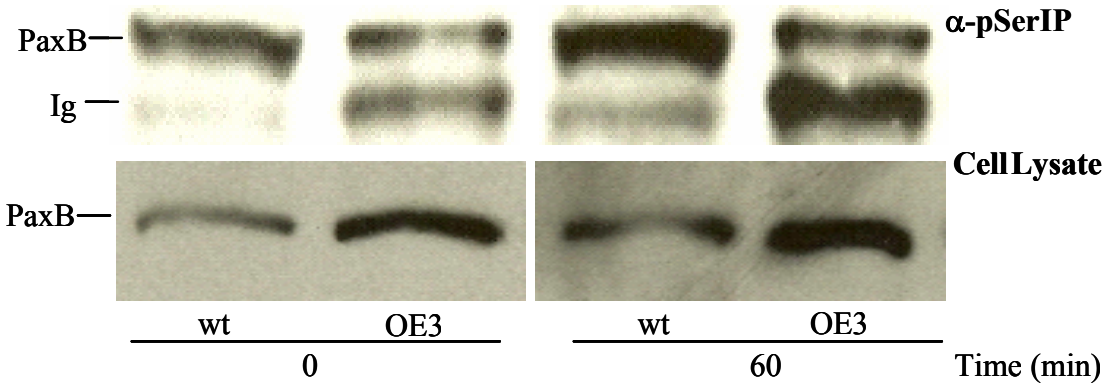
Figure 15. PaxB mediated cell-cell cohesion is calcium dependent
Wild-type and PaxBOE3 cells were starved for four hours and vortexed, and then allowed to readhere in the absence or presence of 10 mM EDTA. Values represent the mean of three independent experiments ± SEM.

5.3 Cell-cell cohesion does not involve changes in PaxB serine phosphorylation

In mammalian cells, a complex series of phosphorylation and dephosphorylation events controls paxillin mediated adhesion [21]. Paxillin has been shown to be heavily phosphorylated on tyrosine residues in focal adhesions [29]. In addition, the phosphorylation of serine/threonine residues in paxillin LIM2/3 domains has been implicated in regulating the localization of paxillin to focal adhesion sites

[13, 21]. To investigate potential methods of regulating PaxB-mediated cohesion, we examined whether PaxB undergoes phosphorylation. To test whether PaxB undergoes serine phosphorylation, cell lysates were collected, immunoprecipitated with α-phospho-serine antibodies, and immunoblotted with α-PaxB antibody. We found that PaxB undergoes serine phosphorylation (Fig. 16A). To test the phosphorylation state of PaxB during cell-cell cohesion we measured the serine phosphorylation levels of cohered and non-cohered cells in wild-type and PaxBOE3 cells. Immunoblot analysis of PaxB serine phosphorylation was done as above with cohered and non-cohered wild-type and PaxBOE3 cells (Fig. 16A). The relative amount of phosphorylation was quantified by comparing phosphorylated PaxB with total PaxB in the lysate. The change in phosphorylation during cohesion was determined by measuring the ratio of phosphorylated PaxB at t= 60 (cohered cells) to t= 0 (non-cohered cells). Thus, a value of 1 means no change in phosphorylation, while a value >1 or <1 means increased or decreased phosphorylation, respectively. In wild-type cells, PaxB serine phosphorylation did not change significantly during cell-cell cohesion (Fig. 16B). Interestingly, PaxBOE3 cells had decreased PaxB serine phosphorylation (Fig. 16B). Taken together, these results suggest that cell-cell cohesion is not regulated by changes in PaxB serine phosphorylation. However, overexpression of *paxB* affects the serine phosphorylation levels of the protein.

A)



B)

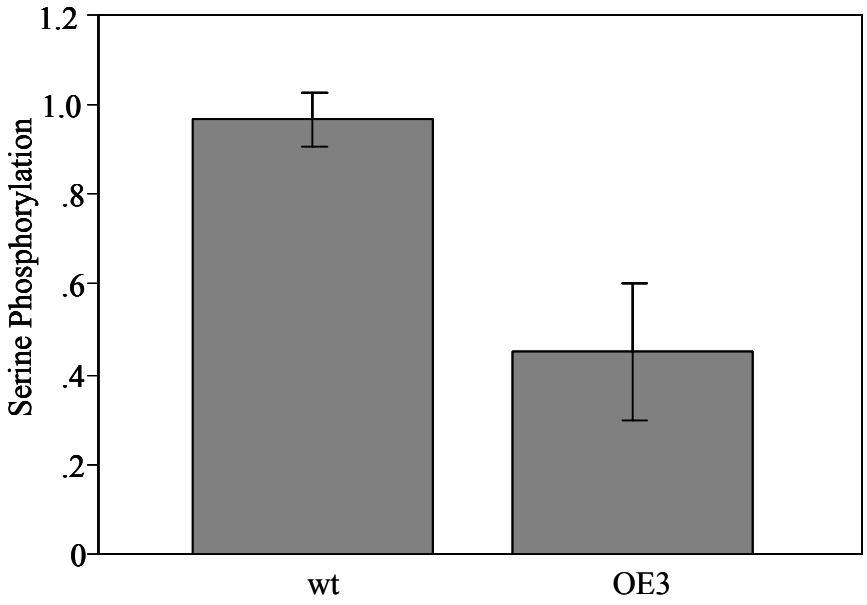
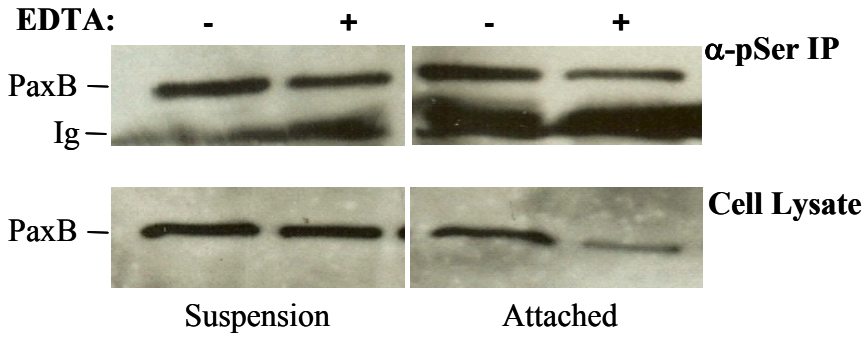


Figure 16. PaxB serine phosphorylation is unchanged during cell-cell cohesion
Wild-type and PaxBOE3 cells, starved for four hours and vortexed, were allowed to readhere. Lysates from cells collected at 0 and 60 minutes were immunoprecipitated with α-phospho-serine antibodies and immunoblot analysis was done with α-PaxB antibody. Densitometric quantification of the relative PaxB serine phosphorylation was determined by the IP to lysate ratio. Changes in phosphorylation with cell-cell cohesion was determined by the ratio of relative PaxB phosphorylation at t=60 (cohered) to t=0 (non-cohered). (A) immunoblot and (B) densitometric quantification of serine phosphorylation. Values represent the mean of three independent experiments ± SEM.

5.4 PaxB serine phosphorylation increases during adhesion

Since serine phosphorylation of paxillin has been associated with cells adhering to a substratum [26, 105, 106], we investigated the serine phosphorylation state of PaxB in wild-type adhered cells versus cells in suspension. Lysates from cells grown in suspension or adhered to a plate were collected, immunoprecipitated with α-phospho-serine antibodies, and immunoblotted with α-PaxB antibody (Fig. 17A). Serine phosphorylation was standardized to the amount of PaxB protein in the cell lysate. Serine phosphorylation of PaxB in attached cells is over 2.5 times greater than cells in suspension (Fig. 17B). This suggests that increased serine phosphorylation is associated with cell-substrate adhesion. The presence of 10 mM EDTA did not affect the level of serine phosphorylation of cells in suspension, but greatly decreased phosphorylation levels in attached cells (Fig. 17B). This implies that regulation of PaxB serine phosphorylation is associated with calcium dependent adhesion to substrate.

A)



B)

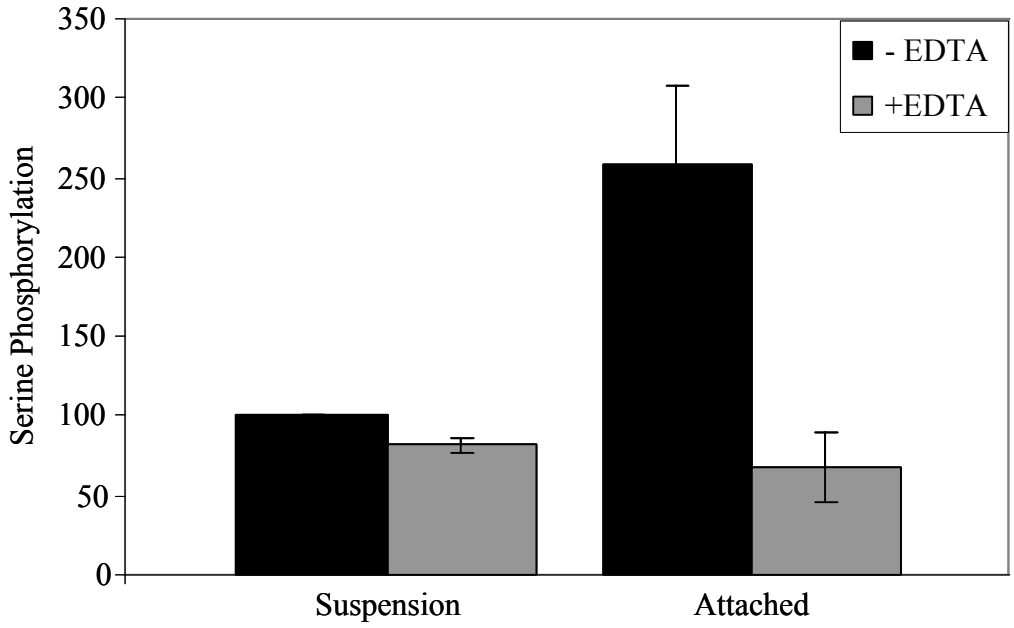


Figure 17. PaxB serine phosphorylation changes during adhesion
Wild-type cells were plated for 2 hours on plastic dishes or left in suspension in the absence or presence of 10 mM EDTA. Cell lysates were immunoprecipitated with α-phospho-serine antibodies and immunoblot analysis was done with α-PaxB antibody. Densitometric quantification of the relative PaxB serine phosphorylation was determined by the IP/lysate ratio. The IP to lysate ratio of cells in suspension in the absence of EDTA was set as 100%. (A) Immunoblot and (B) densitometry quantification of serine phosphorylation. Values represent the mean of three independent experiments ± SEM.

5.5 Cell-substrate adhesion decreases in PaxBOE cells

paxB$^-$ cells have been shown to be less adhesive to various substrates, specifically the deletion strain was considerably less adhesive when exposed to moderate conditions of shear stress [49]. Given that loss of PaxB decreases cell-substrate adhesion, we examined if overexpressing *paxB* has an opposite effect. To test the adhesion of cells to the substratum, we allowed wild-type and PaxBOE3 overexpressing cells to adhere to a glass surface and then counted the number of non-adherent cells after gentle agitation. This was subtracted from the total number of cells to calculate the percentage of adhering cells. The wild-type cells had no difference in the number of cells adhering to the substrate in the presence and absence of Tet, with approximately 40 percent of the cells remaining adhered to the underlying surface (Fig. 18). This suggests that the addition of Tet had no effects on the adhesion of cells to the substratum. The adhesion of PaxBOE3 overexpressing cells in the presence of Tet was similar to that of the wild-type cells (Fig. 18). Surprisingly, in the absence of Tet, adhesion of PaxBOE3 overexpressing cells to the underlying substrate was reduced to approximately 20 percent (Fig. 18). This suggests overexpression of *paxB* disrupts cell-substrate adhesion. However, addition of Tet to the overexpressing cells rescued cell-substrate adhesion to normal levels.

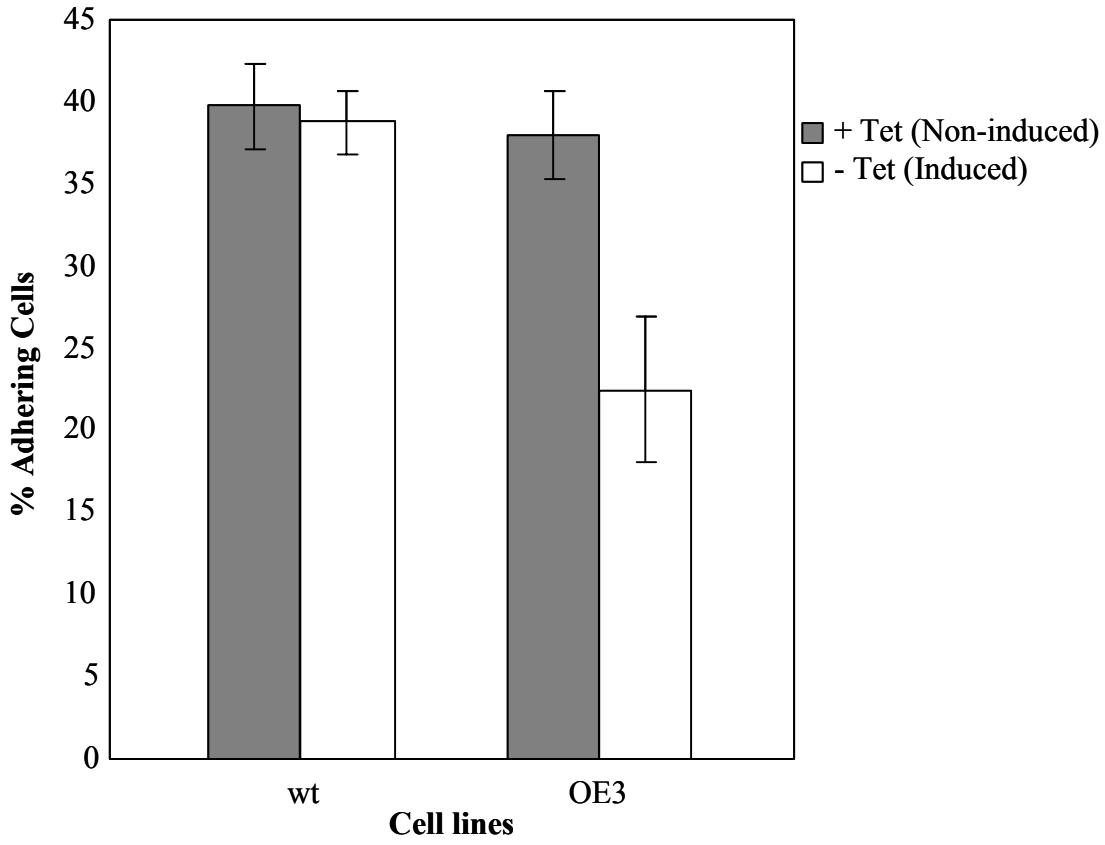


Figure 18. Adhesion of wild-type and PaxBOE3 cells to the underlying substrate
Adhesion of vegetative wild-type and PaxBOE3 overexpressing cells was measured under mild agitation. Values represent the mean of three independent experiments ± SEM.

5.6 Overexpression of *paxB* interferes with actin localization

Given that the actin cytoskeleton is important in cell-substrate adhesion, and that paxillin binds to many proteins that are involved in affecting changes in the organization of the actin cytoskeleton [12], we investigated the localization of F-actin in vegetative and starved PaxBOE3 cells. In vegetative wild-type cells in the absence and presence of Tet, F-actin is localized in the cytoplasm and the periphery of the cell (Fig. 19A, 19B). For vegetative PaxBOE3 cells in the

absence of Tet, F-actin is highly enriched at the periphery of the cell (white arrows), with some scattered F-actin spots (yellow arrows) (Fig. 19C). The F-actin enrichment and spots are less pronounced in the presence of Tet in the vegetative overexpressing cells (Fig. 19D). In wild-type cells under starved conditions in the absence and presence of Tet, F-actin is predominantly localized to the periphery of the cell (Fig. 19E, 19F). In the PaxBOE3 cells under starved conditions in the absence of Tet, a thick band of F-actin is mislocalized to the periphery of the cell, with some scattered F-actin spots (Fig. 19G). In the PaxBOE3 cells under starved conditions in the presence of Tet, the mislocalization of F-actin is less apparent (Fig. 19H). This suggests that PaxB may play a role in the organization of F-actin, and that overexpression of paxB affects F-actin localization more severe in starved cells than vegetative cells.

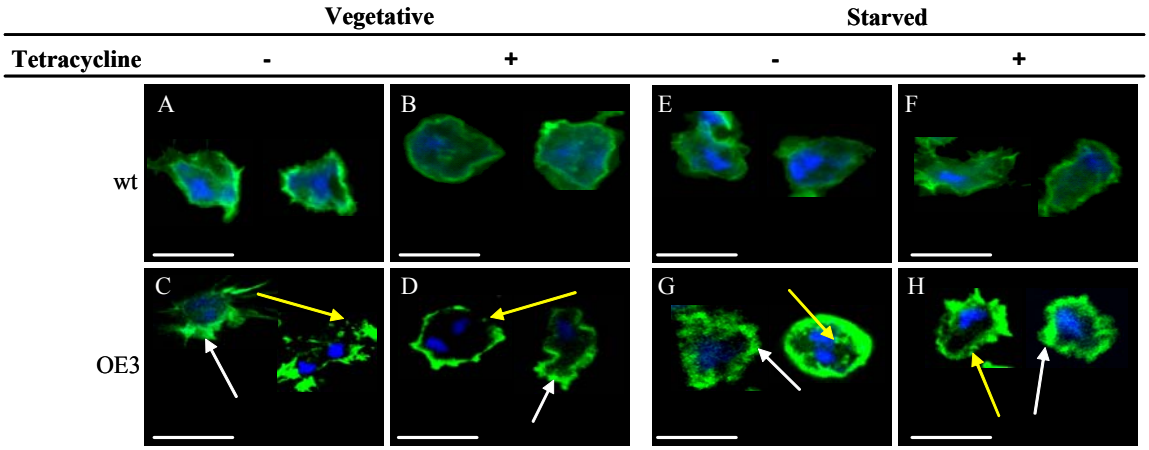


Figure 19. F-actin localization in wild-type and PaxBOE3 cells
Wild-type and PaxBOE3 vegetative and 5 hour starved cells were stained for F-actin with rhodamine phalloidin, and nuclei with TO-PRO stain. Vegetative wild-type and PaxBOE3 cells in the absence (A), (C) and the presence (B), (D) of Tetracycline (Tet), respectively. Starved wild-type and PaxBOE3 cells in the absence (E), (G) and the presence (F), (H) of Tet respectively. Each figure is a composite of two different pictures. White arrows point to F-actin enrichment, yellow arrows point to F-actin spots. Bars 5 µm.

5.7 Overexpression of *paxB* does not affect the speed of the cell

Given that overexpression of *paxB* affects the localization of F-actin, we wanted to investigate the effects of overexpressing *paxB* on motility. Vegetative and starved wild-type and PaxBOE3 overexpressing cells were seeded at low cell density on plastic dishes. The cells were allowed to adhere for one hour, and a time lapse image was captured every minute for 30 minutes. The average speed of wild-type and PaxBOE3 cells was measured in micrometers per minute. The total distance traveled in the 30 minutes was traced and calculated using the Image J software. There is no significant difference in the average speed of vegetative wild-type and PaxBOE3 cells with approximately 5 μm per minute, which is consistent with previous studies (Fig. 20) [107]. However, there is a minor, yet statistically different, decreased motility in starved PaxBOE3 overexpressing cells compared to the wild-type cells (Fig. 20). This suggests the overexpression of *paxB* does not affect the motility rate of the cell under vegetative conditions, but has a minor effect on cells under starved conditions.

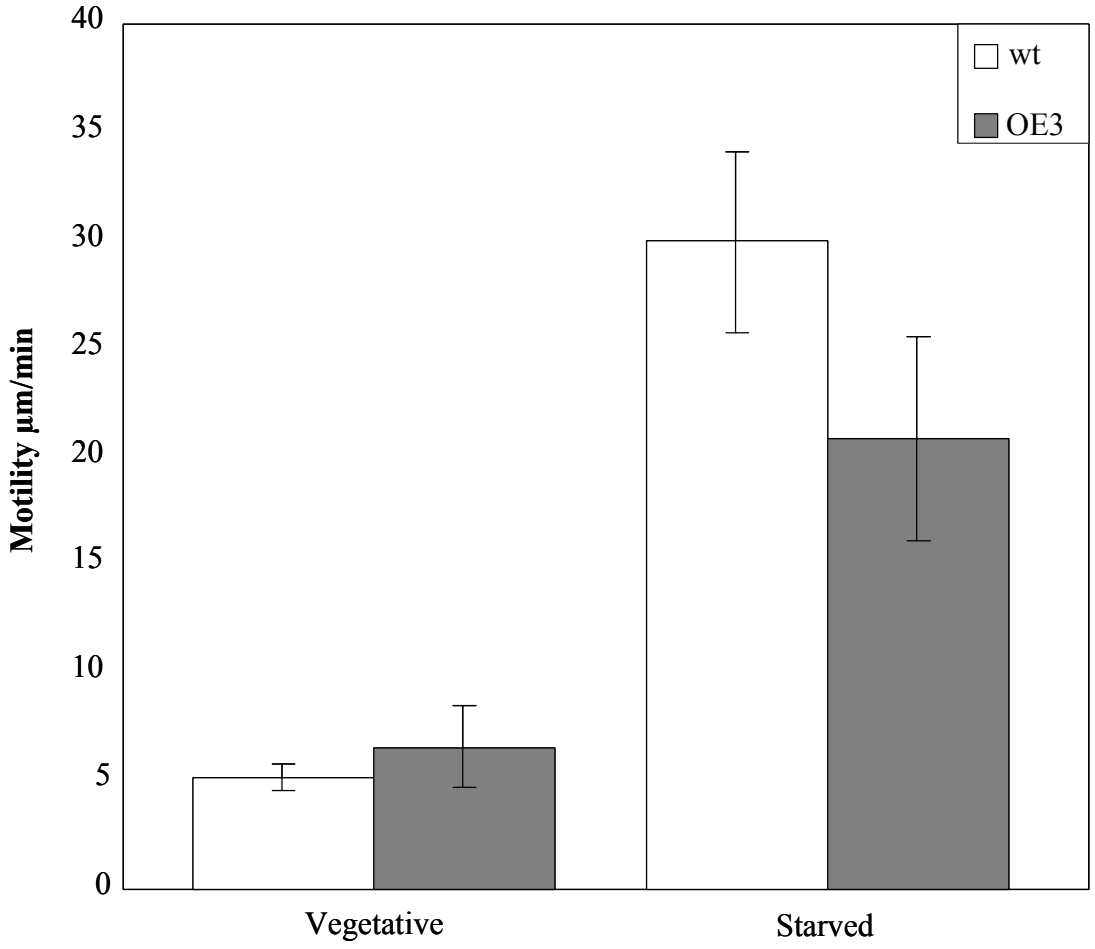


Figure 20. Average speed of wild-type and PaxBOE3 cells
The average speed of vegetative and starved cells was measured in µm/min. Values represent the mean of three independent experiments ± SEM.

5.8 Discussion

It is clear that cell-cell cohesion is an integral part of *Dictyostelium discoideum* development. It is important for initial stream formation, creation of the mound and morphogenesis, and has an influence on cell type differentiation in the multicellular context [51]. Aggregating cells differ from growth-phase cells by their stronger cohesion, which leads to the formation of cell agglomerates while shaking [72]. The overexpression of *paxB* increases cell-cell cohesion and causes larger cell agglomerates compared to wild-type cells. This indicates that *paxB* plays a positive role in cell-cell cohesion. In addition, cells agglomerate faster and to a greater extent when they overproduce PaxB. This is most likely due to an increase in Ca^{2+}dependent adhesion as addition of EDTA totally negates the effects of overexpressing *paxB*. This suggests PaxB-mediated cell-cell cohesion is Ca^{2+}dependent.

Previous studies have shown that PaxB is involved in cell-cell contact sites [49]. One putative PaxB interacting protein is the *D. discoideum* homologue of mammalian cadherin, DdCAD-1. DdCAD-1 is a cell surface protein, and like PaxB, it's involved in Ca^{2+}dependent cell-cell adhesion [72, 108]. In addition, during the vegetative and early developmental stages (3 hours) DdCAD-1 is randomly localized thorough out the cytoplasm, subsequently, by 12 hours of development, it becomes primarily associated with the cell periphery [109]. Specifically, DdCAD-1 has been suggested to form a complex with various membrane and cytoplasmic elements, which may participate in the dynamics of

the cytoskeleton and signal transduction [52, 110]. Moreover, it is involved in adherens junction through interactions with β-catenin [111]. Similar to PaxB, DdCAD-1 is enriched in cell-cell contact region, and disruption of the gene, *cad-A*, results in defective cell sorting [6, 50, 109]. Immunoprecipitation analysis would corroborate whether there is a direct interaction between PaxB and DdCAD-1. Although a direct interaction between paxillin and cadherins has not been proposed, a role for paxillin in cell-cell cohesion has been suggested as paxillin interacts with proteins involved in adherens junctions such as β-catenin [112, 113]. Specifically it was proposed that there is direct interaction between sites of focal adhesions and adherens junctions [112]. Given that DdCAD-1 has been extensively studied in *D. discoideum*, the possibility of PaxB interacting with DdCAD-1 can open new windows into our understanding of its function in cell-cell cohesion.

Previous studies have found changes in paxillin phosphorylation to be involved in adhesion [21]. We present evidence for the first time that a non-mammalian paxillin homologue undergoes serine phosphorylation. Other studies in our lab have shown that PaxB also undergoes tyrosine phosphorylation. Therefore, just like paxillin, PaxB undergoes tyrosine and serine phosphorylation.

The cohesion of cells in *D. discoideum* does not involve changes in PaxB serine and tyrosine phosphorylation. We show that the level of PaxB serine phosphorylation in cohered and non-cohered cells is relatively the same. In

addition, similar studies suggest there is no change in PaxB tyrosine phosphorylation during cell-cell cohesion. This suggests that cell-cell cohesion does not require changes in the PaxB phosphorylation state of these residues. Therefore, PaxB phosphorylation most likely has no effect on cell-cell adhesion. This is in agreement with the fact that overexpression of *paxB* leads to increased adhesion, but decreased PaxB phosphorylation. If the phosphorylation is irrelevant to cell-cell cohesion, the decreased phosphorylation in PaxBOE3 overexpresssing cells is possibly due to the overproduction of PaxB yielding more of the unphosphorylated version of the protein, thereby decreasing the overall phosphorylation ratio. Thus, the change in phosphorylation is most likely not involved in the increased cohesion seen in the PaxBOE3 cells. PaxB concurs with paxillin in that no reports have been published suggesting the association of changes in paxillin phosphorylation with cell-cell cohesion. These results add to our hypothesis of PaxB as the functional *D. discoideum* orthologue of mammalian paxillin

In contrast to our previous results, the levels of PaxB serine phosphorylation change upon attachment to the substratum. The serine phosphorylation of PaxB in suspended cells is less than in cells attached to a surface. Conversely, similar studies in our lab have shown that PaxB is tyrosine phosphorylated to a greater extent in suspended cells than cells attached to a surface, with phosphorylation levels three times lower in adhered cells than cells in suspension. This suggests a correlation between PaxB serine and tyrosine phosphorylation and substrate

adhesion. This effect on phosphorylation is most likely through calcium-dependent adhesion, as the presence of 10 mM EDTA impaired serine phosphorylation of PaxB in attached cells, while it did not affect serine phosphorylation of PaxB in cells in suspension. Similar studies done on PaxB tyrosine phosphorylation yielded analogous results. There is decreased PaxB tyrosine phosphorylation in cells attached to the substratum in the presence of EDTA. Our data parallel analogous investigations performed with mammalian paxillin which found that tyrosine phosphorylation is regulated by adhesion [17] and that paxillin serine phosphorylation is increased by cell adhesion to fibronectin or vitronectin [26, 106]. The phosphorylation state of paxillin has been shown to be dynamically regulated in several cell types by physiological stimuli including bombesin, PDGF, nerve growth factor among others [22, 23]. The rate of focal adhesion disassembly is slower in cells expressing several phosphorylation mutants for domain-deleted forms of paxillin, compared to cells expressing wild-type paxillin [28, 114]. Interestingly, phosphorylated serines 180 and 190 in paxillin, involved in membrane protrusion dynamics, as well as cell migration and spreading without affecting cell adhesion on collagen, are conserved in PaxB [26, 105, 106] and thus may be conserved sites of phosphorylation. In addition, there are 13 tyrosine residues distributed throughout the different PaxB domains, which represent potential sites of phosphorylation. These results suggest that just like paxillin, PaxB is regulated by changes in phosphorylation during cell-substrate adhesion.

While the sites of, and kinases responsible for, tyrosine phosphorylation in paxillin are known, they remain a mystery in *D. discoideum*. The *D. discoideum* genome does not possess canonical tyrosine kinases. It does, however, contain 66 tyrosine kinase-like proteins (TKL) [115] which represent potential kinases for PaxB. A putative *D. discoideum* MEKK-like kinase, SAPKα, has been identified as a possible tyrosine and serine/threonine kinase, which is involved in modulating the actin cytoskeleton [116]. Interestingly, the activation of SAPKα is triggered by the detachment of cells from the substratum, and disruption of the *spkA* gene reduces cell surface adhesion. This suggests that SAPKα might be involved in cell adhesion, possibly through phosphorylation of PaxB at tyrosine, and/or serine/threonine residues.

In addition, a novel serine/threonine kinase, Phg2, involved in adhesion and modulation of focal sites has been found in *D. discoideum* [9]. Phg2 has been proposed to be the mammalian focal adhesions kinase (FAK) for *D. discoideum*, displaying structural and functional similarities to its mammalian counterpart [9, 117, 118]. Phg2 is found in the cytosol, but recruitment to the plasma membrane is required for its activity [9, 118]. Moreover, it is largely accumulated at the cell membrane where it specifically plays a role in the organization of the actin cytoskeleton at actin rich focal sites, and in cell motility [9, 118]. Moreover, Phg2 localizes to cell-substrate interface, a region where PaxB is situated [9, 117, 118]. Given the parallels between mammalian and *D. discoideum* paxillin

phosphorylation, it seems likely that phosphorylation of paxillin may be an evolutionary conserved method of regulating adhesion.

PaxB plays a role in cell-substrate adhesion, since loss of PaxB leads to decreased adhesion [49]. Given that overexpressing *paxB* increases cell-cell cohesion, one would expect that it would similarly increase cell-substrate adhesion. However, we see that overexpressing *paxB* causes a decrease in the adhesion of cells to the underlying substrate. This is most likely caused by inappropriate phosphorylation states of PaxB in overexpressing cells. Cell substrate adhesion is associated with increased paxillin serine phosphorylation [26, 106]. However, we see a decrease in the phosphorylation of PaxB in the overexpressing cells. This may be due to the inability of the kinases responsible for phosphorylating PaxB to phosphorylate the extra protein. Since regulated phosphorylation of PaxB is involved in adhesion, the decreased ratio of phosphorylated PaxB in the overexpressing cells may cause a disruption in the regulation of adhesion, leading to loss of adhesion. Therefore, changes in PaxB serine and tyrosine phosphorylation are linked to cell substrate adhesion.

PaxB may be regulated differently in attached versus suspended cells. Given that PaxB mediated cell-substrate adhesion may require enhanced serine phosphorylation and decreased tyrosine phosphorylation, we suggest the following model. PaxB is both tyrosine and serine phosphorylated when cells are in suspension. Contact with the substrate triggers increased serine

phosphorylation and decreased tyrosine phosphorylation on PaxB. This change in phosphorylation allows the recruitment of PaxB/actin binding proteins to the site of contact, causing the formation of "focal adhesion sites" (Fig. 21).

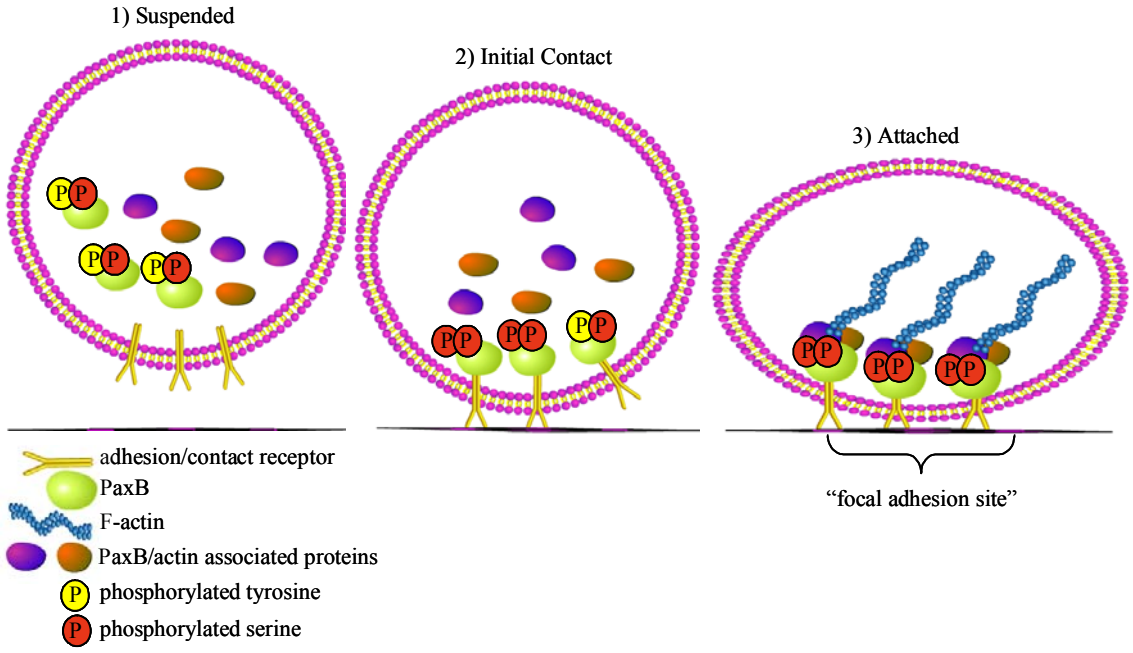


Figure 21. Model for PaxB phosphorylation upon cell-substrate adhesion
(1) PaxB is tyrosine and serine phosphorylated in suspended cells. (2) The initiation of cell contact with the underlying substratum allows an increase in PaxB serine phosphorylation and a decrease in tyrosine phosphorylation. (3) Changes in PaxB phosphorylation trigger the recruitment of PaxB/actin binding proteins to form "focal adhesion sites".

There are some parallels and differences in the phosphorylation state of paxillin and PaxB upon adhesion of cells to the substratum. Blocking phosphorylation through domain deletion of paxillin reduced cell adhesion to fibronectin, whereas the same constitutively expressed phosphorylation site significantly increased the

capacity of cells to adhere to fibronectin [13]. Similar to PaxB, paxillin is serine phophorylated during cell adhesion to the substratum [13]. Several serines are major phosphorylation sites of paxillin in response to adhesion to an extracellular matrix [26]. Moreover, phosphorylation of serines 180/190, which as previously mentioned are conserved residues in PaxB, is required for the increased stability of paxillin on collagen [26].

Paxillin is also highly phosphorylated on tyrosine residues in a developmentally regulated manner [13]. The major tyrosine phosphorylation sites of paxillin, Y31 and Y118, are lacking in PaxB. However, there are 13 tyrosine residues scattered on the various domains of PaxB that are promising phosphorylation sites. In addition, phospho-tyrosine phosphatase activities have been detected in *D. discoideum* which could also regulate PaxB phosphorylation [119-121]. Similar to PaxB, in PC12 neuronal and neuroendocrine cells, paxillin tyrosine phosphorylation is mediated by calcium dependent adhesion [122]. In addition, studies done in different mammalian cells show that paxillin undergoes rapid tyrosine phosphorylation upon adhesion [12, 17, 21]. In contrast, preliminary studies in our lab show decreased PaxB tyrosine phosphorylation during cell-substrate adhesion. These differences in tyrosine phosphorylation upon cell substrate adhesion need to be further explored in PaxB to corroborate the findings above.

The decreased cell-substrate adhesion in *paxB* overexpressing cells could be correlated to the mislocalization of actin. We found that the overexpression of *paxB* affects F-actin localization. PaxBOE3 overexpressing cells display abnormal F-actin enrichment and scattered F-actin spots in both vegetative cells and starved cells. This is in keeping with the role of paxillin and its interacting proteins in mammals, which are known to be involved in the regulation of actin cytoskeleton organization [123]. Paxillin is linked to actin via the cytoskeletal protein talin. *D. discoideum* has two talin orthologues, talin A and talin B. Talin B has two highly conserved F-actin-binding domains, which function in cell-substrate adhesion, and is more abundant in the membrane of prestalk cells than in prespore cells [124, 125]. Similarly, PaxB is localized to sites of cell-cell adhesion and cell-substrate adhesion and is expressed more strongly in prestalk cells than in prespore cells [49]. Just as we found in PaxBOE cells, Tsuijioka and colleagues found that development in *talB*$^-$ mutant cells is arrested at the mound stage [125]. These data are consistent with PaxB and talin B working together to regulate the actin cytoskeleton and may explain the aberrant polymerization of actin in PaxBOE3 cells.

Another protein that plays a role in vegetative cell-substrate adhesion and actin organization is the putative adhesion molecule SadA [126]. SadA is a transmembrane protein with EGF-repeats, like the mammalian tenascins and integrins, which are involved in adhesion. As with PaxBOE, *sadA* null cells give

rise to a more punctate F-actin distribution, suggesting a possible connection between SadA and PaxB in organizing F-actin.

Immunoprecipitation analysis to investigate a possible interaction of PaxB with talin B and/or SadA would be of interest. Given that PaxB, talin B, and SadA potentially interact with actin, investigating the original localization of talin B and SadA in the *paxB*$^{-}$ mutant, and vise versa, could determine if they work in conjunction. Similar investigations employing an overexpression system of *talb* and/or *sadA* in *paxB*$^{-}$ mutant cells, as well as the reciprocal cross-check, could be of interest. If these experiments allow the rescue of the mutant phenotype, the data would indicate these proteins work in the same signaling pathways.

PaxB has been suggested to play a role in cell migration during the multicellular stages of development [49], but its function during cell motility in vegetative and starved conditions have not been explained. Here we show the overexpression of *paxB* does not affect cell motility in vegetative cells. However, we observed a minor decrease in cell motility when the overexpressing cells are starved. In agreement with this, cell expressing a mutated version of paxillin exhibited limited movement during single cell migration [127]. Moreover, paxillin has been shown to play a role in cell motility through interactions with the actin cytoskeleton [11]. Given this information, the effect in cell motility in the PaxBOE3 starved cells could be linked to the F-actin enrichment and mislocalization observed in the overexpressing cells.

Paxillin interacts with numerous proteins [21]. Investigating how and when these proteins associate, and the effect on interacting partners, is of critical importance to comprehending how the cell effectively utilizes paxillin's adapter function. Studying the binding of paxillin to this network of proteins in a simpler model organism, and understanding how signal transduction involving paxillin occurs, can bring new insight to such a complex system. Given that we have established that PaxB parallels many of the functions of paxillin, *D. discoideum* facilitates the ability to study such a tangled process by providing a simpler system. The ability to decode such an intricate network and identify discrete switch points during signal transduction will discern what fails when the inappropriate regulation of paxillin leads to cell transformation and/or metastasis. Additional studies in PaxB are required before investigating this complex system in *D. discoideum*. The preliminary functions of PaxB in processes such as chemotaxis, as well as its binding partners need to be elucidated. In addition, further investigation in the regulation of PaxB through phosphorylation is required by creating a phosphorylation sites map, and point mutations of specific residues.

CHAPTER 6

Summary

We present evidence for the first time that a non-mammalian paxillin orthologue, PaxB, is a positive regulator of adhesion in the model organism *D. discoideum*. The overexpression of *paxB* increases cell-cell cohesion and causes large cell agglomerates to form. This is most likely due to an increase in Ca^{2+} dependent adhesion, as addition of EDTA completely negates the effects of overexpressing *paxB*. Thus, just like paxillin, PaxB is also involved in the cohesion of cells [112, 113].

Studies have found that changes in paxillin phosphorylation regulate its localization [21] and focal adhesion activity [28, 114]. Specifically, there is an increase in paxillin serine phosphorylation upon cell-substrate adhesion [26, 105, 106] . Our data show PaxB undergoes serine and tyrosine phosphorylation. However, the cohesion of cells in *D. discoideum* does not involve changes in PaxB serine and tyrosine phosphorylation. Such a study has not been conducted on paxillin and thus, we show unprecedented evidence that a paxillin orthologue, PaxB, does not involve changes in the phosphorylation of these residues during cell-cell cohesion. Moreover, this further implies that *D. discoideum* is a useful model organism to unveil new characteristics of paxillin. In addition, serine phosphorylation of PaxB increases during cell-substrate adhesion, while tyrosine phosphorylation decreases. This indicates a correlation between PaxB

phosphorylation and cell-substrate adhesion. These findings imply that just like paxillin, PaxB serine phosphorylation is correlated with cell-substrate adhesion.

Paxillin is a focal adhesion protein necessary for the adhesion of cells to the underlying substrate [10, 13]. Similar to paxillin, our studies found that PaxB plays a role in cell-substrate adhesion since overproduction of the protein caused a decrease in adhesion. Since we found that cell-substrate adhesion is associated with increased serine phosphorylation of PaxB and that the overexpressing cells show decreased serine phosphorylation, the decreased adhesion in the overexpressing cells may be due to the inability of the PaxB phosphorylating kinases to phosphorylate the extra protein. This data further suggest the correlation of PaxB phoshorylation and cell-substrate adhesion.

The decreased cell-substrate adhesion could be linked to the mislocalization of actin, since we found that cells overexpressing *paxB* displayed abnormal F-actin enrichment and scattered F-actin spots. This suggests a possible association of PaxB with the actin cytoskeleton. This is in agreement with the role of paxillin, which is known to bind proteins that actively rearrange the actin cytoskeleton [123].

PaxB production peaks during the mound and slug stages. This is consistent with its function in cell-cell cohesion and cell-substrate adhesion, since the interaction of cells with other cells and the substratum are essential for mound formation and

subsequent developmental progression. Furthermore, overexpression of *paxB* arrests development at the mound stage. This phenotypic defect can be rescued with addition of wild-type cells. Moreover, in chimeric fruiting bodies there is a decrease in the number of spores developing from *paxB* overexpressing cells. These results delineate the importance of normal expression of *paxB* in the development and morphogenesis of *D. discoideum*. Like PaxB, paxillin has been shown to play an essential role during development. Murine paxillin knockout arrests development due to the inability of cells to migrate (51). These results further link the similarity of paxillin and PaxB.

This work leads to several conclusions of particular interest to the *D. discoideum* and paxillin fields. An intracellular signaling network links local cellular adhesion events to the more general regulation of cellular physiology. Growth, differentiation, motility, and death are all regulated by adhesion. Today, more than 50 mammalian proteins have been reported to be associated with focal contacts and related ECM adhesions [9]. Investigating the individual role of each of these proteins, as well as their functional partners, is vital to elucidate the organization and function of such a complex network. Paxillin interacts with numerous proteins [21]. Investigating how and when these proteins associate is essential to comprehending how the cell effectively utilizes paxillin's adapter function.

Studying the binding of paxillin to proteins in this network is a daunting prospect. Thus, the ability to examine paxillin and understand how signal transduction involving paxillin occurs in a simple model organism can bring new insight to such a complex system. Given that we have established that PaxB parallels many of the functions of paxillin, *D. discoideum* facilitates the study of such a tangled process by providing a simpler system with fewer interacting proteins. Paxillin and PaxB appear to be intricately involved in the signaling pathways that govern cell-cell adhesion and cell-substrate adhesion. Finding the proteins that interact with PaxB will give insight into how these signaling networks are regulated, and ultimately how PaxB is involved in the normal function and physiology of the cell. Given the exciting finding that like paxillin, PaxB is possibly regulated by adhesion, *D. discoideum* is set to be used to illuminate these signaling networks. With its genetic tractability and simple differentiation and development, we are poised to gain greater understanding of the regulation of paxillin and its role in adhesion and cellular physiology.

REFERENCES

1. Schwartz, M.A., and R.K. Assoian, *Integrins and cell proliferation: regulation of cyclin-dependent kinase via cytoplasmic signaling pathways.* J Cell Sci, 2001. **114**: p. 2553-60.

2. Katsumi, A., et al., *Integrins in mechanotransduction.* J Biol Chem, 2004. **279**(13): p. 12001-4.

3. Gomer, R., et al., *Cell motility mediates tissue size regulation in Dictyostelium.* J Muscle Res Cell Motil, 2002. **23**(7-8): p. 809-15.

4. Dinc, S., et al., *Methylene blue prevents surgery-induced peritoneal adhesions but impairs the early phase of anastomotic wound healing.* Can J Surg, 2006. **49**(5): p. 321-8.

5. Shtutman, M., et al., *Cell adhesion molecule L1 disrupts E-cadherin-containing adherens junctions and increases scattering and motility of MCF7 breast carcinoma cells.* Cancer Res, 2006. **66**(23): p. 11370-80.

6. Lin, Z., et al., *Solution structures of the adhesion molecule DdCAD-1 reveal new insights into Ca(2+)-dependent cell-cell adhesion.* Nat Struct Mol Biol, 2006. **13**(11): p. 1016-22.

7. Liu, C.I., et al., *LrrA, a novel leucine-rich repeat protein involved in cytoskeleton remodeling, is required for multicellular morphogenesis in Dictyostelium discoideum.* Dev Biol, 2005. **285**(1): p. 238-51.

8. Ginger, R.S., et al., *A novel Dictyostelium cell surface protein important for both cell adhesion and cell sorting.* Development, 1998. **125**(17): p. 3343-52.

9. Gebbie, L., et al., *Phg2, a kinase involved in adhesion and focal site modeling in Dictyostelium.* Mol Biol Cell, 2004. **15**(8): p. 3915-25.

10. Brown, M.C., K.A. West, and C.E. Turner, *Paxillin-dependent paxillin kinase linker and p21-activated kinase localization to focal adhesions involves a multistep activation pathway.* Mol Biol Cell, 2002. **13**(5): p. 1550-65.

11. West, K.A., et al., *The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL).* J Cell Biol, 2001. **154**(1): p. 161-76.

12. Turner, C.E., *Paxillin interactions.* J Cell Sci, 2000. **113 Pt 23**: p. 4139-40.

13. Brown, M.C., J.A. Perrotta, and C.E. Turner, *Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin.* Mol Biol Cell, 1998. **9**(7): p. 1803-16.

14. Salgia, R., et al., *Molecular cloning of human paxillin, a focal adhesion protein phosphorylated by P210BCR/ABL.* J Biol Chem, 1995. **270**(10): p. 5039-47.

15. Breen, J.J., A.D. Agulnick, H. Westphal, and I.B. Dawid., *Interactions between LIM domains and the LIM domain-binding protein Ldb1.* J Biol Chem, 1998. **273**: p. 4712-7.

16. Yagi, R., et al., *A novel muscle LIM-only protein is generated from the paxillin gene locus in Drosophila.* EMBO Rep, 2001. **2**(9): p. 814-20.

17. Turner, C.E., *Paxillin: a cytoskeletal target for tyrosine kinases.* Bioessays, 1994. **16**(1): p. 47-52.

18. Zamir, E. and B. Geiger, *Molecular complexity and dynamics of cell-matrix adhesions.* J Cell Sci, 2001. **114**(Pt 20): p. 3583-90.

19. Schaller, M.D.a.S., E.M., *Multiple stimuli induce tyrosine phosphosrylation of the Crk-binding sites of paxillin.* Biochem. J., 2001. **360**: p. 57-66.

20. Tumbarello, D.A., M.C. Brown, and C.E. Turner, *The paxillin LD motifs.* FEBS Lett, 2002. **513**(1): p. 114-8.

21. Brown, M.C. and C.E. Turner, *Paxillin: adapting to change.* Physiol Rev, 2004. **84**(4): p. 1315-39.

22. Turner, C.E., et al., *Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells.* J Cell Sci, 1995. **108 (Pt 1)**: p. 333-42.

23. Melamed, I., et al., *Nerve growth factor triggers microfilament assembly and paxillin phosphorylation in human B lymphocytes.* J Exp Med, 1995. **181**(3): p. 1071-9.

24. Rankin, S., et al., *Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin.* FEBS Lett, 1994. **354**(3): p. 315-9.

25. Nakamura, K., et al., *Tyrosine phosphorylation of paxillin alpha is involved in temporospatial regulation of paxillin-containing focal adhesion formation and F-actin organization in motile cells.* J Biol Chem, 2000. **275**(35): p. 27155-64.

26. Zeid, N.A., A. M. Valles, and B. Boyer, *Serine phosphorylation regulates paxillin turover during cell migration.* Cell Communication and Signaling, 2006. **4**(8): p. 1478-.

27. Webb, D.J., Kovalenko, M., Whitmore, L., and Horwitz, A.F., *Phosphorylation of serine 709 in GIT1 regulates protrusive activity in cells.* Biochem. and Biophysical Communications, 2006. **346**: p. 1284-1288.

28. Yamaguchi, R., et al., *Mitosis specific serine phosphorylation and downregulation of one of the focal adhesion protein, paxillin.* Oncogene, 1997. **15**(15): p. 1753-61.

29. Turner, C.E., *Paxillin is a major phosphotyrosine-containing protein during embryonic development.* J Cell Biol, 1991. **115**(1): p. 201-7.

30. Zachary, I., et al., *Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells.* J Biol Chem, 1993. **268**(29): p. 22060-5.

31. Salgia, R., J.L. Li, S.H.Lo, B. Brunkhorst, G.S. Kansas, E.S. Sobhany, Y. Sun, E. Pisick, M. Hallek, T. Ernst, R. Tantravahi, L.B. Chen and, J.D. Griffin, *Molecular Cloning of Human Paxillin, a Focal Adhesion Protein Phophorylated by P210 BCR/ABL.* 1994.

32. McWhirter, J.R. and J.Y. Wang, *Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins.* Mol Cell Biol, 1991. **11**(3): p. 1553-65.

33. McWhirter, J.R. and J.Y. Wang, *An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias.* Embo J, 1993. **12**(4): p. 1533-46.

34. Glenney, J.R., Jr. and L. Zokas, *Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton.* J Cell Biol, 1989. **108**(6): p. 2401-8.

35. Fernandez-Valle, C., et al., *Paxillin binds schwannomin and regulates its density-dependent localization and effect on cell morphology.* Nat Genet, 2002. **31**(4): p. 354-62.

36. Das, K., J. Bohl, and S.B. Vande Pol, *Identification of a second transforming function in bovine papillomavirus type 1 E6 and the role of E6 interactions with paxillin, E6BP, and E6AP.* J Virol, 2000. **74**(2): p. 812-6.

37. Hood, J.D. and D.A. Cheresh, *Role of integrins in cell invasion and migration.* Nat Rev Cancer, 2002. **2**(2): p. 91-100.

38. Schwartz, M.A. and M.H. Ginsberg, *Networks and crosstalk: integrin signalling spreads.* Nat Cell Biol, 2002. **4**(4): p. E65-8.

39. Schaller, M.D., et al., *Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains.* J Cell Biol, 1995. **130**(5): p. 1181-7.

40. Vadlamudi, R., et al., *Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells.* Cancer Res, 1999. **59**(12): p. 2843-6.

41. Scibelli, A., et al., *Expression levels of the focal adhesion-associated proteins paxillin and p130CAS in canine and feline mammary tumors.* Vet Res, 2003. **34**(2): p. 193-202.

42. Yano, H., et al., *Paxillin alpha and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9076-81.

43. Jackson, J.L. and M.R. Young, *Protein phosphatase-2A regulates protein tyrosine phosphatase activity in Lewis lung carcinoma tumor variants.* Clin Exp Metastasis, 2003. **20**(4): p. 357-64.

44. Salgia, R., et al., *Expression of the focal adhesion protein paxillin in lung cancer and its relation to cell motility.* Oncogene, 1999. **18**(1): p. 67-77.

45. Ye, L., et al., *Endogenous bone morphogenetic protein-7 controls the motility of prostate cancer cells through regulation of bone morphogenetic protein antagonists.* J Urol, 2007. **178**(3 Pt 1): p. 1086-91.

46. Jones, R.J., Boyce, T., Fennell, M., Jacobs, V., Pinto, F., Duffield, E., Clark, G., Green, T., Kelly, J. and, Robertson, J. (2007) *The impact of delay in cryo-fixation on biomarkers of Src tyrosine kinase in human breast and bladder cancer*. **Volume**,

47. Ma, P.C., Tretiakova, M.S., Nallasura, V., Jagadeeswaran, R., Husain, A.N., and Salgia, R., *Downstream signalling and specific inhibition of c-MEt/HGF pathway in small cell lung cancer: implications for tumor invasion.* Br J Cancer, 2007.

48. Hagel, M., George E.L., Kim, A., Tamimi, R., Opitz, S.L., Turner, C.E., Imamoto, A., and Thomas, S.M., *The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling.* Mol Cell Biol, 2002. **22**: p. 901-915.

49. Bukharova, T., G. Weijer, L. Bosgraaf, D. Dormann, P.J. van Haastert, and C.J. Weijer., *Paxillin is required for cell-substrate adhesion, cell sorting and*

slug migration during Dictyostelium development. J Cell Sci, 2005. **118**: p. 4295-310.

50. Wong, E., et al., *Disruption of the gene encoding the cell adhesion molecule DdCAD-1 leads to aberrant cell sorting and cell-type proportioning during Dictyostelium development.* Development, 2002. **129**(16): p. 3839-50.

51. Siu, C.H., et al., *Regulation of cell-cell adhesion during Dictyostelium development.* Semin Cell Dev Biol, 2004. **15**(6): p. 633-41.

52. Bowers-Morrow, V.M., S.O. Ali, and K.L. Williams, *Cell adhesion during the migratory slug stage of Dictyostelium discoideum.* Cell Biol Int, 2002. **26**(11): p. 951-62.

53. Bracco, E., B. Pergolizzi, B. Peracino, E. Ponte, A. Balbo, A. Mai, A. Ceccarelli, and S. Bozzaro, *Cell-cell signaling and adhesion in phagocytosis and early development of Dictyostelium.* Int J Dev Biol, 2000(44): p. 733-42.

54. Bonner, J.T., *The Cellular Slime Molds*. 2 ed. Vol. 1. 1967: Princeton University Press.

55. Loomis, W.F., *Dictyostelium discoideum. A developmental System*. 1 ed. Vol. 1. 1975, New York: Academic Press.

56. Kessin, R.H., *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity*. 1 ed. Vol. 1. 2001, New York: Cambridge University Press. 294.

57. Escalante, R., et al., *The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of Dictyostelium development.* Dev Biol, 2001. **235**(2): p. 314-29.

58. Kuspa, A. and W.F. Loomis, *Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA.* Proc Natl Acad Sci U S A, 1992. **89**(18): p. 8803-7.

59. Blanton, R.L., *Phylum Acrasea. In Jones & Bartlett Series in Life Sciences: Handbook of Protoctista: The structure, cultivation, habitats and life histories of the eukaryotic microorganisms and their descendents exclusive of animals, plants and fungi: a guide to algae, ciliates, foraminifera, sporozoa, slime molds, and the other protoctista*. 1990, Boston: Jones and Bartlett. 75-87.

60. Strmecki, L., D.M. Greene, and C.J. Pears, *Developmental decisions in Dictyostelium discoideum.* Dev Biol, 2005. **284**(1): p. 25-36.

61. Maree, A.F., and P. Hogeweg, *How amoeboids self-organize into a fruiting body: Multicellilar coordinations in Dictyostelium.* 2001. **98**(7): p. 3879-3883.

62. Stege, J.T., G. Shaulsky, and W.F. Loomis, *Sorting of the initial cell types in Dictyostelium is dependent on the tipA gene.* Dev Biol, 1997. **185**(1): p. 34-41.

63. Clow, P.A., et al., *Three-dimensional in vivo analysis of Dictyostelium mounds reveals directional sorting of prestalk cells and defines a role for the myosin II regulatory light chain in prestalk cell sorting and tip protrusion.* Development, 2000. **127**(12): p. 2715-28.

64. Vasiev, B. and C.J. Weijer, *Modeling chemotactic cell sorting during Dictyostelium discoideum mound formation.* Biophys J, 1999. **76**(2): p. 595-605.

65. Gerisch, G., *Cyclic AMP and other signals controlling cell development and differentiation in Dictyostelium.* Annu Rev Biochem, 1987. **56**: p. 853-79.

66. Parent, C.A.a.P.N.D., *Molecular genetics of signal transdution in Dictyostelium.* Annu Rev Biochem, 1996. **65**: p. 411-440.

67. Bozzaro, S. and E. Ponte, *Cell adhesion in the life cycle of Dictyostelium.* Experientia, 1995. **51**(12): p. 1175-88.

68. Fisher, P.R., *Directional movement of migrating slugs in Dictyostelium, a model system for cell and developmental biology*. 1997, Tokyo: Universal Acad. Press. 437-452.

69. Williams, J.G., *Transcriptional regulation of Dictyostelium pattern formation.* EMBO Rep, 2006. **7**(7): p. 694-8.

70. Williams, J.G., et al., *Origins of the prestalk-prespore pattern in Dictyostelium development.* Cell, 1989. **59**(6): p. 1157-63.

71. Weeks, G., *Signaling molecules involved in cellular differention during Dictyostelium morphogenesis.* Current Opinion in Microbiology, 2000. **3**: p. 625-630.

72. Secko, D.M., et al., *An activated Ras protein alters cell adhesion by dephosphorylating Dictyostelium DdCAD-1.* Microbiology, 2006. **152**(Pt 5): p. 1497-505.

73. Yuan, A., C.H. Siu, and C.P. Chia, *Calcium requirement for efficient phagocytosis by Dictyostelium discoideum.* Cell Calcium, 2001. **29**(4): p. 229-38.

74. Beug, H., F.E. Katz, and G. Gerisch, *Dynamics in antigenic membrane sites relating to cell aggregation in Dictyostelium discoideum.* J. Cell Biol., 1973. **56**: p. 647-658.

75. Knecht, D.A., D.L. Fuller, and W.F. Loomis, *Surface glycoprotein, gp24, involved in early adhesion of Dictyostelium discoideum.* Dev Biol, 1987. **121**(1): p. 277-83.

76. Brar, S.K., C.H. Siu, *Characterization of cell adhesion molecule gp24 in Dictyostelium-mediation lf cell-cell adhesion via a calcium dependent mechanism.* J.Biol. Chem., 1993. **268**: p. 24902-24909.

77. Kamboj, R.K., J. Gariepy, and C.H. Siu, *Identification of an octapeptide involved in homophilic interaction of the cell adhesion molecule gp80 of dictyostelium discoideum.* Cell, 1989. **59**(4): p. 615-25.

78. Ponte, E., et al., *Detection of subtle phenotypes: the case of the cell adhesion molecule csA in Dictyostelium.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9360-5.

79. Gao, E.N., P. Shier, and C.H. Siu, *Purification and partial characterization of a cell-adhesion molecule (gp150) involved in post-aggregation stage cell-cell binding in Dictyostelium discoideum.* J.Biol. Chem., 1992. **267**: p. 9409-9415.

80. Siu, C.H., T.J.C. Harris, E.F.S. Wong, C. Yung, H. Sesaki, and J. Wang, *Cell adhesion molecules in Dictyostelium- A model system for cell and developmental biology*. 1997, Tokyo: Univesity Academy Press. 111-144.

81. Dynes, J.L., A.M. Clark, G. Shaulsky, A. Kuspa, W.F. Loomis, and R.A. Firtel, *LagC is required for cell-cell interactions that are essential for cell-type differentiation in Dictyostelium.* Genes Dev, 1994. **8**: p. 948-58.

82. Varney, T.R., et al., *A novel disintegrin domain protein affects early cell type specification and pattern formation in Dictyostelium.* Development, 2002. **129**(10): p. 2381-9.

83. Blumberg, D.D., H.N. Ho, C.L. Petty, T.R. Varney, and S. Gandham, *AmpA, a modular protein containing disintegrin and ornatin domains, has multiple effects on cell adhesion and cell fate specification.* J Muscle Research and Cell Motility, 2002. **23**: p. 817-828.

84. Aubry, L., et al., *The Dictyostelium mitogen-activated protein kinase ERK2 is regulated by Ras and cAMP-dependent protein kinase (PKA) and mediates PKA function.* J Biol Chem, 1997. **272**(7): p. 3883-6.

85. Weber, I., et al., *Two-step positioning of a cleavage furrow by cortexillin and myosin II.* Curr Biol, 2000. **10**(9): p. 501-6.

86. Van Haastert, P.J., *Transduction of the chemotactic cAMP signal across the plasma membrane of Dictyostelium cells.* Experientia, 1995. **51**(12): p. 1144-54.

87. Noegel, A.A. and J.E. Luna, *The Dictyostelium cytoskeleton.* Experientia, 1995. **51**(12): p. 1135-43.

88. Wong, E.F., et al., *Molecular cloning and characterization of DdCAD-1, a Ca2+-dependent cell-cell adhesion molecule, in Dictyostelium discoideum.* J Biol Chem, 1996. **271**(27): p. 16399-408.

89. Matsunaga, T. and N. Mori, *The origin of the immune system. The possibility that immunoglobulin superfamily molecules and cell adhesion molecules of chicken and slime mould are all related.* Scand J Immunol, 1987. **25**(5): p. 485-95.

90. Maeda, M. and R.A. Firtel, *Activation of the mitogen-activated protein kinase ERK2 by the chemoattractant folic acid in Dictyostelium.* J Biol Chem, 1997. **272**(38): p. 23690-5.

91. Loomis, W.F., A. Kuspa, and G. Shaulsky, *Gene discovery in Dictyostelium.* Genet Eng (N Y), 1994. **16**: p. 49-64.

92. Sussman, M., *Cultivation and synchronous morphogenesis of Dictyostelium under controlled experimental conditions.* Methods cell Biol, 1987. **28**: p. 9-29.

93. Howard, P.K., K.G. Ahern, and R.A. Firtel, *Establishment of a transient expression system for Dictyostelium discoideum.* Nucleic Acids Res, 1988. **16**(6): p. 2613-23.

94. Heikoop, J.C., P.D. Grootenhuis, M. Blaauw, J.S. Veldema, P.J. van Haastert, and M.H. Linskens, *Expression of a bioactive, single-chain choriogonadotropin in Dictyostelium discoideum.* Eur J Biochem, 1998. **256**: p. 359-63.

95. Engler-Blum, G., et al., *Reduction of background problems in nonradioactive northern and Southern blot analyses enables higher sensitivity than 32P-based hybridizations.* Anal Biochem, 1993. **210**(2): p. 235-44.

96. Jermyn, K.A. and J.G. Williams, *An analysis of culmination in Dictyostelium using prestalk and stalk-specific cell autonomous markers.* Development, 1991. **111**(3): p. 779-87.

97. Chen, C.F. and E.R. Katz, *Mediation of cell-substratum adhesion by RasG in Dictyostelium.* J Cell Biochem, 2000. **79**(1): p. 139-49.

98. Bishop, J.D., et al., *A second UDP-glucose pyrophosphorylase is required for differentiation and development in Dictyostelium discoideum.* J Biol Chem, 2002. **277**(36): p. 32430-7.

99. Lim, C.J., et al., *Loss of the Dictyostelium RasC protein alters vegetative cell size, motility and endocytosis.* Exp Cell Res, 2005. **306**(1): p. 47-55.

100. Blaauw, M., M.H. Linskens, and P.J. van Haastert, *Efficient control of gene expression by a tetracycline-dependent transactivator in single Dictyostelium discoideum cells.* Gene, 2000. **252**(1-2): p. 71-82.

101. Gossen, M. and H. Bujard, *Tight control of gene expression in mammalian cells by tetracycline-responsive promoters.* Proc Natl Acad Sci U S A, 1992. **89**(12): p. 5547-51.

102. Gaudet, P., H. MacWilliams, and A. Tsang, *Inducible expression of exogenous genes in Dictyostelium discoideum using the ribonucleotide reductase promoter.* Nucleic Acids Res, 2001. **29**(2): p. E5.

103. Weijer, C.J., G. Duschl, and C. N. David, *Dependence of cell-type proportioning and sorting on cell cylce phase in Dictyostelium discoideum.* J. Cell Sci., 1984. **70**: p. 133-145.

104. Fosnaugh, K.L.a.W.F.L., *Coordinate regulation of the spore coat genes in Dictyostelium discoideum.* Dev Genet, 1991. **12**: p. 123-32.

105. De Nichilo, M.O. and K.M. Yamada, *Integrin alpha v beta 5-dependent serine phosphorylation of paxillin in cultured human macrophages adherent to vitronectin.* J Biol Chem, 1996. **271**(18): p. 11016-22.

106. Bellis, S.L., et al., *Adhesion of fibroblasts to fibronectin stimulates both serine and tyrosine phosphorylation of paxillin.* Biochem J, 1997. **325 (Pt 2)**: p. 375-81.

107. Song, L., Nadkarni, S.M., Bodeker, H.U., Beta, C., Bae, A., Franck, C., Rappel, W.J., Loomis, W.F., and Bodenschatz, E., *Dictyostelium discoideum chemotaxis: Threshold for directed motion* Eur J Cell Biol, 2006. **85**: p. 891-989.

108. Yang, C., Brar, S. K., Desbarats, L., and Siu, C. H., *Synthesis of the calcium-dependent adhesion molecule DdCAD-1 is regulated by multiple factors during Dictyostelium development.* Diffentiation, 1997. **61**: p. 275-284.

109. Sesaki, H. and C.H. Siu, *Novel redistribution of the Ca(2+)-dependent cell adhesion molecule DdCAD-1 during development of Dictyostelium discoideum.* Dev Biol, 1996. **177**(2): p. 504-16.

110. Desbarats, L., S.K. Brar, and C.H. Siu, *Involvement of cell-cell adhesion in the expression of the cell cohesion molecule gp80 in Dictyostelium discoideum.* J Cell Sci, 1994. **107 (Pt 6)**: p. 1705-12.

111. Grimson, M.J., et al., *Adherens junctions and beta-catenin-mediated cell signalling in a non-metazoan organism.* Nature, 2000. **408**(6813): p. 727-31.

112. Birukova, A.A., et al., *Paxillin-beta-catenin interactions are involved in Rac/Cdc42-mediated endothelial barrier-protective response to oxidized phospholipids.* Am J Physiol Lung Cell Mol Physiol, 2007. **293**(1): p. L199-211.

113. Palovuori, R. and S. Eskelinen, *Role of vinculin in the maintenance of cell-cell contacts in kidney epithelial MDBK cells.* Eur J Cell Biol, 2000. **79**(12): p. 961-74.

114. Webb, D.J., M.J. Schroeder, C.J. Brame, L. Whitmore, J. Shabanowitz, D.F. Hunt, and A.R. Horwitz, *Paxillin phosphorylation site mapped by mass spectrometry.* J Cell Sci, 2005. **118**: p. 4925-29.

115. Goldberg, J.M., et al., *The dictyostelium kinome--analysis of the protein kinases from a simple model organism.* PLoS Genet, 2006. **2**(3): p. e38.

116. Sun, B., H. Ma, and R.A. Firtel, *Dictyostelium stress-activated protein kinase alpha, a novel stress-activated mitogen-activated protein kinase kinase kinase-like kinase, is important for the proper regulation of the cytoskeleton.* Mol Biol Cell, 2003. **14**(11): p. 4526-40.

117. Cherix, N., R. Froquet, S.J. Charette, C.Blanc, F. Letourneur, and P. Cosson, *A Phg2-Adrm1 pathway participates in the nutrient-controlled developmental response in Dictyostelium.* Mol Biol Cell, 2006. **17**: p. 4982-7.

118. Blanc, C., et al., *A novel phosphatidylinositol 4,5-bisphosphate-binding domain targeting the Phg2 kinase to the membrane in Dictyostelium cells.* Eur J Cell Biol, 2005. **84**(12): p. 951-60.

119. Howard, P.K., B.M. Sefton, and R.A. Firtel, *Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in Dictyostelium.* Cell, 1992. **71**(4): p. 637-47.

120. Howard, P.K., et al., *Regulation by protein-tyrosine phosphatase PTP2 is distinct from that by PTP1 during Dictyostelium growth and development.* Mol Cell Biol, 1994. **14**(8): p. 5154-64.

121. Ramalingam, R., D.R. Shaw, and H.L. Ennis, *Cloning and functional expression of a Dictyostelium discoideum protein tyrosine phosphatase.* J Biol Chem, 1993. **268**(30): p. 22680-5.

122. Khan, M.A., N. Okumura, and M. Okada, *Depolarization-induced tyrosine phosphorylation of paxillin in PC12h cells.* Eur J Biochem, 1996. **235**(3): p. 579-84.

123. Chen, H.Y., et al., *Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin.* Mol Cell Biol, 2004. **24**(24): p. 10558-72.

124. Tsujioka, M., L.M. Machesky, S.L. Cole, K. Yahata, and K. Inouye, *A unique talin homologue with a villin headpiece-like domain is required for multicellular morphogenesis in Dictytostelium.* Curr Biol, 1999. **9**: p. 389-92.

125. Tsujioka, M., K. Yoshida, and K. Inouye, *Talin B is required for force transmission in morphogenesis of Dictyostelium.* Embo j, 2004. **23**: p. 2216-25.

126. Fey, P., et al., *SadA, a novel adhesion receptor in Dictyostelium.* J Cell Biol, 2002. **159**(6): p. 1109-19.

127. Huang, C., Rajfur, Z., Borchers, C., Schaller, M.D., and Jacobson, K., *JNK phosphorylates paxillin and regulates cell migration.* Nature (letters to nature), 2003. **424**: p. 219-223.