CHARACTERIZATION OF PAXILLIN, PHOSPHOLIPASE D AND THEIR FUNCTIONAL INTERACTION

by

JELENA PRIBIC

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requirement for the degree of Doctor of Philosophy.

Dr. Derrick Brazill

Date | Chair of Examining Committee

Dr. Edward J. Kennelly

Date | Executive Officer

Dr. David Foster

Dr. Diana Bratu

Dr. Jeffrey E. Segall

Dr. Anne Bresnick
Supervision Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

Characterization of paxillin, phospholipase D and their functional interaction

by

Jelena Pribic

Advisor: Dr. Derrick Brazill

The actin cytoskeleton plays a fundamental role in various processes including differentiation, migration, endocytosis and exocytosis. An adapter protein, paxillin, as well as an enzyme, Phospholipase D (PLD), have been associated with processes based on actin cytoskeleton regulation. Such regulation is critical for the development of *Dictyostelium discoideum*. To gain better insight into the roles of paxillin and PLD and to investigate their potential interactions, we study the paxillin and PLD orthologs, PaxB and PldB, respectively. Previous work showed that in *Dictyostelium discoideum*, paxillin (PaxB) and Phospholipase D (PldB) colocalize and co-immunoprecipitate, suggesting that they physically interact. We found that the phenotypes observed during development, cell sorting and several actin-required processes including cAMP chemotaxis, cell-substrate adhesion, actin polymerization, phagocytosis, and exocytosis reveal a genetic interaction between *paxB* and *pldB* suggesting a functional interaction between gene products. Taken together, our data point to PldB being a required binding partner of PaxB during processes involving actin reorganization.

Based on our study in the model organism *Dictyostelium discoideum*, we examined whether a similar relationship between paxillin and PLD exists in the highly aggressive human breast cancer cell line MDA-MB-231. We investigated the role of PLD activity on paxillin

regulation, Erk activation and formation of a paxillin-Erk and paxillin-FAK complex. Inhibition of PLD activity led to an increase in paxillin tyrosine phosphorylation, a decrease in Erk activation, and enhanced association of paxillin with Erk. In addition, we found that paxillin tyrosine phosphorylation depends upon Erk activity and may be a consequence of an increased association with FAK. Taken together, our results suggest that Erk activity is governed by PLD activity and regulates the tyrosine phosphorylation of paxillin, potentially explaining its role in cell motility. This study indicated that PLD, paxillin, FAK and Erk participate in the same signaling pathway in this breast cancer cell line. The proposed studies will allow further insight into the role of these proteins in cancer and better understanding of the clinical course of disease.

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The thesis is dedicated to my parents.

Овај рад посвећујем својим родитељима.

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Chapter 1: Introduction

1.1 The actin cytoskeleton

Cytoskeletal networks determine cell shape, organelle distribution and the cytoplasmatic consistency of cells. Dynamic remodeling of the cytoskeleton is responsible for cell behavior and has to be precisely regulated in space and time, in response to both external and internal signals. The internal cytoskeleton of eukaryotic cells is composed of actin microfilaments, microtubules, and intermediate filaments which in turn interact with each other, as well as with a number of associated proteins.

One important component of the eukaryotic cytoskeleton is actin. Actin is only found in eukaryotes and forms highly conserved family of proteins that fall into three classes: α, β and γ-isoforms. Different actin isoforms perform different functions, even though the detailed mechanisms involved are not clear yet. Actin is mainly located in the cytoplasm, but it is also present in the nucleus [1]. It exists both as monomeric (globular or G-actin) and polymeric (filamentous or F-actin) forms, which is an active form of actin. G-actin and F-actin are present roughly at 1:1 ratio.

1.1.1 Actin polymerization

The actin cytoskeleton is a dynamic system that constantly changes through processes of actin polymerization and depolymerization of existing filaments. Polymerization consists of three phases: 1) slow initial association to a dimer that is more likely to rapidly dissociate to monomers than to assemble; 2) the formation of a stable trimer that represents the nucleus for

polymerization, a state where actin assembly is more likely than is disassembly; and 3) the elongation phase during which actin monomers are rapidly assembled by addition of subunits to both ends of the filament. The steady-state phase is characterized by a dynamic equilibrium where the length of the actin filaments remains constant, while actin monomers continually associate with and dissociate from the ends (Fig. 1).

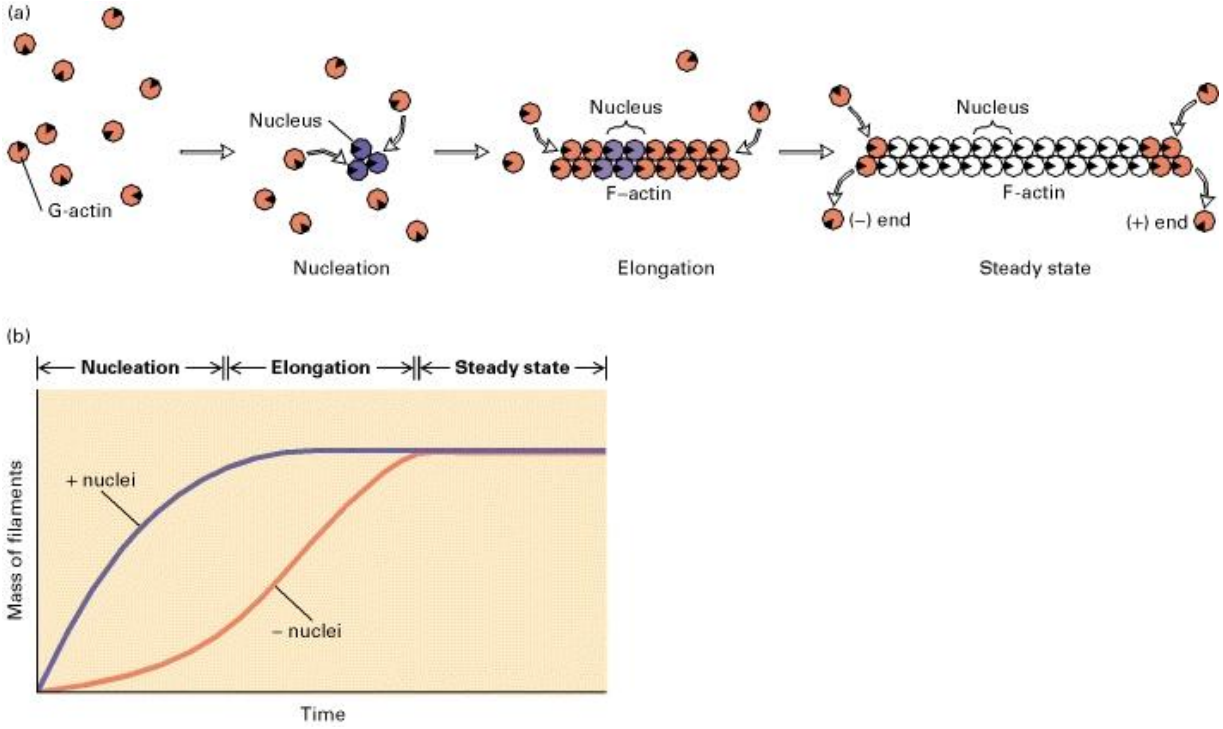

Figure 1. **The three phases of G-actin polymerization.**
a) During the nucleation phase ATP-G-actin monomers form complexes of actin. These nuclei are elongated in elongation phase. b) Time course of actin polymerization reaction reveals the initial lag period [2].

In cultured cells, conversion between G-actin and F-actin is regulated by G-actin sequestering proteins, which bind G-actin and prevent uncontrolled polymerization by lowering the concentration of free G-actin (Fig. 2).

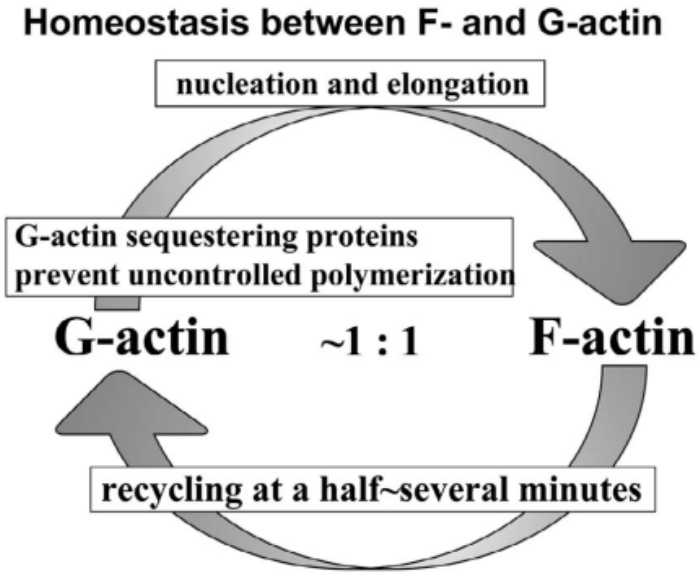

Figure 2: Homeostasis between G-actin and F-actin [3].

1.1.2 Regulation

Actin polymerization is tightly controlled by actin binding proteins such as profilin, ADF/cofilin, capping proteins, sequestering proteins etc. Signaling pathways that regulate these processes therefore regulate the actual ratio between G- and F-actin resulting in assembly or disassembly of the actin filaments [4, 5]. More than 70 types of actin-binding proteins have been identified. They modulate the function of the actin cytoskeleton in terms of polymerization and dynamics, cross-linking and bundling, nucleation and branching, actin-membrane interaction, cell-ECM interaction, cell-cell interaction, scaffolding, and signaling [6]. The activities of actin-binding proteins are often modulated by phosphorylation, by signaling molecules such as Ca^{2+} and phosphoinositides, and by small GTPases including Rac, Rho, and Cdc42 [7]. Rac, activated by the receptor tyrosine kinase pathway, including focal adhesion molecules such as integrins and growth factors, controls the formation of membrane ruffles and lamellipodia (sheet-like extensions in the leading edge of a moving cell). Rho controls the generation of adherens

junctions and focal adhesions in polarized epithelial cells, whereas Cdc42 plays a role in the formation of filopodia (finger-like protrusions consisting of actin bundles) and the reorientation of the Golgi apparatus in the direction of movement.

1.1.3 Function

Actin reorganization is required cell for attachment to the substratum and other cells. Epithelial actin-associated membrane structures include: 1) tight junctions, which cross-link cells to form a ‘‘fence’’ and regulate the paracellular pathway and epithelial permeability; 2) adherens junctions, which form a continuous adhesion belt around each interacting cell in the epithelial sheet; 3) gap junctions which connect neighboring cells by intercellular channels and 4) focal adhesions which connect the actin cytoskeleton to the ECM through the integrins and their associated actin-binding proteins. Cell-cell and cell-matrix interactions are mediated by transmembrane proteins. These proteins interact with neighboring cells or with ECM through their extracellular domains, and with cytoplasmic adaptor molecules through their intracellular domains. In focal adhesions, the extracellular domain of integrins binds to ECM, while the intracellular domain binds to many actin-binding proteins such as talin, vinculin, paxillin, and α-actinin, which in turn interact with actin [8]. Assembly of the cytoskeleton is intimately linked with integrin signaling. As integrins bind to ECM, they become clustered in the plane of the cell membrane and associate with a cytoskeletal and signaling complex that promotes the assembly of actin filaments. The reorganization of actin filaments into larger stress fibers, in turn, causes more integrin clustering, thus enhancing the matrix binding and organization by integrins in a positive feedback system (Fig. 3).

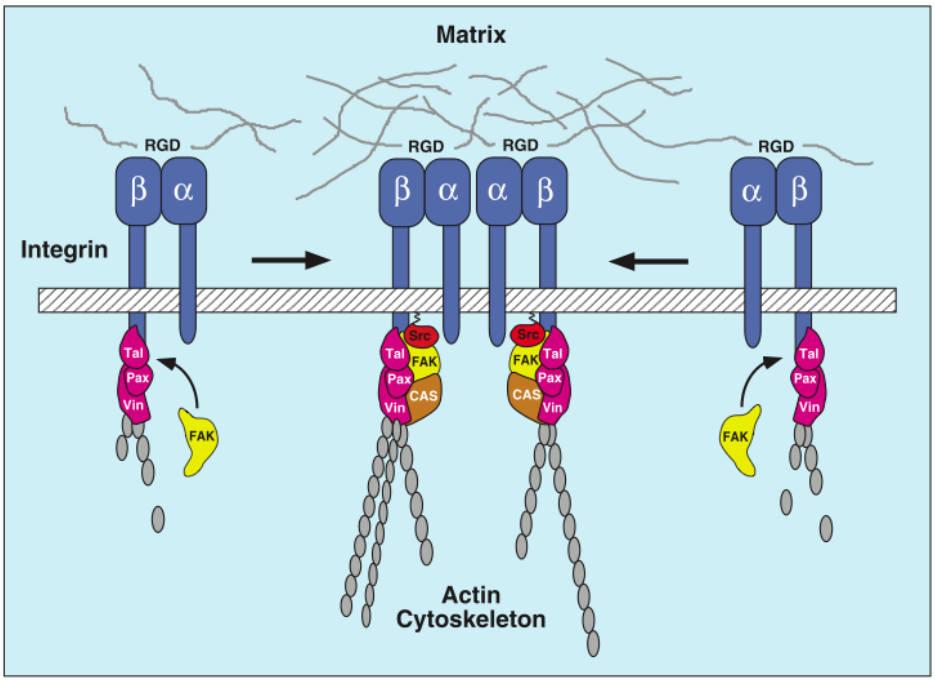

Figure 3. Integrin signaling and actin cytoskeleton assembly are intimately linked. RGD, Arg-Gly-Asp integrin-binding motif; Tal, talin; Pax, paxillin; Vin, vinculin; CAS, p130CAS. [8].

Cell-cell and cell-matrix interactions are essential for maintaining cell morphology and tissue integrity. Abnormalities in these contacts are associated with a variety of diseases, including diarrhea, carcinogenesis, and metastasis. In addition, cell-cell and cell-matrix interactions play crucial roles in cell migration. Migration through tissues and endothelial barriers is a complex series of events which requires precisely regulated processes such as cell protrusion, retraction, adhesion and detachment, all facilitated through major dynamic rearrangements of the actin cytoskeleton. Abnormal actin cytoskeleton reorganization can cause defects in cell polarity and chemotaxis, and inhibit formation of normal protrusions such as filopodia. Thus, actin by controling cell adhesion and detachment from surfaces is intimately linked to cell motility.

Filamentous actin, in cooperation with accessory proteins, forms differently organized cytoskeletal networks that play crucial role in cell adhesion, migration, cell shape changes, endocytosis and exocytosis. Mutations of these accessory proteins can cause defects in cellular processes that are based on actin cytoskeleton reorganization. It is crucial to understand how actin can change its conformation for various biological processes, which cellular components are involved in this transition and what mechanisms actin uses to maintain its various biological functions.

Proper actin polymerization/depolymerization is necessary for in a variety of cellular processes, such as cell migration and cellular morphogenesis. Highly motile tumor cells are characterized by increased actin polymerization, and thereby increased amounts of F-actin [9]. However, an increased proportion of F-actin has also been observed in cells displaying active locomotion, such as epithelial cells moving over an open wound and regenerating liver cells, indicating that changes in actin polymerization are characteristic of cell activation and movement rather than of malignant transformation [10]. Thus, increased amounts of F-actin in tumor cells suggest that actin in these cells might be organized in order to interact with myosin and participate in contractile events.

The actin cytoskeleton regulation is crucial for many cellular functions that are involved in immune response, such as cell migration, extravasation, antigen recognition and uptake, and cell activation. At the inflammatory site, phagocytosis of particulate antigens and uptake of soluble antigens through pinocytosis by neutrophils and macrophages requires protrusion and retraction of the plasma membrane, which is regulated by the actin cytoskeleton [11]. Thus, misregulation of the proteins which are key regulators of the actin cytoskeleton and hence

essential for multiple cell functions, including cell motility, may lead to immunodeficiency disorders [12-18].

1.2 *Dictyostelium discoideum*

Like other eukaryotes, *Dictyostelium discoideum* depends upon proper actin regulation for survival. *Dictyostelium discoideum* undergoes many processes characteristic of complex eukaryotes, including chemotaxis, endocytosis, exocytosis and adhesion. It also undergoes a relatively simple program of multicellular development, which in many ways resembles animal development. Signaling in *Dictyostelium* involves extracellular cues that coordinate changes in cell behavior, cell fate, and cell-cell communication. Given its similarity to mammalian cells, *Dictyostelium* is a well-established model for amoeboid cell movement, like that seen in leukocytes [19]. Both types of cells use G protein–mediated signaling to regulate chemotaxis and the second messenger pathways activated are similar between the two types of cells. However, unlike in leukocytes, various genetic approaches can be used in *Dictyostelium discoideum* to identify genes implicated in actin-required processes as well as the functions of the gene products. Generation of null mutants in cytoskeletal protein genes by homologous recombination has allowed illumination of the roles of myosin and other actin-binding proteins in actin dynamics [20].

1.2.1 *Dictyostelium discoideum* life cycle

Chemotaxis is a key mechanism in both the vegetative and the multicellular development cycles of *Dictyostelium discoideum*. In the vegetative stage, cells rely on chemotaxis to folate in order to hunt bacteria. Upon starvation, up to 10^5 cells use chemotaxis to cAMP to aggregate and

form a multicellular organism. This stage is called developmental phase and culminates in the formation of fruiting body structures (Fig. 4) [21]. The fruiting body consists of dead vacuolated stalk cells that support a mass of spore cells. The spores disperse and each spore can germinate to start a new colony under favorable conditions supporting live, viable spore cells which are eventually dispersed to areas with more abundant sources of food [22].

Figure 4. Fruiting body.

A few hours after starvation, cells become sensitive to cAMP due to expression of cell-surface cAMP receptors. The cells show a chemotactic movement in the direction of higher cAMP concentration. The aggregation process is governed by periodic cAMP signal generation by cells in the aggregation center, cAMP signal relay by all other cells and chemotactic movement of cells up the cAMP gradient.

Chemotaxing cells, which are highly polarized and elongated, form aggregation streams. They make specific cell-cell interactions via the EDTA-resistant cell adhesion molecule, contact site A (CSA) which is induced by pulses of cAMP [23, 24]. Cells forming the aggregation stream eventually migrate to the aggregation center. The cells entering the aggregation center pile on top of each other to form three dimensional hemispherical structure, called a mound [25].

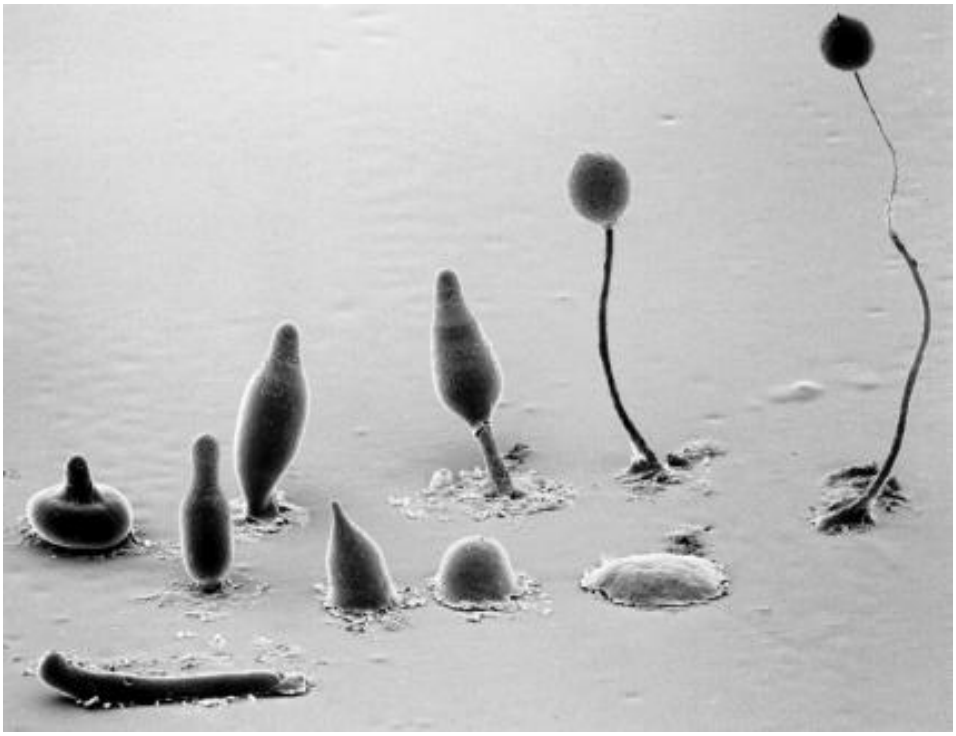

Figure 5. The *Dictyostelium discoideum* developmental cycle involves aggregates of starving cells progressing through various developmental stages to form fruiting body structures [21].

Each cell in the mound moves as an individual, but the behavior of all cells is coordinated. Once the hemispherical aggregates are formed, the cells start to differentiate into a prestalk and prespore population, after which, cell sorting takes place. During cell sorting, a dynamic movement of differentiated cells leads to the formation of tipped mounds [26]. The tip is formed on top of the mound from prestalk cells and acts as an organizer for cell migration [25]. The precise mechanism of the cell sorting is still unclear but may involve cell-type specific differences in cAMP chemotaxis [27], in the function of the actin cytoskeleton [28] and/or in adhesion [29]. Tipped mounds extend into finger-like structures and development proceeds until the culmination phase is reached and fruiting body formed (Fig. 5).

Development of *Dictyostelium discoideum* is highly dependent upon proper actin cytoskeleton regulation. Two proteins known to play a role in such regulation are paxillin and phospholipase D (PLD).

1.3 Paxillin

The mammalian protein paxillin is a 68 kDa molecule which serves as an adapter and anchor protein at the plasma membrane but has no intrinsic enzymatic activity. It is a cytoskeletal protein that localizes to areas of cell/matrix contact called focal adhesions. Deletion of paxillin in mice leads to early embryonic lethality [30]. Analysis of these mutants has shown paxillin to be essential for the development of mesodermal tissues [31], formation of focal adhesions and cell migration [32, 33]. It has been also suggested that paxillin is involved in the dynamics of focal adhesions and consequently cell migration in primary foreskin fibroblast and glioblastoma cell lines [34]. Taken together, these data imply that paxillin plays a crucial role in adhesion and cell movement. However, the role of paxillin in cell motility has not been well established, as the reports on this are controversial.

1.3.1 Protein interactions

As a scaffolding protein, paxillin contains multiple domains that interact with cytoskeletal and signaling molecules (Fig. 6). The amino terminus contains five leucine-rich LD motifs that function in protein recognition [35]. They provide specific protein interaction interfaces. LD1 mediates interactions with actopaxin [36], the integrin-linked kinase (ILK) [37],

vinculin [38] and papillomavirus protein E6 [39]. LD2 binds to vinculin and FAK/PYK2 [38, 40]. LD4 binds to actopaxin [36], FAK/PYK2, clathrin [38, 40]. The carboxy terminus of paxillin consists of four LIM domains. LIM domains are double-zinc finger motifs that mediate protein-protein interactions [41]. They allow specific subcellular localization to the actin cytoskeleton and focal adhesions. Specifically, LIM3 (with LIM2 cooperating) mediates localization of paxillin to focal adhesions [40] and adhesion to fibronectin [42]. Tubulin is identified as a binding partner for LIM2 and LIM3 domains [43] and PTP-PEST as a binding partner for LIM3 and LIM4 of paxillin [44]. Thus, paxillin binds to many proteins that are involved in actin cytoskeleton reorganization, which is necessary for cell motility events associated with embryonic development, wound repair and tumor metastasis.

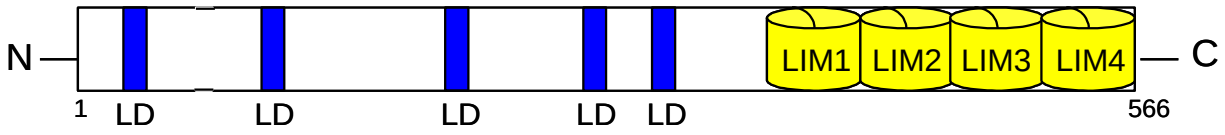

Figure 6. Mammalian paxillin [40].

1.3.2 Function and regulation

Paxillin is mostly regulated through tyrosine and serine phosphorylation. Paxillin is tyrosine phosphorylated via activation of FAK and SRC kinases [45, 46] with tyrosine residues 31 and 118 as primary targets [45, 46]. Various agents trigger paxillin tyrosine phosphorylation through a range of transmembrane receptors and provoke significant changes in the organization of the cytoskeleton. Epidermal growth factor (EGF) [47], growth hormone [48], hepatocyte growth factor [49], insulin-like growth factor I (IGF-1) [50, 51], vascular endothelial growth

factor [52] each stimulate paxillin tyrosine phosphorylation. In addition, agonists of 7 transmembrane domains serpentine family receptors induce paxillin tyrosine phosphorylation, including: acetylcholine [53], epinephrine [54], angiotensin II, thrombin [55], bombesin, vasopressin, endothelin [56], bradykinin [57] and lysophosphatidic acid (LPA) [58]. Cellular stress such as membrane depolarization [59], tissue injury [60], shear stress [61], increased cell density [62] also induce paxillin tyrosine phosphorylation. A major consequence of paxillin tyrosine phosphorylation is the generation of binding sites for SH2 domain-containing proteins and consequently regulation of protein interactions.

In addition to tyrosine phosphorylation, paxillin serine/threonine phosphorylation is also well documented. MAP kinase, JNK, has been reported to directly phosphorylate paxillin S178 to regulate cell migration [63] while p38 MAP kinase phosphorylates S85 and regulates neurite outgrowth [64]. ERK1/2 has been reported to directly phosphorylate paxillin [65, 66]. Paxillin serine phosphorylation increases when fibroblasts attach to fibronectin or when macrophages adhere to vitronectin [67]. While these results may provide the first clues that phosphorylation of these sites regulates adhesion and migration, additional studies are required to fully elucidate the function of phosphorylation of paxillin on serine/threonine residues.

Paxillin phosphorylation regulates cell spreading and cell migration [46]. While paxillin tyrosine phoshorylation is involved in generation of binding sites for FAK and vinculin and subsequent localization of these proteins to focal adhesions [68], serine/threonine phosphorylation of the LIM domains is important for localization [42]. Studies in NBTII bladder tumor cells have shown that induced paxillin phosphorylation affects cell adhesion, spreading and motility [69]. Paxillin phosphorylation by integrin and growth factor receptors [30, 70, 71], known players in tumorigenesis and invasion [72-74], suggests a role for paxillin in cancer.

Metastatic lung cancer has been correlated with a decrease in paxillin expression and tyrosine phosphorylation [75, 76]. However, paxillin upregulation is reported in metastatic renal [77] and prostate carcinomas [78]. Observed contradictions regarding a direct correlation between paxillin expression/phosphorylation and cancer aggressiveness suggest tissue-specific and context-specific roles for paxillin in cellular function. Thus, further research is necessary to elucidate the precise role of paxillin in cancer and metastasis.

1.4 Phospholipase D

Like paxillin, Phospholipase D (PLD), the enzyme responsible for the hydrolysis of phosphatidylcholine (PC) resulting in the production of choline and the second messenger phosphatidic acid (PA), has also been functionally linked to the actin cytoskeleton [79]. It is implicated in a variety of actin-required cellular processes including endocytosis, exocytosis, cell migration, and cancer development [9, 80].

There are two mammalian PLD genes: PLD1 and PLD2. PLD2 is exclusively localized on the plasma membrane in light membrane "lipid raft" fractions and is constitutively active [81, 82]. PLD1 is localized throughout the cell, particularly in perinuclear, Golgi, and heavy membrane fractions [81-83]. Although it is primarily associated with intracellular membranes, it can also be found in lipid rafts [82, 84]. It has low basal rate of catalysis and is regulated by PKCα, ARF, and RhoA family members [85, 86]. Protein kinase C (PKC) may phosphorylate PLD or some associated regulatory component [87, 88]. In addition, a phosphorylation-independent activation mechanism has been suggested [89]. ARF is an activator of PLD activity

in HL-60 cells [90, 91], and is GTP dependent [86]. The role of Rho in PLD activation is not clear. Rho may use PLD to mediate cell morphology and motility.

1.4.1 Function and regulation

It has been shown that PA, the product of PLD anzymatic activity, affects actin cytoskeletal rearrangement and hence lamellipodium extension as well as migration. PLD activity has been found in detergent-insoluble membrane fractions in which a wide variety of cytoskeletal proteins, such as F-actin, α-actinin, vinculin, paxillin, and talin, were enriched [79]. Furthermore, the stimulation of PLD with physiologic and pharmacologic agonists results in its association with actin filaments [79]. In addition, actin polymerization is tightly coupled to the activation of PLD [92]. However, the mechanisms that regulate the activation and localization of PLD as well as pathways downstream of PLD are still poorly understood.

There are a number of proteins identified that negatively regulate PLD activity. Synaptojanin inhibits the activity of phosphoinositide dependent PLD enzymes including PLD1 in vitro by hydrolysis of the activator PIP_2 [93]. Fodrin (a non-erythroid form of spectrin) inhibits ARF activated PLD, possibly through the sequestration of PIP_2 [94]. Clathrin assembly protein-3 (AP-3) interacts with and inhibits PLD1. This interaction may play a role in endocytosis of released synaptic vesicles [95]. Synucleins are proteins involved in familial Parkinson's and Alzheimer's diseases [96] and are potent inhibitors of PLD2 activity in vitro. PLD1 is less sensitive to inhibition [97]. Although there are a number of intriguing possibilities that remain to be fully explored experimentally, so far it seems that negative regulation activities

could play an important role in modulation of PLD1 activity and might be most important in regulation of PLD2 activity.

Taken together, several lines of evidence suggest that PLD is functionally associated with actin cytoskeleton, just like paxillin is. However, the mechanism responsible for this association and whether it is mediated by paxillin is unclear. In addition, growth factors/mitogens such as epidermal growth factor (EGF), platelet derived growth factor, insulin, and serum that implicate tyrosine kinases are important regulators of PLD activity [98-101]. However, several gaps in knowledge still exist in spite of its importance in cell signaling. Our work revealed a functional interaction between paxillin and PLD that depends upon Erk activity. This knowledge is important because understanding of the precise role of PLD in actin-based processes as well as its regulation should lead to new directions for the development of PLD regulators as potential therapeutics.

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Chapter 2: Paxillin and Phospholipase D interact to regulate actin-based process in *Dictyostelium discoideum*

2.1 Introduction

Cytoskeletal networks determine cell shape, organelle distribution and the cytoplasmatic consistency of cells. Dynamic remodeling of the cytoskeleton is responsible for cell behavior and has to be precisely regulated in space and time, in response to both external and internal signals. One important component of the eukaryotic cytoskeleton is actin. Filamentous actin, in cooperation with accessory proteins, forms differently organized cytoskeletal networks that play a crucial role in cell adhesion, migration, cell shape changes, phagocytosis and exocytosis [1-3]. Structural and signaling proteins facilitate organization of actin filaments into appropriate networks in response to external signals. Mutations of these accessory proteins can cause defects in cellular processes that are based on actin cytoskeleton reorganization [4-6].

Two such proteins involved in actin-dependent processes are paxillin and phospholipase D (PLD). Paxillin acts as an adapter and anchor protein by recruiting diverse structural and signaling proteins into a complex to assist transmission of downstream signals [7]. Paxillin provides binding sites at the plasma membrane for tyrosine kinases such as FAK and SRC, and cytoskeletal proteins, such as vinculin and parvin [8-11]. Paxillin also serves as a docking protein to bring enzymes, PTP-PEST and CSK, into close proximity to their targets [12-14]. Paxillin is implicated in cell migration, but its precise role in this process has not been well established [15-17]. In addition, it is a proposed player in phagocytosis and exocytosis [18, 19].

PLD hydrolyzes phosphatidylcholine, resulting in the production of choline and the second messenger phosphatidic acid. Like paxillin, it has been implicated in cytoskeletal

reorganization, endocytosis, exocytosis and cell migration [20]. While paxillin and PLD both regulate the actin cytoskeleton, it has yet to be determined whether these proteins function separately or together to perform this task.

2.1.1 *Dictyostelium discoideum*

Like other eukaryotes, *Dictyostelium discoideum* depends upon proper actin regulation for survival. *Dictyostelium discoideum* is a soil amoeba that feeds on bacteria by phagocytosis and can grow axenically in nutrient broth by macropinocytosis, both processes which depend upon actin [21-23]. Upon nutrient depletion, the solitary amoebae enter a multicellular developmental program. Starving cells start attracting each other by sensing and secreting cAMP. Highly polarized and elongated cells, connected head to tail, forming aggregation streams. The cells entering the aggregation center become ameboid again and pile on top of each other to form a three dimensional hemispherical structure called a mound [24]. Cells differentiate into prestalk and prespore populations, after which cell sorting takes place. During cell sorting, prestalk cells form a tip on the top of the mound which acts as an organizer for culmination and fruiting body formation [25]. Thus, normal development requires proper cell shape, cell-substrate adhesion, cell-cell adhesion and locomotion, all of which depend upon proper actin regulation. Like mammalian cells, *Dictyostelium discoideum* has paxillin and PLD.

2.1.2 *Dictyostelium discoideum* paxillin

Dictyostelium discoideum paxillin, PaxB, has 33% amino acid identity and 46% homology with mammalian paxillin. *Dictyostelium discoideum* PaxB contains four of the five conserved LD domains and all four conserved LIM domains found in mammalian paxillin. LD

domains correspond closely to LD1, LD2, LD3 and LD5 domains of vertebrate paxillin, although the spacing between these domains is not conserved. PaxB is produced at low level in the vegetative state, but production rises dramatically by 8 to 12 hours, corresponding to the mound and tipped mound developmental stages. Production remains high at the slug stage and then decreases gradually [26]. The expression pattern of the *paxB* gene suggests the protein is highly produced during the formation of mound structures, the developmental stage where cell-cell contacts, cell-substrate adhesion and differential cell movement occurs. It has been shown that PaxB is required for proper cell-substrate adhesion in *Dictyostelium discoideum* and PaxB-GFP localized to small stationary spots at the sites of cell-substrate contacts [26]. PaxB foci were formed at the leading edge of cells and stayed present as long as the cells were attached to the substrate. In *Dictyostelium* cells, adhesion sites are formed at the distal ends of filopodia at the cell-substrate contact. PaxB is recruited to these sites, after which they are rapidly turned over [27]. Loss of PaxB leads to cells forming fewer cell-substrate adhesions and becoming less adherent to the substrate [26]. Thus PaxB is required for the correct regulation of cell substrate adhesion, which is critical for directed cell migration. It has been shown that *paxB* knockout cells are able to aggregate, stream and form flat mounds of normal morphology. However, upon reaching the mound stage, further development was arrested [26].

Previous work done in our laboratory has shown that *paxB* overexpressing cells undergo the initial stages of development normally and that they reach the mound stage at the same time as the wild-type cells. However, while wild-type cells form fruiting bodies by 24 hours, *paxB* overexpressing cells remain arrested at the mound stage [28]. The developmental phenotypes of *paxB* knockout and *paxB* overexpressing cells suggest that PaxB may play a role in cell migration during the multicellular stages of development.

2.1.3 *Dictyostelium discoideum* Phospholipase D

Dictyostelium discoideum also contains a homologue of mammalian Phospholipase D, PldB. Like paxillin, PLD has been implicated in cytoskeletal reorganization, endocytosis, exocytosis and cell migration [29], all of which depend from actin. A recent report states that PLD regulates integrins that support stable adhesion during neutrophil migration [20]. PldB may also have an important role in these processes. Furthermore, it has been demonstrated that PLD activity is essential for normal motility, F-actin distribution, endocytosis and phagocytosis of *Dictyostelium* cells [30]. In addition, PldB is required for cell migration and has been observed at protruding areas of moving cells [31]. Thus, like their mammalian counterparts, PaxB and PldB are implicated in regulating the actin cytoskeleton.

Dictyostelium discoideum is a well established model for studying the mechanisms of actin cytoskeleton rearrangement. Various genetic approaches allow the identification and functional characterization of genes involved in actin-dependent processes [4, 5, 32, 33]. Given the fact that homologous proteins exist, *Dictyostelium discoideum* serves as an excellent model system for studying the roles of paxillin and PLD and their potential interactions. Here we used genetic and biochemical approaches to elucidate a functional relationship between PaxB and PldB. We show that PaxB and PldB physically interact, and functionally interact in a number of developmental and actin-based processes.

2.2 Materials and methods

2.2.1 Strains and culture conditions

Dictyostelium discoideum cells of the wild-type strain AX2 were grown axenically at 22^0C in HL5 nutrient medium (0.5% (w/v) yeast extract, 0.5% protease peptone, 0.5% thiotone

peptone, 1% dextrose, 4.7 mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH 6.5) [34]. HR30 cells (AX2 cells expressing β-galactosidase) [35] and *pldB*OE cells (AX2 cells overexpressing *pldB*) [36] cells were grown in HL5 supplemented with 20 µg/ml G418. *paxB*$^-$ cells were grown in HL5 supplemented with 10 µg/ml Blasticidin [26]. *paxB*$^-$/*pldB*OE cells were created by transforming a plasmid containg the *pldB* gene under the control of the constitutively active actin 15 promoter [36] into *paxB*$^-$ cells [26] by electroporation as described previously [37, 38]. Transformed cells were selected in the presence of 10 µg/ml Blasticidin and 20 µg/ml G418. Individual clones were isolated on GYP plates (0.2% peptone, 0.025% yeast extract, 2.2% agar, 0.1% dextrose, 19 mM Na_2HPO_4, 30 mM KH_2PO_4) containing 10 µg/ml Blasticidin and 20 µg/ml G418. To initiate development, cells at mid-log phase (2-5 X 10^6 cells/ml) were washed twice with PBM (20 mM KH_2PO_4, 10 µM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1 with KOH) and plated on to nitrocellulose filter pads 2.4 cm diameter (Millipore, Billerica, MA) at 1 X 10^7 cells/pad at 22^0C [39].

2.2.2 Fluorescence microscopy

Cells were washed twice with PBM and starved on an 8 well chambered slide for 6 hours. Cells were fixed (3.7% formaldehyde solution in PBS for 5 min) and permeabilized (0.2% Triton X-100 solution in PBS for 5 minutes). F-actin was labeled with 8 U/ml Rhodamine phalloidin (Invitrogen, Eugene, OR) for 1 hour in the dark. Slow Fade Gold (Invitrogen, Eugene, OR) mounting solution was added prior to placing a coverslip onto the slide. Confocal fluorescence microscopy was performed with a Leica laser-scanning microscope equipped with a 100X (HCX PLAN APO, CS 1.40) objective and Leica Confocal Software.

2.2.3 Western blot analysis

1 X 10^7 cells were collected and boiled for 3 minutes in SDS-PAGE sample buffer in a final volume of 500 µl. Proteins were separated by SDS-PAGE, transferred to Hybond-P membrane (Amersham Bioscience, Piscataway, NJ), immunoblotted with a peptide-purified anti-PaxB or anti-PldB antibodies [28, 36] and visualized by enhanced chemiluminescent substrate for HRP detection (Pierce, Rockford, IL).

2.2.4 Chimeras

Chimeras were created and stained as previously described with some adjustments [40]. Chimeras consisting of 10% HR30 cells and 90% *paxB*$^-$, *pldB*OE or *paxB*$^-$/*pldB*OE cells were developed on white filter pads, 0.8 mm pore size (Millipore, Billerica, MA). Chimeric structures were fixed with glutaraldehyde solution (0.1% glutaraldehyde, 0.1% TritonX-100 in Z-Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$)), washed twice for 10 minutes in Z-Buffer and then incubated in staining solution (5 mM $K_3[Fe(CN)_6]$, 0.4 mg/ml X-gal, and 0.5% Tween 20 in Z Buffer) at 37^0C overnight. Images were taken with a dissecting microscope equipped with a 4.5X objective, utilizing SPOT Advanced program with a SPOT insight color camera 3.2.0 (Diagnostics Instruments, USA).

2.2.5 Chemotaxis assay

Under agarose chemotaxis assays were performed as previously described with some adjustments [41]. In folate chemotaxis, vegetative cells were collected and resuspended to 1 X 10^6 cells/ml. 100 µl of cells were used in the assay. For cAMP chemotaxis, 1 X 10^7 cells were starved on filter pads for 6 hours prior to the assay. Cells were collected and resuspended in 1 ml

PBM. A 100 µl sample of cells was used in the assay. 1hour after the cells were placed in the wells, images of the cells migrating under the agarose were taken every 30 seconds for 20 minutes on an inverted Nikon TE 200 Eclipse microscope using Metafluor Image System (Molecular Devices, Downingtown, PA) viewed through a 10X objective. The individual cells were tracked using Image J software. Directionality is defined as (the absolute displacement)/ (the total path). Chemotaxis index is the cosine of the angle between the line up the gradient and the line drawn from the cell's start point to its end point.

2.2.6 Actin polymerization assay

cAMP-induced F-actin formation was measured as described previously with some adjustments [42]. 1 X 10^7 cells were starved on filter pads for 6 hours prior to the assay. Cells were collected and resuspended in 1 ml PBM. A 100 µl sample of cells was used in the assay. The cells were fixed (3.7% formaldehyde solution in PBS) before and after stimulation with cAMP. After staining with 8 U/ml Rhodamine phalloidin (Invitrogen Eugene, OR) in the dark for 1 hour, the fluorescence of individual cells was analyzed by flow cytometry. Cells were resuspended to 1 X 10^6 cells/ml in cold PBS containing 0.5% BSA and passed through a fluorescence-activated cell sorter (FACSCalibur, Beckton Dickinson, San Jose, CA, USA). Cells were identified based on forward and side scatter, and their fluorescence was measured (excitation 488 nm, emission 572 nm). The mean fluorescence value was determined for at least 10,000 cells, after background auto fluorescence of unstained cells was determined and subtracted.

2.2.7 Phagocytosis and exocytosis assays

Phagocytosis was measured using a modification of a previously described assay [43]. Cells grown to 2 X 10^6 cells/ml were harvested and resuspended in HL5. After 15 minutes of shaking, 1-µm latex beads conjugated with TRITC (Sigma, MO) were added to a final concentration of 100 beads / cell. 2 X 10^5 cells were collected at specific time points and 2% formaldehyde was added to stop the uptake. The cells were then washed three times in PBS and lysed in 5 mM glycine-NaOH (pH 8.5) containing 100 mM sucrose and 0.2% Triton X-100. The fluorescence of the lysate was measured in a Typhoon 9410 Imager (Amersham Bioscience, Piscataway, NJ) with excitation and emission wavelengths 532 nm and 610 nm, respectively. Relative fluorescence against time was plotted and the phagocytosis rate determined. That of wild-type cells was set as 100%. The exocytosis assay was essentially performed as previously described [33]. Cells grown to 2 X 10^6 cells/ml were harvested, resuspended in HL5 containing FITC-Dextran (Sigma, MO) at 2 mg/ml and allowed to internalize it for 3 hours. The cells were then collected and resuspended in fresh HL5. At various time points, 1 ml of cells was collected and 2% formaldehyde was added to stop the uptake. The cells were then washed once in HL5 and twice in 5 mM glycine-NaOH (pH 8.5) containing 100 mM sucrose. Then the cells were lysed in the same buffer containing 0.2% Triton X-100. The fluorescence of the lysate was measured in a Versa Fluor^{TM} Fluorometer (Biorad, Hercules, CA) with excitation and emission wavelengths 495 nm and 520 nm, respectively. Relative fluorescence against time was plotted and exocytosis rate determined. That of wild-type cells was set as 100%.

2.2.8 Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA), followed by t-test for pair-wise comparisons. A p value < 0.05 was considered statistically significant. Absence of a star means no significant difference from the wild-type.

2.3. Results

2.3.1 PaxB and PldB are part of a protein complex

Paxillin and PLD co-localize in leukocytes [44]. To determine whether their orthologs in *Dictyostelium discoideum* also co-localize, we labelled starved *Dictyostelium* cells with antibodies to PaxB or PldB, and the F-actin stain Rhodamine-phalloidin (Fig 1).

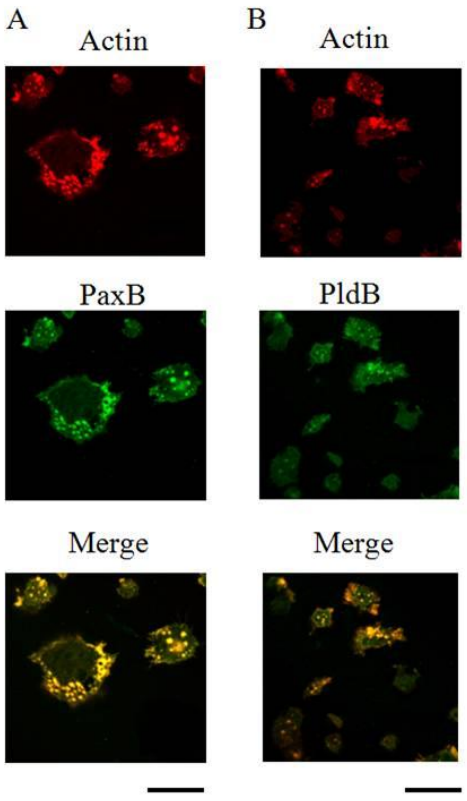


Figure 1. Co-localization of PaxB and PldB with F-actin by immunofluorescence.
6h starved vegetative cells were double-labeled with PaxB (A) or PldB Ab (B) and Rhodamin-phalloidin (A and B) to visualize F-actin. Bars, 10 µm.

We found overlapping localization of PaxB with F-actin rich spots at the cell-substrate interface (Fig. 1A). In addition, we found co-localization of PldB with F-actin rich spots at the cell-substrate interface (Fig. 1B). Thus, both PaxB and PldB are found associated with F-actin at the cell-surface interface.

The common localization of PaxB and PldB with F-actin is consistent with these proteins physically interacting. To investigate this possibility, co-immunoprecipitation assays were performed. Cells were starved for 14 hours and the lysates were immunoprecipitated with antibodies to PaxB and probed for the presence of PldB. As seen in Figure 2, PldB was detected in wild type Ax2 cells immunoprecipitated with PaxB antibodies, suggesting these proteins exist in a complex. PldB was not present in *paxB*$^-$ immunoprecipitated samples, demonstrating that co-immunoprecipitation of PldB is dependent upon PaxB. This suggests a physical interaction, either direct or indirect, between PaxB and PldB.

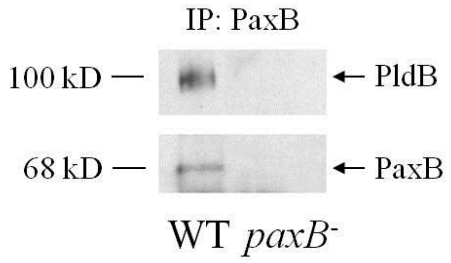


Figure 2. PaxB and PldB co-immuniprecipitate.
Wild-type and *paxB*$^-$ cells were lysed, and the samples immunoprecipitated with α-PaxB antibodies. The immnoprecipitates were analyzed by Western blotting with α-PldB antibodies (A) or α-PaxB antibodies (B).

2.3.2 Overexpression of *pldB* rescues the developmental block and cell sorting defect of *paxB*$^-$ cells

Upon starvation *Dictyostelium discoideum* cells enter a developmental phase that culminates in the formation of a fruiting body, consisting of dead stalk cells supporting live,

viable spore cells. The process begins when starved cells use cAMP to aggregate, forming a mound which eventually undergoes morphogenesis to form the fruiting body. PaxB is required for this process as *paxB*$^-$ cells are not able to progress fully through development and are arrested at the mound stage (Figure 3A) [26]. As previously reported, overexpression of *pldB* prevents cells from aggregating [36]. Interestingly, overexpression of *pldB* in *paxB*$^-$ cells resulted in multicellular development comparable to that seen in wild-type cells (Figure 3A). Therefore, PaxB and PldB may act in a shared process during development, suggesting a functional interaction between PaxB and PldB.

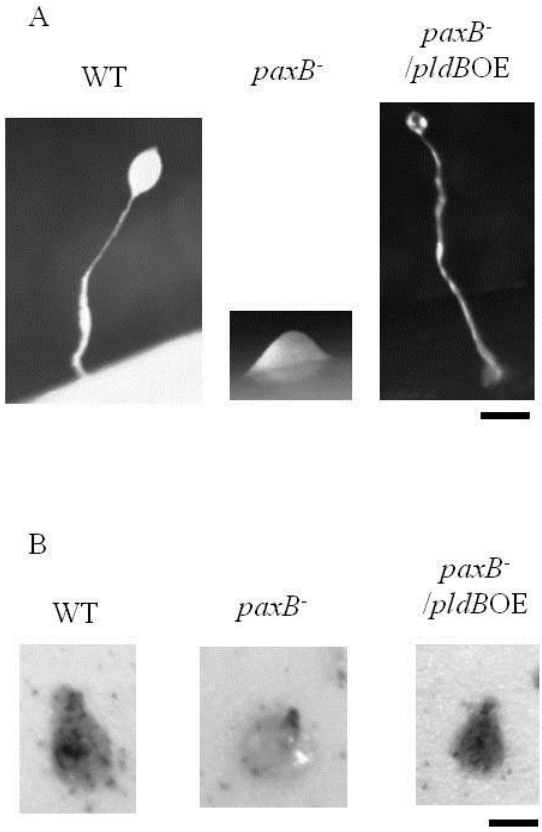


Figure 3. Development and cell sorting of *paxB*-/*pldB*OE is normal.
(A) Logarithmically growing wild-type, *paxB*- and *paxB*-/*pldB*OE cells were starved on filters. Pictures of structures were taken after 24 hours. Bar, 0.2 mm. (B) Wild-type cells carrying the actin 15:lacZ reporter construct (HR30) were mixed in a 10:90 ratio with wild-type, *paxB*- and *paxB*-/*pldB*OE cells and starved on filters. Tipped mounds were fixed, stained and photographed after 16 hours. Bar, 0.2 mm.

In addition to their developmental arrest, *paxB*$^{-}$ cells are also defective in cell sorting, in that when mixed with wild-type cells they are unable to sort to the tip of the mound [26]. This sorting is essential for morphogenesis into the fruiting body. To determine whether the overexpression of *pldB* rescues this defect in *paxB*$^{-}$ cells, we examined the localization of HR30 (wild-type AX2 cells expressing β-galactosidase) cells in chimeras with wild-type, *paxB*$^{-}$, *pldB*OE and *paxB*$^{-}$/*pldB*OE cells. As expected, chimeras composed of 90% wild-type cells showed random distribution of the HR30 in the tipped mound, demonstrating that expression of β-galactosidase had no effect on cell sorting (Figure 3B). In chimeras consisting of 90% *paxB*$^{-}$ cells, HR30 cells were found in the tip of the mound (Figure 3B), consistent with the defect in cell sorting previously demonstrated in *paxB*$^{-}$ cells [26]. Chimeras with 90% *pldB* OE cells failed to aggregate. Interestingly, in chimeras with 90% *paxB*$^{-}$/*pldB*OE cells, HR30 cells were randomly distributed in the tipped mound, suggesting that the cells carrying the double mutation are able to sort similarly to wild-type cells (Figure 3B). Thus, much like with fruiting body formation, overexpression of *pldB* rescues the inability of *paxB*$^{-}$ cells to sort properly in the mound.

2.3.3 The production of PaxB and PldB are independent of each other

The ability of *pldB* overexpression to rescue the developmental defect observed in *paxB*$^{-}$ cells could be due to *paxB*$^{-}$ cells not expressing *pldB*. To test this possibility, we examined production of PaxB and PldB in wild-type, *paxB*$^{-}$, *pldB*OE and *paxB*$^{-}$/*pldB*OE cells. Western blot analysis confirmed that the levels of PldB in *paxB*$^{-}$ cells and *paxB*$^{-}$/*pldB*OE cells were similar to those in wild-type and *pldB*OE cells, respectively (Figure 4). In addition, the levels of PaxB were unchanged in *pldB*OE cells as compared with wild-type cells (Figure 4).

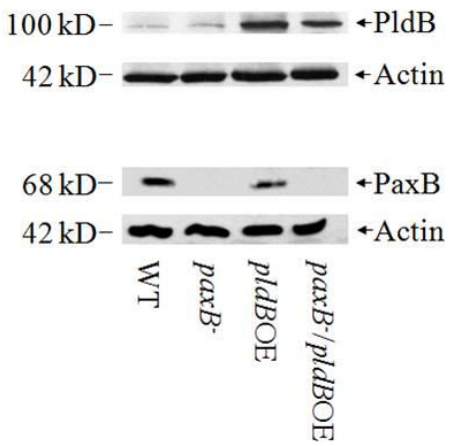


Figure 4. Production of PaxB and PldB is independent of each other.
Total cell lysates from wild-type, *paxB*-, *pldB*OE and *paxB*-/*pldB*OE cells were analyzed by Western blot for the presence of PldB and PaxB. Actin from the same lysates was probed to confirm equal loading.

This indicated that loss of *paxB* does not affect the production of PldB, nor does overexpression of *pldB* affect the production of PaxB. Thus the rescue in development is not due to restoration of PldB production and is most likely indicative of a functional interaction between PaxB and PldB.

2.3.4 PaxB and PldB functionally interact during cAMP chemotaxis

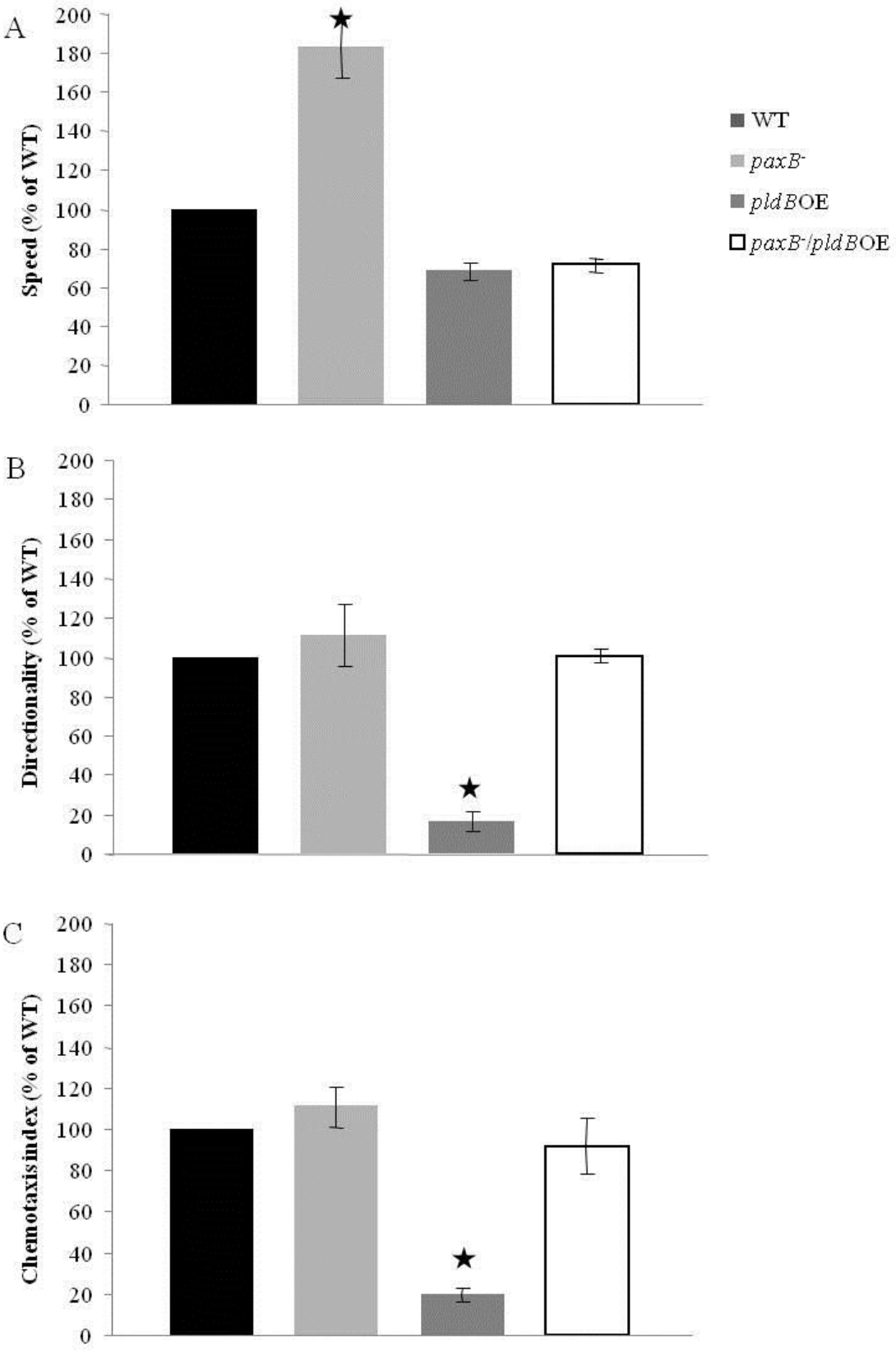
As cAMP chemotaxis is required for aggregation and tip formation, the developmental block seen in *pldB* OE and *paxB*$^-$ cells could be due to inefficient chemotactic movement. To examine this possibility, we measured cAMP chemotaxis in wild-type, *paxB*$^-$, *pldB*OE and *paxB*$^-$/*pldB*OE cells using an under agarose assay. In this assay, cells move up a stable chemotactic gradient by crawling underneath a thin layer of agarose. Wild-type cells moved towards the cAMP source with an average speed of 7.9 ± 0.4 μm/min, which is consistent with previous reports [45-47]. The speed of *pax*B$^-$ cells was increased over that of wild-type cells by 80%. *pldB*OE cells had a speed similar to wild-type cells. Interestingly, *paxB*$^-$/*pldB*OE cells also had a

speed similar to wild-type cells (Figure 5A), suggesting that overexpression of *pldB* rescues the defect in speed in *pax*B$^-$ cells.

To analyze the efficiency of chemotaxis, we determined the directionality of the cell movement as well as the chemotaxis index. Directionality is a measure of the deviation of the cell movement from the straight path and is calculated as the ratio of the distance of a straight line from the cell's start point to its end point to the length of the total path followed by the cell. Thus, cells that move in a straight line have the maximal directionality value of 1, while cells that make a random walk and end up back at their starting point will have a directionality of 0. In accordance with previous work, wild-type cells moved with a directionality of 0.65 ± 0.05 [45, 47]. In *pax*B$^-$ cells, the directionality was similar to wild-type cells. In *pldB*OE cells it was drastically reduced by 82% when compared to wild-type cells. Surprisingly, *paxB*$^-$/*pldB*OE cells moved with directionality comparable to the wild-type cells (Figure 5B).

The chemotaxis index (CI) is a measure of how well the cell moves up the gradient and is defined as the cosine of the angle between the line up the gradient and the line drawn from the cell's start point to its end point. Thus, cells that move directly towards the cAMP source will have CI of 1. Cells that move perpendicular to the gradient will have CI of 0. Cells that move directly away from cAMP source will have a CI of -1. Wild-type and *pax*B$^-$ cells had similar CI's of 0.80 ± 0.05 and 0.90 ± 0.01, respectively. In *pldB*OE cells, the CI was significantly decreased to just 20% of the wild-type cells. *paxB*$^-$/*pldB*OE cells had CI comparable to wild-type cells (Figure 5C). Thus, as measured by directionality and chemotaxis index, overexpression of *pldB* leads to an impaired efficiency of chemotaxis to cAMP. This is rescued by removal of *paxB*. Taken together, this suggests that overexpression of *pldB* rescues defects caused by loss of *paxB*, and loss of *paxB* rescues defects caused by overexpression of *pldB*.

To determine whether the observed genetic interaction between *paxB* and *pldB* was specific to cAMP chemotaxis or is a general, chemotaxis-related interaction, we assayed folate chemotaxis. Both single mutants as well as the double mutant were able to chemotax towards folate with a speed (Figure 5D), directionality (Figure 5E) and chemotaxis index (Figure 5F) comparable to that of wild-type cells. This indicated that PaxB and PldB do not play roles in chemotaxis towards folate, and that the observed genetic interaction between *paxB* and *pldB* is specific to developing cells responding to cAMP.


A
Speed (% of WT)
200
180
160
140
120
100
80
60
40
20
0
WT
paxB-
pldBOE
paxB-/pldBOE
B
Directionality (% of WT)
C
Chemotaxis index (% of WT)

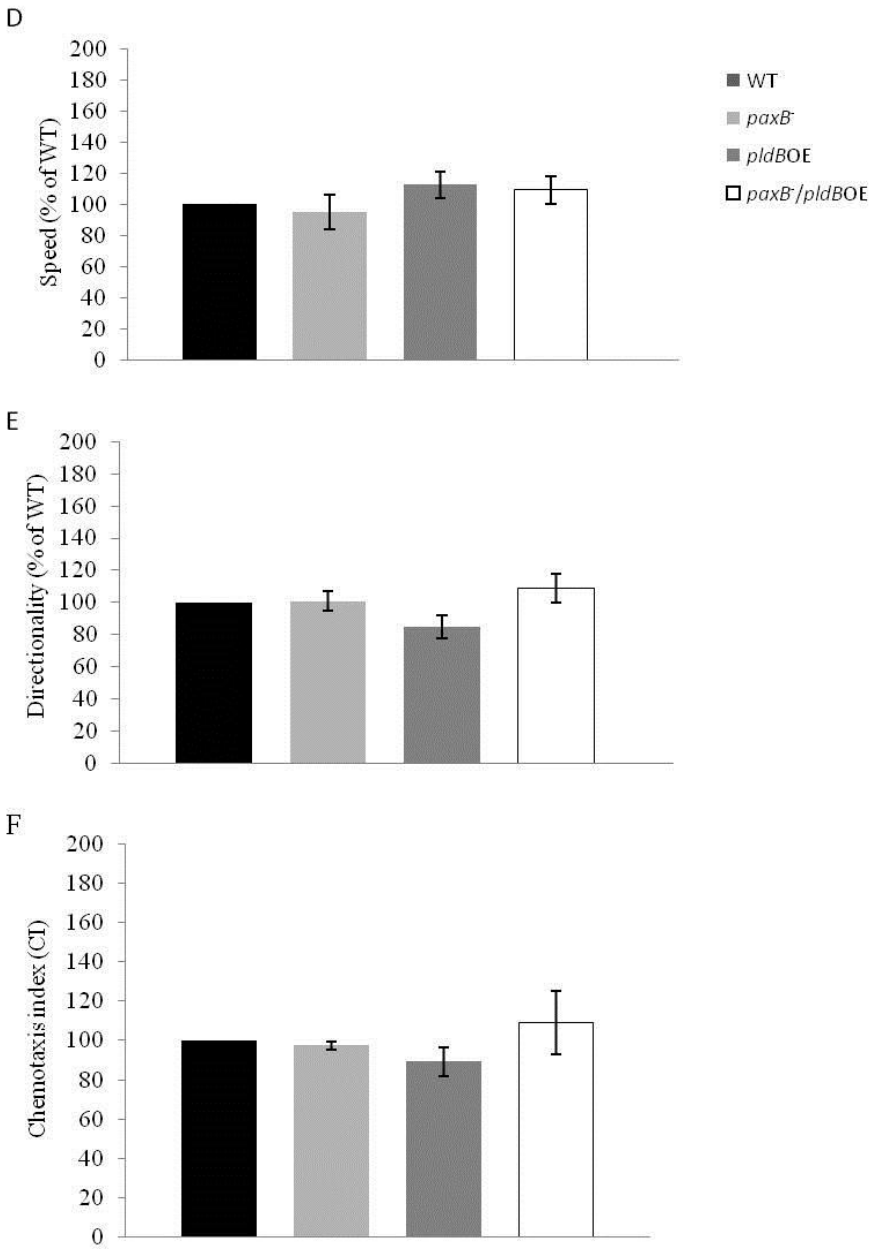


Figure 5. **PaxB and PldB functionally interact during cAMP chemotaxis, but not during folate chemotaxis.**

Cells starved for 6 hours were exposed to cAMP gradient and the speed (A), directionality (B) and chemotaxis index (C) of individual cells were measured. Vegetative cells were exposed to a folate gradient and the speed (D), directionality (E) and chemotaxis index (F) of individual cells were measured. Data presented are the means and SEM of at least three independent experiments, with each experiment tracking 15 cells. * indicates a significant difference compared to wild-type with $p<0.05$.

2.3.5 PaxB and PldB functionally interact to regulate actin

As proper organization of the actin cytoskeleton is necessary for chemotaxis and adhesion, one possible cause for the altered cAMP chemotaxis of our mutant cells could be a defective actin cytoskeleton. To examine the distribution of F-actin in starved cells, wild-type and mutant cells were fixed and stained with Rhodamine-phalloidin. In wild-type cells, F-actin was mainly localized to broad membrane protrusions and filopodia, as previously reported (Figure 6) [27]. *paxB*$^-$ cells seem to have impaired cortical staining and increased filopodia. In contrast, *pldB*OE cells had cortical staining, but did not project filopodia at all. *paxB*$^-$/*pldB*OE cells were able to project filopodia and had normal cortical staining. Thus, while *pldB*OE cells exhibited an obvious defect in modulating the actin cytoskeleton, disruption of *paxB* in these cells restored a wild-type phenotype, indicating a functional interaction between PaxB and PldB in actin regulation.

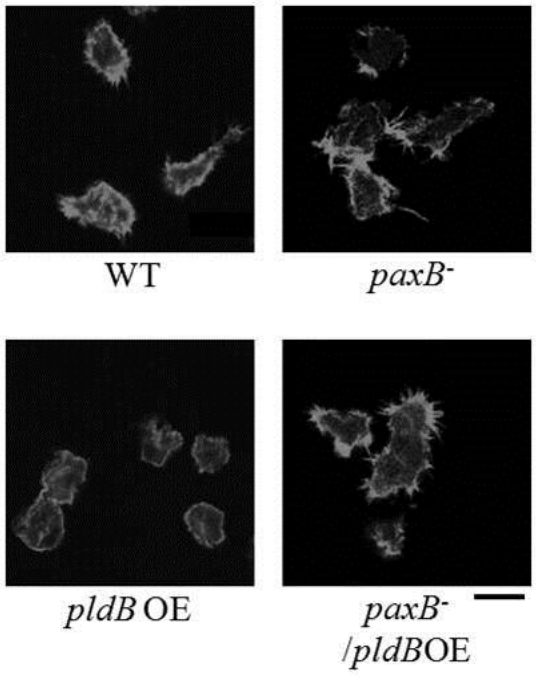


Figure 6. **Starved *paxB*-/*pldB*OE cells have normal cell morphology and F-actin staining.** Wild-type, *paxB*-, *pldB*OE and *paxB*-/*pldB*OE cells were starved in submerged culture, fixed and stained with Rhodamin-phalloidin. Bar, 10 µm.

To further study F-actin regulation, we examined F-actin formation in response to cAMP stimulation. cAMP stimulation causes polymerization and reorganization of actin, which is crucial for the extension of new pseudopods during chemotaxis [42]. To examine actin polymerization in response to cAMP, starved wild-type and mutant cells were fixed before or after cAMP stimulation. The amount of polymerized actin was determined by staining with Rhodamine-phalloidin and measuring the fluorescence of individual cells by flow cytometry. The increased staining seen in wild-type cells upon cAMP stimulation was defined as 100%. Upon cAMP stimulation, *paxB*$^-$ cells exhibited slightly less stimulation than that seen in wild-type cells. This difference was not statistically significant ($p>0.05$). *pldB*OE cells showed drastically reduced stimulation compared to that seen in wild-type cells. The level of stimulation in the double mutant was similar to that seen in *paxB*$^-$ cells ($p>0.05$) (Figure 7).

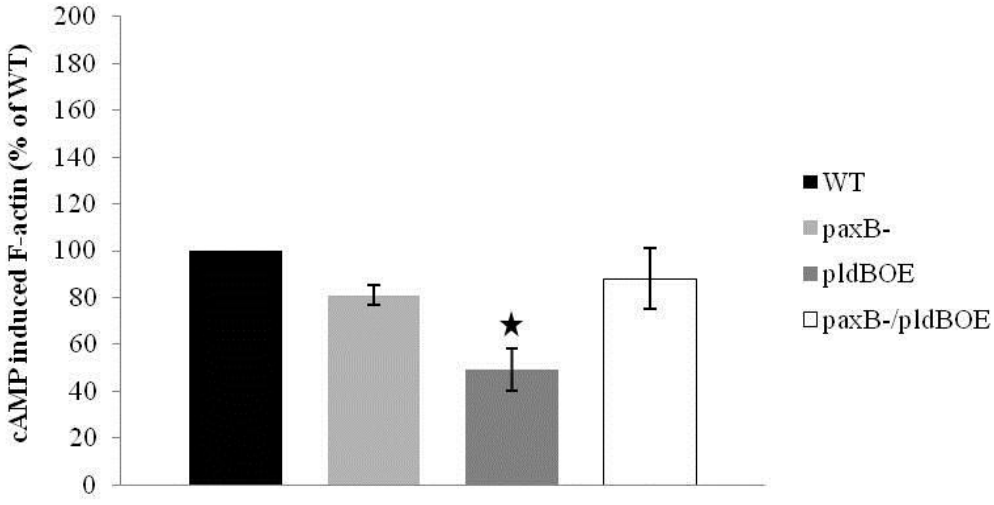


Figure 7. PaxB and PldB functionally interact to regulate cAMP-induced actin polymerization.
6 hours starved wild-type, *paxB*-, *pldB*OE and *paxB*-/*pldB*OE cells were fixed and stained with Rhodamin-phalloidin before and after cAMP stimulation. Fluorescence was measured by flow cytometry. The data presented are the means and SEM of at least three independent experiments, with each experiment counting 10,000 cells. * indicates a significant difference compared to wild-type with $p<0.05$.

These data suggest that lack of *paxB* does not seem to have a significant effect on actin polymerization in response to cAMP while overexpression of *pldB* impairs the process. In addition, loss of *paxB* is able to rescue the defect seen in *pldB*OE cells, implying a functional interaction between PaxB and PldB in cAMP-stimulated F-actin formation**.**

2.3.6 PldB positively regulates PaxB function during phagocytosis and exocytosis

As our experiments suggested that PaxB and PldB functionally interact to regulate proper F-actin localization and actin polymerization, we wanted to determine whether this interaction also exists in two other actin-based processes, phagocytosis and exocytosis. Phagocytosis was measured by monitoring the uptake of 1µm fluorescent latex beads over 30 min. The rate of uptake was calculated and that of wild-type cells was set at 100%. As shown in Figure 8, the rate of phagocytosis in *paxB*$^-$ cells was 6 times higher than that of wild-type cells. *pldB*OE cells exhibited a phagocytosis rate similar to that of wild-type cells. The phagocytosis rate in *paxB*$^-$/*pldB*OE cells was similar to that seen in *paxB*$^-$ cells, 6 times higher than that of wild-type cells. Thus, while PaxB negatively regulates phagocytosis, overexpression of *pldB* does not affect this process. In addition, the *paxB*$^-$ phenotype is able to override the *pldB*OE phenotype in the double mutant during phagocytosis.

Exocytosis was examined by loading cells with FITC-Dextran and then observing its release over the course of 60 min. The rate of decreased fluorescence was calculated and that of wild-type cells was set at 100%. *paxB*$^-$ cells displayed an impaired exocytosis rate compared to that of wild-type cells, whereas *pldB*OE cells had an increased exocytosis rate. The rate of exocytosis in *paxB*$^-$/*pldB*OE cells was similar to that in *paxB*$^-$ cells ($p>0.05$) (Figure 8). This suggests that PaxB and PldB are positive regulators of exocytosis, and that these functionally

interact to regulate this process. Consistent with phagocytosis, the *paxB*$^{-}$ phenotype is able to override the *pldB*OE phenotype in the double mutant during exocytosis.

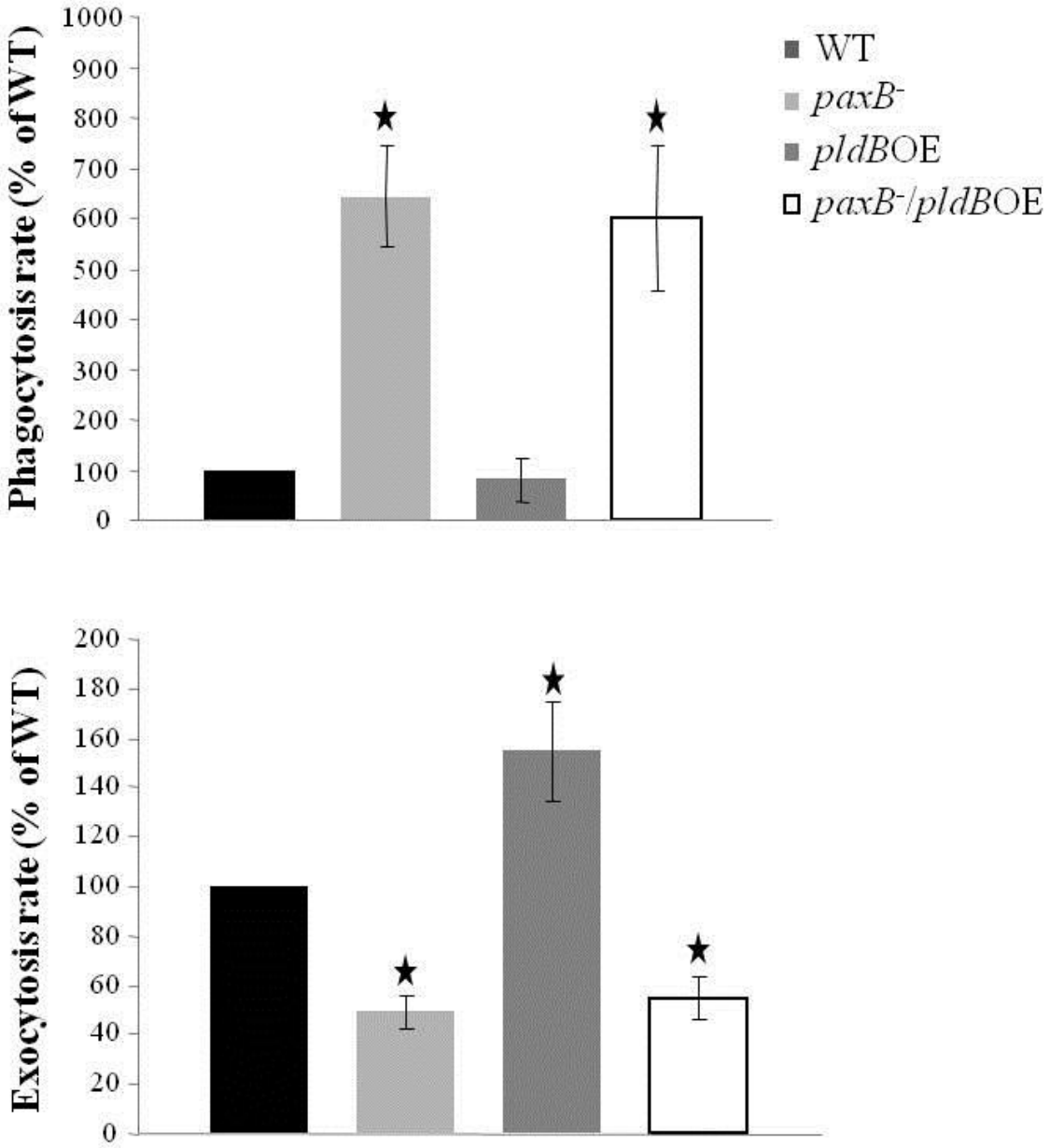


Figure 8. ***paxB*- phenotype overrides *pldB*OE phenotype during phagocytosis and exocytosis.**

The rate of uptake of 1µm fluorescent latex beads was determined in wild-type, *paxB*-, *pldB*OE and *paxB*-/*pldB*OE cells. That of wild-type cells was set at 100%. The rate of release of FITC-dextran was determined in wild-type, *paxB*-, *pldB*OE and *paxB*-/*pldB*OE cells. That of wild-type cells was set at 100%. The data presented are the means and SEM of at least three independent experiments. * indicates a significant difference compared to wild-type with $p<0.05$.

2.4 Discussion

Previous studies in mammalian cell lines implied that paxillin and PLD functionally associate with the actin cytoskeleton [44, 48]. Both paxillin and PLD are critical for efficient cellular migration, requiring the coordination of membrane trafficking and the reorganization of the actin cytoskeleton [29, 49], suggesting a possible interaction. And while PLD has not been shown to bind paxillin, PLD activity has been associated with cell membrane fractions containing paxillin, and PLD1 partially colocalizes with paxillin at the cell-substrate interface in human macrophages, opening the possibility that these proteins do form a complex [44]. Given the role of paxillin as an adapter molecule that provides a platform for an array of signaling and structural proteins [50], it is quite possible that PLD binds paxillin either directly or indirectly. However, the molecular mechanisms by which such paxillin-containing protein complexes function remain to be fully elucidated.

In this study we provide evidence that, much like in mammalian cells, paxillin and PLD regulate the actin cytoskeleton in *Dictyostelium discoideum*. We show here that loss of PaxB (paxillin) leads to defects in cAMP chemotaxis, adhesion, F-actin localization, phagocytosis and exocytosis. We also demonstrate that overproduction of PldB (PLD) disrupts proper cAMP chemotaxis, F-actin formation and localization, and exocytosis. More interesting is the fact that the results from several experiments support the possibility that the two proteins function together to regulate the actin cytoskeleton. Previously reported protein expression profiles of *paxB* and *pldB* showed that the two proteins are expressed during the same period of development and in the same cell type (6, 10, 40). In addition, previous work done in our lab has shown that both proteins localize to F-actin rich areas within the cell. More telling, however, was the discovery that PaxB and PldB can be coimmunoprecipitated. Thus, PaxB and PldB are

present at the same time, in the same cells, in the same locations, and can form a complex, all of which are consistent with the two proteins working together.

Evidence that the two proteins are functioning in the same pathways is found by examining the phenotypes of the *paxB*$^{-}$*/pldB*OE double mutants. Classical epistasis analysis can be used to determine whether two gene products function in the same pathway, and if so, their order of action. If the phenotypes associated with two mutations are different, then the phenotype of the double mutant dictates which gene product acts downstream of the other. For example, cells lacking *paxB* have decreased adhesion. Cells overexpressing *pldB* have normal adhesion. The double mutants, which lack *paxB* and overexpress *pldB*, have normal adhesion, just like the *pldB* overexpressing cells. Epistasis analysis argues that since the *pldB* overexpressing cell phenotype is dominant, PldB must be acting downstream of PaxB. According to this logic, PldB acts downstream of PaxB in regulating speed of chemotaxis to cAMP and adhesion. In contrast, PldB acts upstream of PaxB in controlling directionality, chemotactic index, cAMP-induced F-actin polymerization, phagocytosis and exocytosis. Simple epistasis cannot explain the fact that the order of action of PaxB and PldB varies depending on the process measured. Thus, while it is clear that PaxB and PldB work together to regulate actin, it cannot be in a simple pathway where one protein activates the other.

To explain how PaxB and PldB are working together to regulate cytoskeletal functions, both their physical and functional interactions must be taken into account. Our observations provide evidence in favor of the hypothesis that PaxB and PldB form a complex. PLD catalyzes the hydrolysis of phosphatidylcholine, producing phosphatidic acid (PA), a second messenger [51]. PldB uses PaxB as an adaptor to be recruited to specific subcellular locations, where production of PA can facilitate proper actin responses to environmental changes (Figure 9). This

could trigger recruitment of other proteins, such as Phg2 and SadA by PaxB at focal sites, which in turn controls adhesion, motility and actin cytoskeleton organization [52, 53]. In *paxB*$^{-}$ cells, PldB is unable to properly localize, leading to improper actin reorganization. In *pldBOE* cells, an inordinate amount of PldB gets recruited to sites of action by PaxB, leading to hyperactive signaling and therefore cytoskeletal defects. These phenotypes are ameliorated in the *paxB*$^{-}$*/pldBOE* double mutant because even though there is an abundance of PldB, there is no PaxB to efficiently localize it. A relatively small percentage of the overproduced PldB is able to localize to its sites of action, allowing it to perform its signaling function and regulate the actin cytoskeleton (Figure 9).

From this point of view, the cAMP chemotaxis results can easily be explained. Efficient chemotaxis requires PaxB to recruit PldB and other proteins into a complex at sites of action at the plasma membrane and cell-substrate interface. Without PaxB, these complexes cannot be formed. When *pldB* is overexpressed recruitment of these protein complexes by PaxB is increased, to the point where they are mislocalized throughout the cell, preventing pseudopod extension and disrupting directed migration. In the absence of PaxB, PldB is not efficiently recruited so that even when overexpressed, only a small portion is localized properly. This allows rescued efficiency of cAMP chemotaxis and hence full development of *paxB*$^{-}$/*pldB*OE cells. Interestingly, the role of PaxB and PldB in chemotaxis appears to be specific to cAMP, as all mutants were able to chemotax towards folate with efficiency comparable to that of wild-type cells. Thus, the functional interaction between PaxB and PldB is specific to cAMP chemotaxis and does not occur in the vegetative cells.

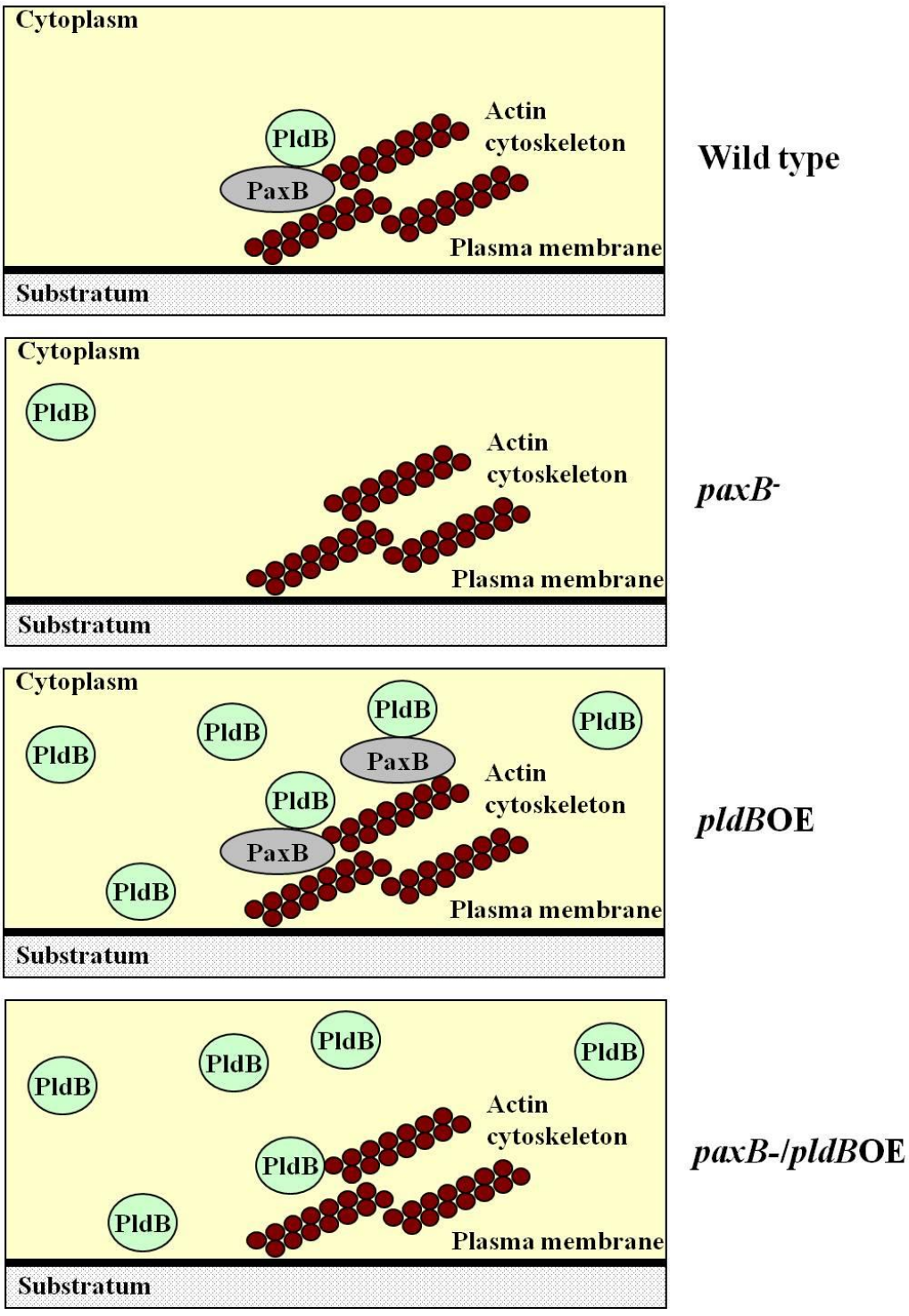


Figure 9. **Model of PaxB involvement in recruiting PldB to the actin sites.**
In wild type cells, PaxB serves as a scaffold to recruit PldB to sites of actin organization and cell-substrate adhesion. In the absence of PaxB, PldB may not properly localize to areas of adhesion. When PldB is overexpressed in cells lacking PaxB (*paxB*-/*pldB*OE) excessive levels of PldB are generated which likely localize to adhesion areas without PaxB.

We found that cell overexpressing *pldB* have a dramatic defect in cAMP-induced actin polymerization. This could explain inefficient cAMP chemotaxis of these cells. As seen during cAMP chemotaxis, inefficient response to cAMP stimulation was rescued in *paxB*$^{-}$/*pldB*OE cells, since in the absence of PaxB, PldB was not efficiently recruited to the site of action, masking the phenotype seen during overexpression of *pldB.*

The rescued chemotaxis can also explain the developmental phenotypes that the mutants exhibit. Previously reported expression profiles of *paxB* and *pldB* showed that both proteins reached their peak production during mound and slug formation, suggesting their importance at these stages. In accordance with this interpretation, *pldB*OE cells cannot form mounds, and *paxB*$^{-}$ strains are not able to fully develop, arresting at the mound stage (Figure 3A) [26]. The inability of *pldB*OE cells to form mounds could be explained by their low directionality and chemotactic index in response to cAMP. *paxB*$^{-}$ cells have normal directionality and chemotactic index, thus they can form mounds. However, once in the mound, they cannot form tips (6). In fact, we find that in chimeric mounds with wild-type cells, the *paxB*$^{-}$ cells are specifically excluded from the tip. During *Dictyostelium* development, the tip forms by the sorting of prestalk cells toward the top of the mound [54, 55]. The tip seems to direct all subsequent morphogenesis, exhibiting the properties of an organizer. Thus, the *paxB*$^{-}$ arrest at the mound stage is most likely due to an inability to form a tip. The details of the sorting mechanism are still unknown but may involve cell-type specific differences in cytoskeletal function [56, 57], adhesion [58] or chemotaxis to cAMP signals [59, 60]. Interestingly, the *paxB*$^{-}$ cells have increased chemotactic speed, which could explain why they are not able to form a tip. In contrast to the *pldB*OE and *paxB*$^{-}$ cells, the *paxB*$^{-}$/*pldB*OE double mutants have normal speed, directionality and chemotactic index. They are thus able to form mounds, sort properly to form a tip, and eventually form a fruiting body.

2.5 Conclusion

Using *Dictyostelium discoideum* we have for the first time described a functional and physical interaction between paxillin and PLD. We provide evidence that this interaction regulates multicellular development, cAMP chemotaxis, cell-substrate adhesion, actin distribution and polymerization, phagocytosis and exocytosis. We hypothesize that PaxB controls PldB action by shuttling it to specific cellular localizations. This is not to say that PaxB is the only protein regulating PldB. In mammals, PLD activity is regulated by protein kinases, Arf and Rho family GTP-ases [51]. The *Dictyostelium* genome contains several of these protein, some of which regulate the cytoskeleton [61]. While their modes of action are still unknown some of them may function through regulating PldB activity. Given that *Dictyostelium discoideum* has proven to be an excellent cellular model for studying the cytoskeleton and its regulation, it can be used to uncover other interactions involved in signaling to the cytoskeleton.

2.6 References

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Chapter 3: PLD activation by removal of serum alters paxillin phosphorylation and complex formation with Erk and FAK in MDA-MB-231 cells

3.1 Introduction

The metastatic process consists of tumor cell intravasation, survival in circulation, extravasation into a distant organ, angiogenesis and unconstrained tumor growth [1]. The specificity of the tumors for particular distant targets is known as tissue tropism, but its mechanism and molecular basis are not fully understood. Specific gene expression patterns are correlated with various metastases and recent studies are focused on elucidating genetic factors critical for tumor metastasis [2, 3].

3.1.1 Breast carcinoma

Adenocarcinoma of the breast, and its associated metastasis, is the second leading cause of cancer mortality among women in the United States [4]. Metastasis occurs when malignant cells migrate to an organ distant from the primary tumor, adjust to the new microenvironment and expand secondary tumors. The most common target for breast cancer metastases is bone, followed by lung, liver and brain [5]. The successful treatment of breast cancer requires greater understanding of the molecular and cellular basis of breast cancer phenotypes as well as modification of the treatment regimen for an individual patient.

Highly aggressive human breast cancer is often modeled using MDA-MB-231 cells. These cells are derived from a human adenocarcinoma, originally isolated from pleural fluid [6]. Invasiveness of these cells depends on phospholipase D (PLD) activity which is known to play an important role in cell proliferation, tumorigenesis and migration [7, 8]. In fact, PLD activity

has been reported to be elevated in a majority of breast cancers examined [9]. Increased PLD activity has also been observed in cancer cell lines in response to the stress of serum removal, which is associated with increased cell motility and invasiveness [8].

3.1.2 Phospholipase D

PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA is metabolically convertible to the mitogenic lipids, diacylglycerol and lysophosphatidic acid. There are two mammalian PLD genes: PLD1 and PLD2. PLD1 is localized throughout the cell, particularly in perinuclear, Golgi, and heavy membrane fractions [10-12]. Although it is primarily associated with intracellular membranes, it can also be found in lipid rafts [11, 13]. It has a low basal rate of catalysis and is regulated by PKCα, ARF, and RhoA family members [14, 15]. PLD2 is exclusively localized on the plasma membrane in light membrane "lipid raft" fractions and is constitutively active [10, 11]. Both PLD1 and PLD2 are implicated in mitogenic signaling: PLD1 by functional association with Ral GTP-ase, a downstream target of Ras, and PLD2 by localization to light membrane fractions containing many signaling molecules, including EGFR [16-18]. However, the precise mechanism of the involvement of PLD activity in mitogenic signaling as well as associated proteins still remain to be revealed.

3.1.3 Erk

Previous studies have demonstrated that mitogen-activated protein kinases (MAPKs) are essential for cell migration. The MAPK family can be divided into three groups: extracellular-signal-regulated protein kinase (Erk/MAPK), p38 and Jun N-terminus kinase (JNK). It has been

suggested that signaling through the Erk1/2 pathway is very important for breast cancer progression [19]. Erk has two isoforms; p44 (Erk-1) and p42 (Erk-2). Their activities are stimulated by various growth factors and mitogens which activate the Ras\Raf-1\MEK-1/2\Erk-1/2 signaling module [20]. Ras, activated by extracellular signals, recruits MAPKKK, Raf-1, to the membrane. Raf-1 phosphorylates and activates the MAPKKs, MEK-1 and MEK-2, which phosphorylate the Thr and Tyr residues of Erk1/2. Erk has been implicated in the migration of numerous cell types [21-26], leaving open the possibility that PLD affects motility through the regulation of Erk in tumor cells.

3.1.4 Paxillin

Migration and invasion of tumor cells is also highly dependent on adhesion molecules that regulate focal contacts with matrix components. The adaptor and scaffold protein paxillin is a component of focal adhesion complexes and mediates focal adhesion assembly and turnover and thus motility [27]. Dispersed throughout paxillin are many serine and threonine and tyrosine phosphorylation sites. It is known that various extracellular factors are able to induce tyrosine phosphorylation of paxillin [28-31]. One kinase known to phosphorylate paxillin on Tyr 31 and Tyr 118 is Focal adhesion kinase (FAK) [32, 33]. It is a paxillin binding partner in focal adhesions and is overexpressed in breast, ovary and colon cancers [27, 34-36]. A major consequence of paxillin tyrosine phosphorylation is the generation of binding sites for SH2 domain-containing proteins. Therefore, it is not surprising that phosphorylation of paxillin leads to its recruitment of numerous signaling molecules, allowing it to regulate focal adhesion dynamics and cell migration. Interestingly, contradictions in the correlation between paxillin expression, paxillin phosphorylation and cancer aggressiveness indicate that the roles of paxillin

in various tumors are tissue-specific and context-specific [37-41]. Therefore, the investigation of paxillin expression along with the co-expression of other paxillin binding partners may be important in determining the full biological impact of paxillin on tumor phenotype and thus on selecting appropriate therapies.

It has been suggested that Erk, by phosphorylation of FAK and paxillin, affects the formation of a complex between paxillin and FAK and thus plays a role in the regulation of focal adhesion dynamics [42, 43]. However, the precise role of Erk and paxillin in controlling cell motility, along with their correlation with PLD remain unclear. In this study, we described the effect of PLD activity on paxillin phosphorylation, Erk activity and complex formation between paxillin and Erk in the MDA-MB-231 breast cancer cell line. Interestingly, our data show that Erk activity is governed by PLD activity and regulates the tyrosine phosphorylation of paxillin. Thus, Erk, PLD, and paxillin may act in concert to regulate cell motility in MDA-MB-231 cells.

3.2 Materials and methods

3.2.1 Cells, cell culture conditions, and transfection

MDA-MB-231 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified eagle's medium (DMEM) with 10% bovine calf serum both purchased from Sigma (St. Louis, MO). Transfections were performed using Lipofectamine^{TM} LTX and Plus reagent purchased from Invitrogen (Eugene, OR) according to the manufacturer's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 purchased from Clonetech (Mountain View, CA), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 70%.

3.2.2 Materials

1-butanol and tertiary-butanol were from Sigma (St. Lous, MO). [^{3}H] myristate was obtained from New England Nuclear (Newton, MA). U0126 was obtained from Promega (Fitchburg WI). Anti-paxillin mouse antibody was obtained from Calbiochem (La Jolla, CA). Anti-phosphotyrosine antibody, anti-phospho-ERK1/2 antibody, anti-FAK antibody and anti-HA-tag antibody were obtained from Cell Signaling Technology (Beverly, MA). ERK 1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was obtained from Sigma (St. Louis, MO). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA).

3.2.3 PLD assays

MDA-MB-231 cells were plated in 60 mm culture dishes at 1 X 10^5 cells/dish. Two days later, cells were shifted into DMEM containg 0.5% bovine calf serum and kept overnight. Cells were then prelabeled for 4 h with [^{3}H] myristate (3 µCi, 40 Ci/mmol) in 3 ml of medium. The cells were then incubated for 20 min with of 1-butanol or tertiary-butanol to achieve 0.8% final concentration. The extraction and characterization of lipids by thin layer chromatography were performed as described previously [44]. Radioactivity corresponding to the tertiary-butanol reaction (background) was subtracted from that of the butan-1-ol reaction to determine PLD activity.

3.2.4 Immunoprecipitation and immunoblotting

MDA-MB-231 cells were lysed in ice-cold RIPA buffer (65 mM Tris base, 154 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA, pH 7.4) that contained 1 M Na-

othovanadate, 100 mM NaF, 1X Roche Complete Protease Inhibitor Cocktail, Mini (Indianapolis, IN), 1X Phosphatase inhibitor cocktail set I obtained from Calbiochem (La Jolla, CA) and rotated for 20 min at 4^0 C. Lysates were clarified by centrifugation at 10,000xg for 10 min. Supernatants were normalized for total protein by Bio-Rad assay (Hercules, CA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Hybond-P membrane obtained from Amersham Bioscience (Piscataway, NJ) and immunoblotted with antibodies as described in the text. Depending upon the origin of the primary antibodies, either HRP-conjugated anti-mouse or anti-rabbit immunoglobulin G was used for visualization by enhanced chemiluminescent substrate for HRP detection obtained from Pierce (Rockford, IL).

For immunoprecipitations, precleared lysates were incubated with 1 µg antibody overnight on a rotating shaker at 4°C. Pure Proteome Protein A magnetic beads purchased from Millipore (Temecula, CA) were added to the sample and incubated on a rotatory shaker for 1 hour at 25°C. Beads were collected with a magnetic rack obtained from Millipore (Temecula, CA) and washed 3 times with PBS containing 0.1% Tween. Beads were resuspended in 50 µl PBS + 0.1% Tween and 10 µl 6X protein sample buffer, then boiled for 10 minutes at 90°C. Beads were then removed from suspension with the magnetic rack. The samples were electrophoretically separated on SDS-polyacrylamide gels and immunoblotted as described above.

3.2.5 Statistical analysis

For quantitative analysis, data are presented as mean ± SEM from at least three independent experiments. The significance of differences was analyzed by the Student's t-test for

pair-wise comparisons. A p value < 0.05 was considered statistically significant. Absence of a star means no significant difference from the control was found.

3.3 RESULTS

3.3.1 Paxillin production level does not depend upon PLD activity

It has been reported that serum withdrawal leads to increased PLD activity in MDA-MB-231 cells [8]. To confirm the elevated PLD activity in MDA-MB-231 cells upon serum removal, we performed PLD activity assay. As shown in Fig. 1, PLD activity was higher in MDA-MB-231 cells in 0.5% serum than in cells maintained in 10% serum, which is consistent with previous reports.

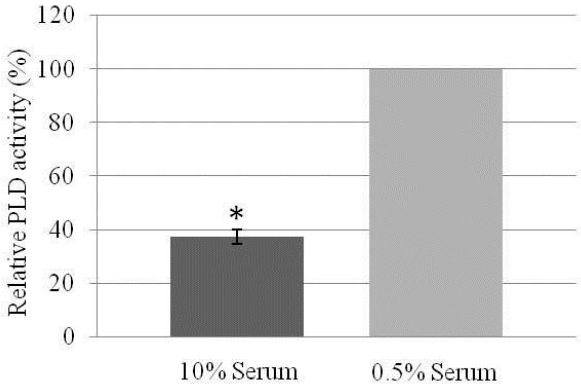

Figure 1. MDA MB-231 cells have elevated PLD activity in low serum.
Data presented are the means and SEM of at least three independent measurements.

Given the confusion of whether paxillin expression correlates with cell motility and invasiveness, we examined whether serum removal and PLD activity, both of which stimulate motility and invasiveness in MDA-MB-231 cells [8], affect the level of paxillin production. The dependence of paxillin production on PLD activity was assessed using an “alcohol trap” assay. In this assay, primary alcohols are preferentially utilized over water by PLD to catalyze the formation of phosphatidylalcohol rather than phosphatidic acid, thus blocking signaling via

phosphatidic acid. Western blot analysis of whole cell lysates revealed similar paxillin levels in cells maintained in 10% serum, where PLD activity is low (Fig. 2, lane 1), as in cells exposed 0.5% serum, where PLD activity is high (Fig. 2, lane 2). In addition, paxillin levels were not affected by treatment with 1-Butanol that inhibits PLD signaling (Fig. 2, lane 3) nor tertiary-Butanol, which is a poor substrate of PLD and is used as a negative control for 1-Butanol as it does not prevent phosphatidic acid formation (Fig. 2, lane 4). These data indicated that paxillin levels are independent from serum levels and PLD activity.

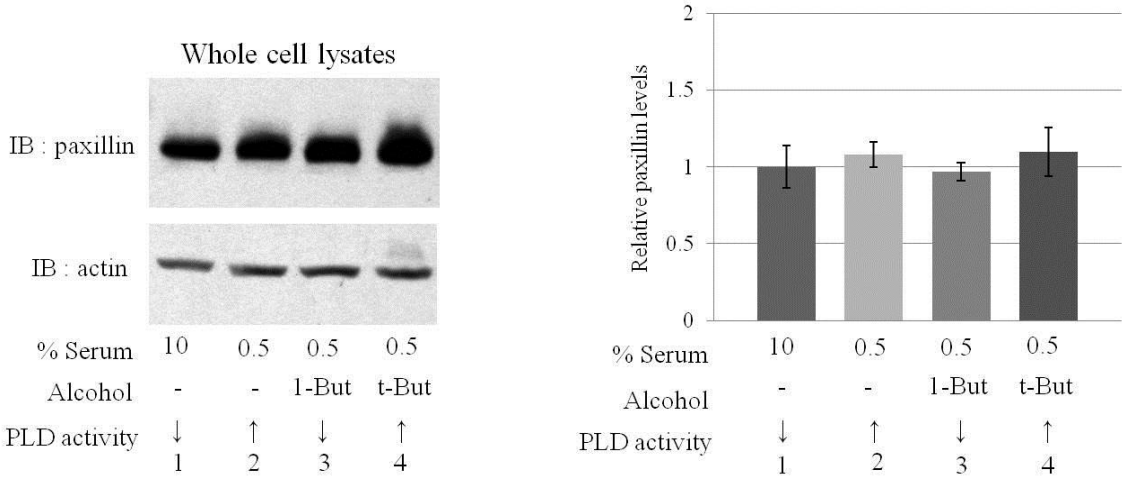

Figure 2. Paxillin levels do not depend upon PLD activity.
Cell lysates were subjected to Western blot analysis with α-paxillin Ab followed by Western blotting using α-actin Ab as a control for equal loading. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. Representative blot is shown.

To demonstrate that paxillin production is independent specifically of PLD activity, we suppressed PLD activity in MDA-MB-231 cells by introducing catalytically inactive PLD1(K898R) or PLD2(K758R), which have been used previously as dominant interfering mutants [45, 46]. Expression of the dominant negative PLD proteins was determined by Western blot analysis using an antibody raised against HA tag (Fig. 3).

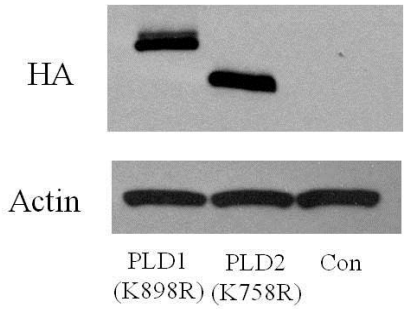

Figure 3. MDA-MB-231 cells were transiently transfected with PLD1 (K898R), PLD2 (K758R) or vector control (Con).
Cell lysates were subjected to Western blot analysis with antibodies raised against the hemagglutinin (HA) tag on PLD1-K898R or PLD2-K758R and actin.

The introduction of PLD1(K898R) or PLD2(K758R) via transient transfection suppressed the PLD activity in MDA-MB-231 cells to 50% of a vector control (Fig. 4). This is similar to what has been reported previously [8].

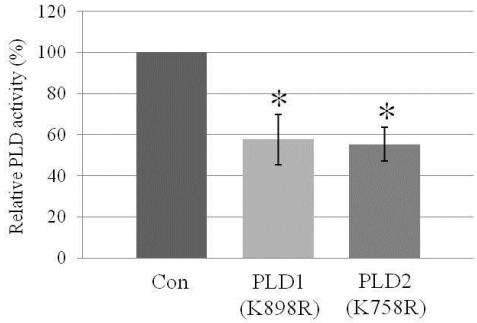

Figure 4. PLD activity is reduced in PLD1 (K898R) or PLD2 (K758R) expressing cells.
MDA-MB-231 cells that had been transiently transfected with either a vector control (Con) or with pCGN-PLD1-K898R or pCGN-PLD2-K758R were placed in media containing 0.5% serum, and 24 h later, PLD activity was determined using the transphosphatidylation reaction. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to control.

Paxillin production was further examined in cells expressing catalytically inactive PLD1(K898R) or PLD2(K758R) mutants. As expected, paxillin levels were not affected by expression of dominant negative PLD2 (Fig. 5, lane 2), nor of dominant negative PLD1 (Fig. 5, lane 3) demonstrating that paxillin production does not depend upon PLD activity.

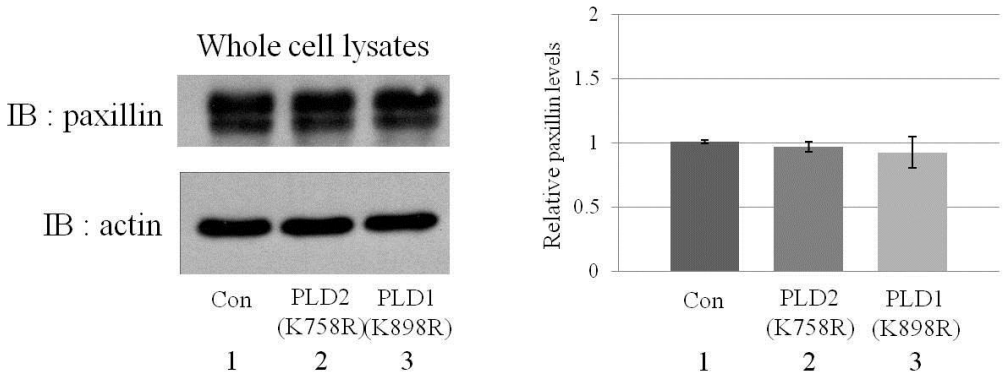

Figure 5. Paxillin production does not depend upon PLD activity.
MDA-MB-231 cells that had been transiently transfected with either pCGN-PLD1-K898R or pCGN-PLD2-K758R or vector control (Con) were placed in media containing 0.5% serum. 24 h later, cell were lysed and subjected to Western blot analysis with α-paxillin Ab followed by Western blotting using α-actin Ab as a control for equal loading. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. Representative blot is shown.

3.3.2 Paxillin tyrosine phosphorylation parallels PLD activity in MDA-MB-231 cells

Given that serum removal affects the activity of PLD and increases cell motility [8], we were interested in observing the effect of serum withdrawal on the tyrosine phosphorylation of paxillin, which is known to regulate motility [37, 41]. To examine the effect of PLD activity on paxillin phosphorylation, antiphosphotyrosine blotting of paxillin immunoprecipitates from MDA-MB-231 cells was performed. As shown in Fig. 6A, in 10% serum, where PLD activity is low (lane 1), paxillin primarily runs as one band at a molecular mass of 68 kDa. Following serum removal, where PLD activity is high (lane 2), the amount of paxillin at 68 kDa decreased, and a new band appeared at ~70 kDa, reminiscent of tyrosine phosphorylation. This reduction in gel mobility was only detectable if the gels were run for prolonged periods until paxillin nearly exited the gel. Addition of 1-Butanol which inhibits PLD signaling, repressed paxillin phosphorylation (lane 3), while tertiary-Butanol, which does not affect PLD, did not have an effect (lane 4). This suggested that paxillin tyrosine phosphorylation parallels PLD activity.

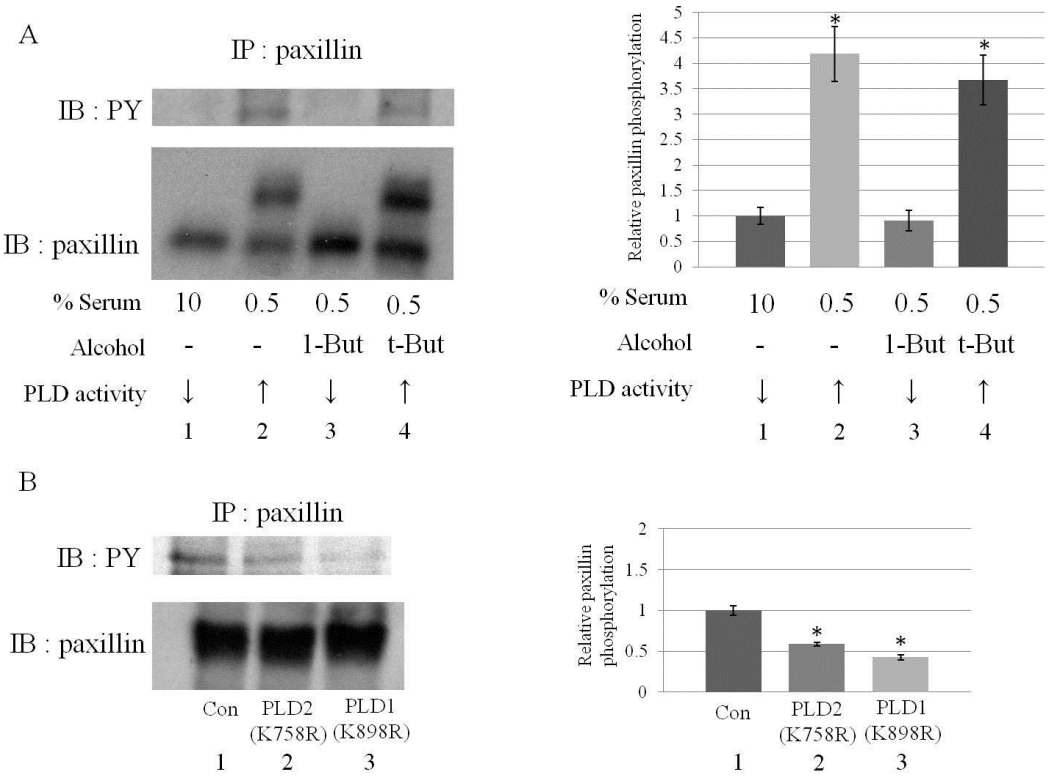

Figure 6. Paxillin tyrosine phosphorylation parallels PLD activity in MDA-MB-231 cells. A) Cell lysates were immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phosphotyrosine Ab, followed by stripping and reprobing with α-paxillin Ab. B) MDA-MB-231 cells that had been transiently transfected with either vector control (Con), or pCGN-PLD2-K758R or pCGN-PLD1-K898R were placed in media containing 0.5% serum. 24 h later, cell were lysed, immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phosphotyrosine Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or contol (B) with $p < 0.05$. Representative blots are shown.

Paxillin tyrosine phosphorylation was further examined in cells expressing catalytically inactive PLD2(K758R) or PLD1(K898R) mutants. As seen in Fig. 6B, paxillin tyrosine phosphorylation was suppressed in both dominant negative PLD2 (lane 2) and PLD1 (lane 3) mutants compared to the control cells (lane 1) suggesting that paxillin tyrosine phosphorylation is dependent upon PLD activity, and that the effect we observed with serum removal and alcohol

addition were due to disrupted PLD signaling. Thus, increase in PLD activity led to an increase in tyrosine phosphorylated paxillin, while having no effect on total paxillin levels.

3.3.3 PLD activity inhibits Erk phosphorylation in MDA-MB-231 cells

PLD activity has been implicated in mitogenic signaling [18, 47-50]. In addition, EGF-induced MAP kinase phosphorylation is dependent upon PLD activity [45]. To determine whether there is a correlation between Erk activity and PLD activity in MDA-MB-231 cells, we examined the effect of serum removal and PLD inhibition (alcohol trap) on the level of Erk1/2 activation, as measured by its phosphorylation. Western blot analysis of whole cell lysates revealed that compared to the cells maintained in 10% serum, where PLD activity is low (Fig. 7A, lane 1), there was a decrease in Erk1/2 phosphorylation in serum deprived cells, where PLD activity is high (Fig. 7A, lane 2), which is consistent with previous finding in MDA-MB-231 cells [51]. Moreover, Erk1/2 phosphorylation in serum deprived cells was amplified by 1-Butanol, when PLD signaling is inhibited (Fig. 7A, lane 3) but not by tertiary-Butanol, which does not inhibit PLD signaling (Fig. 7A, lane 4). This suggested that PLD activity inhibited Erk phosphorylation and thus, activation.

We further examined Erk1/2 phosphorylation in cells expressing dominant negative PLD1 and PLD2 mutants. When compared to the control cells (Fig. 7B, lane 1), the level of phosphorylated Erk1/2 was enhanced in cells expressing dominant negative PLD1 (Fig. 7B, lane 2) and PLD2 (Fig. 7B, lane 3) mutants. Along with the level of phosphorylated Erk1/2 seen in cells exposed to 1-Butanol, these results suggested that Erk activity, as measured by Erk phosphorylation, is inhibited by PLD activity.

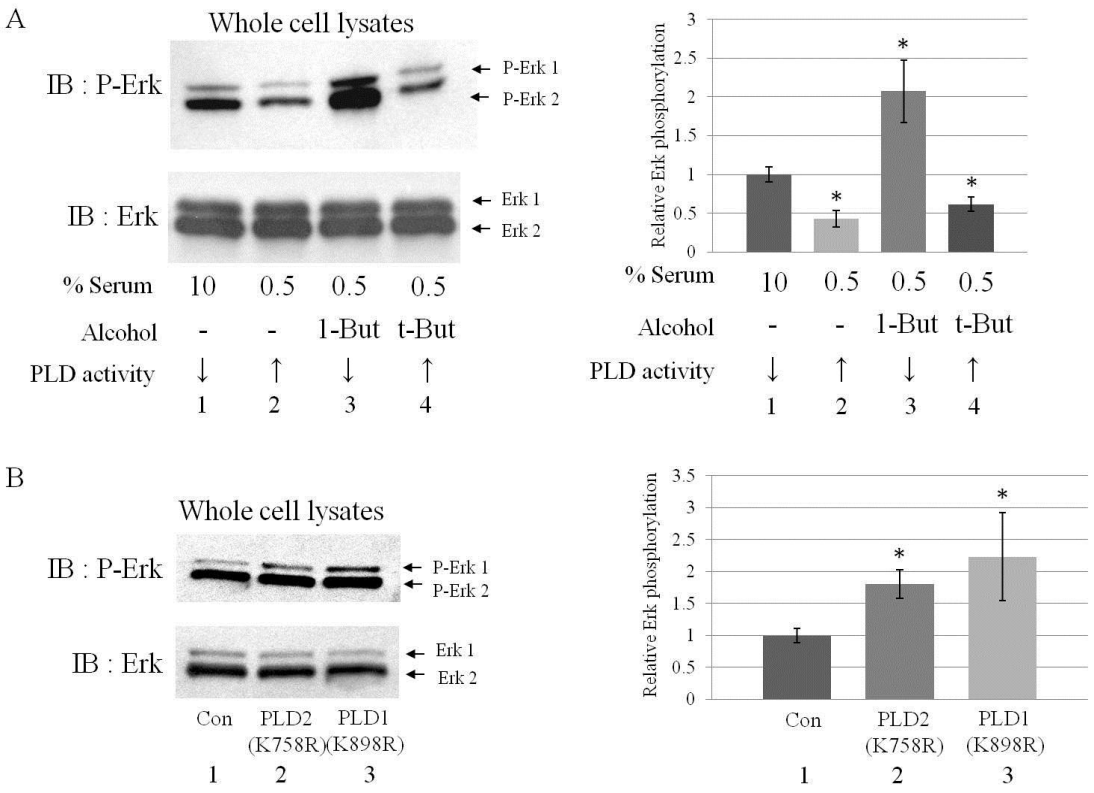

Figure 7. Erk activity negatively correlates with PLD activity.
A) Cell lysates were subjected to Western blot analysis with α-phospho-Erk 1/2 Ab. The blots were stripped and reprobed with α-Erk 1/2 Ab. B) MDA-MB-231 cells that had been transiently transfected with either vector control (Con) or pCGN- PLD2-K758R or pCGN- PLD1-K898R were placed in media containing 0.5% serum. 24 h later, cell were lysed and subjected to Western blot analysis with α-phospho-Erk 1/2 Ab. The blots were stripped and reprobed with α-Erk 1/2 Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or contol (B) with $p < 0.05$. Representative blots are shown.

3.3.4 PLD activity inhibits complex formation between Erk and paxillin in MDA-MB-231 cells

Finding that both paxillin tyrosine phosphorylation and Erk1/2 phosphorylation depend upon PLD activity prompted us to further explore a connection between paxillin and Erk. A physical association between paxillin and Erk has been described previously in renal medullary cells [42, 52]. To elucidate whether there is a similar association between paxillin and Erk in MDA-MB-231 cells, we performed co-immunoprecipitation studies (Fig 8).

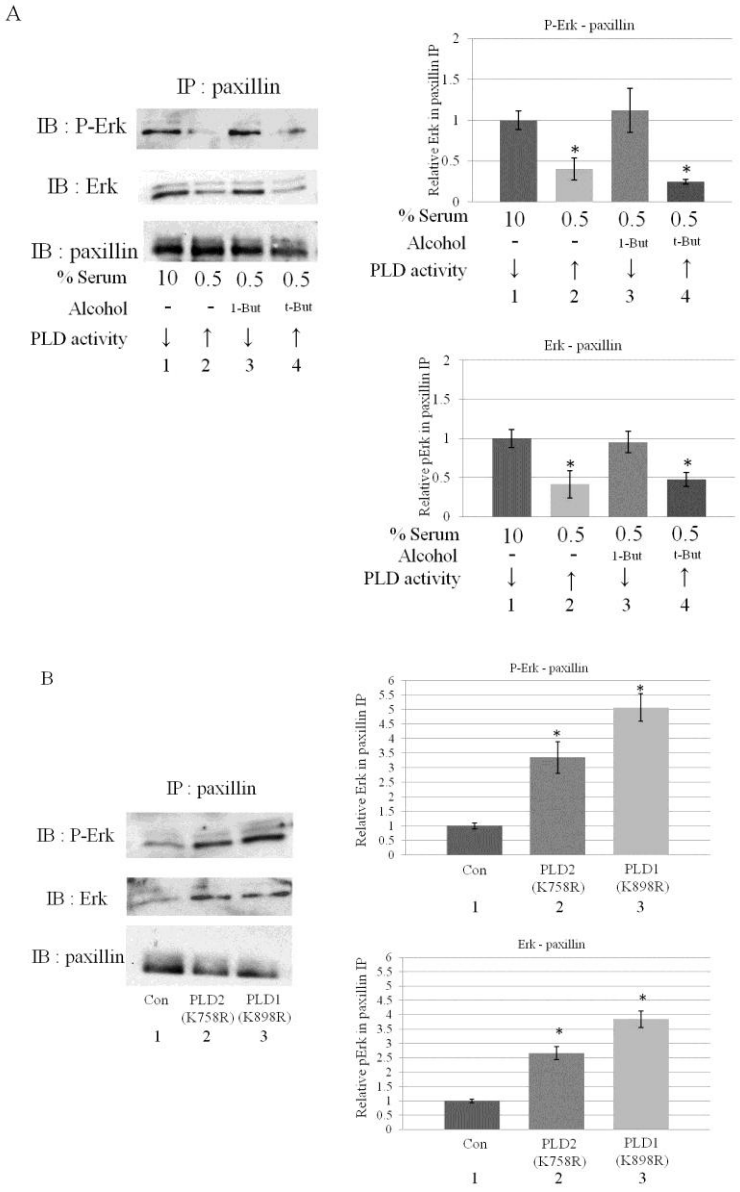

Figure 8. Erk - paxillin association is dependent upon PLD activity.
A) Cell lysates were immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phospho-Erk 1/2 Ab, followed by stripping and reprobing with α-Erk 1/2 Ab as well as stripping and reprobing with with α-paxillin AB. B) MDA-MB-231 cells that had been transiently transfected with either vector control (Con) or pCGN- PLD2-K758R or pCGN- PLD1-K898R were placed in media containing 0.5% serum. 24 h later, cell were lysed, immnuoprecipitated with α-paxillin Ab and immunoblotted with α-P-Erk 1/2 Ab followed by stripping and reprobing with α-Erk 1/2 Ab as well as stripping and reprobing with with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or contol (B) with $p < 0.05$. Representative blots are shown.

Paxillin immunoprecipitates were examined for the presence of total and activated Erk. As indicated in Fig. 8A, compared to the cells maintained in 10% serum, where PLD activity is low (Fig. 8A, lane 1), there is less Erk (either total or phosphorylated) associated with paxillin in serum deprived cells, where PLD activity is high (Fig. 8A, lane 2). Furthermore, complex formation between Erk and paxillin was improved when PLD signaling was inhibited by addition of 1-Butanol to the serum deprived cells (Fig. 8A, lane 3), while tertiary-Butanol did not have an effect (Fig. 8A, lane 4). Thus, paxillin and Erk form a complex in MDA-MB-231 cells which depends upon PLD activity.

To demonstrate that complex formation between Erk and paxillin is dependent upon PLD activity, we used MDA-MB-231 cells transiently transfected with catalytically inactive mutants of either PLD1 or PLD2 (Fig. 8B). When compared to the control cells (lane 1), the amount of total Erk and phosphorylated Erk associated with paxillin was enhanced in dominant negative PLD2 (lane 2) and PLD1 (lane 3) mutants. Along with the results presented earlier, these results suggested that PLD activity inhibits complex formation between paxillin and Erk.

3.3.5 Inhibition of Erik enhances paxillin tyrosine phosphorylation

Since our experiments showed an association between paxillin and Erk, we investigated the effect of Erk activity on paxillin phosphorylation. We measured paxillin tyrosine phosphorylation in the absence and presence of the selective MEK inhibitor U0126. This compound has been shown to inhibit MEK1 and MEK2 [53, 54], thus preventing Erk phosphorylation and activation. As shown in Fig. 9, U0126 completely suppressed Erk phosphorylation in the cells in 10% serum. In addition, U0126 induced paxillin tyrosine

phosphorylation (Fig. 10), indicating that paxillin tyrosine phosphorylation is mediated through an Erk-dependent pathway.

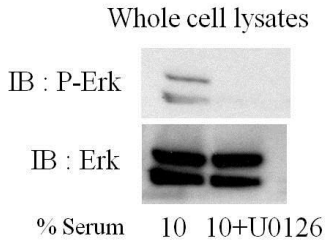

Figure 9. Phospho-Erk was not detected after treatment with U0126.
MDA-MB-231 cells exposed to high serum (10%) were incubated overnight with U0126. Whole cell lysates were immunoblotted with with α-P-Erk1/2 Ab. The blots were stripped and reprobed with α-Erk Ab.

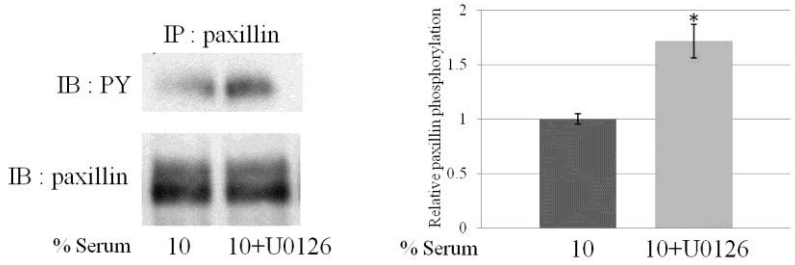

Figure 10. Paxillin tyrosine phosphorylation increases after treatment with U0126.
MDA-MB-231 cells exposed to high serum (10%) were incubated overnight with U0126. Cells were lysed, immnuoprecipitated with α-paxillin Ab and immunoblotted with α-P-Y Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to U0126 untreated cells with $p < 0.05$. Representative blot is shown.

3.3.6 Association of paxillin with FAK increases after serum removal

To address the cause of paxillin tyrosine phosphorylation, we examined the association of paxillin with FAK, which is known to tyrosine-phosphorylate paxillin [32, 33], in the presence and absence of serum. As shown in Fig. 11, paxillin is able to co-immunoprecipitate FAK in 10% serum, suggesting that these proteins form a complex. When the cells are serum deprived,

the amount of FAK associating with paxillin is increased, arguing that serum removal favors the formation of a complex between FAK and paxillin. This suggests that the increased tyrosine phosphorylation seen in paxillin with serum removal may be due to improved association of FAK with paxillin.

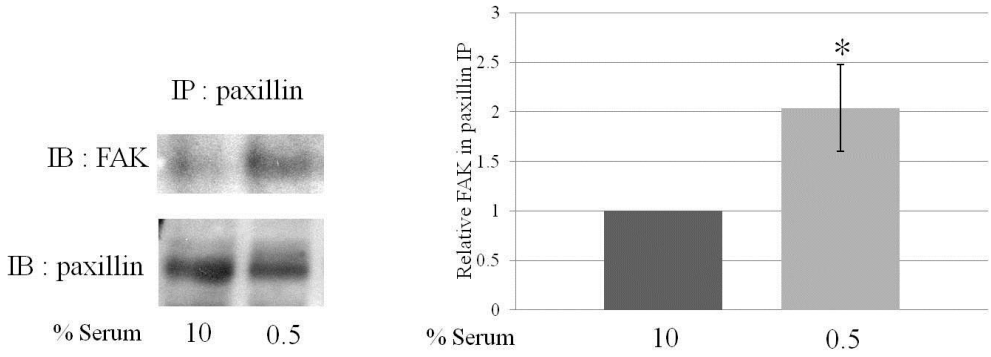

Figure 11. Association of paxillin with FAK increases after serum removal.
Cell lysates were immnuoprecipitated with α-paxillin Ab and immunoblotted with α- FAK Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum with $p < 0.05$. Representative blot is shown.

3.4 Discussion

Metastasis occurs when malignant cells migrate to an organ distant from the primary tumor, adjust to the new microenvironment and expand secondary tumors. The metastatic process consists of tumor cell intravasation, survival in circulation, extravasation into a distant organ, angiogenesis and finally unconstrained tumor growth [1]. Specific gene expression patterns are correlated with various metastases and recent studies have focused on elucidating genetic factors critical for tumor metastasis [2, 3].

In the center of a growing tumor, cells need to deal with the absence of serum growth factors. In order to survive under these conditions, cells need to bypass the immediate need for growth and survival factors and eventually obtain serum either by stimulating vascularization or

by inducing migration to sites where nutrients are available. Hence, the lack of vascularization in a developing tumor can be modeled in culture by serum withdrawal. In accordance with this, survival and migration have been linked to the stress of serum removal in MDA-MB-231 cells [8], making them an excellent model for metastatic breast cancer.

When starved for serum, and thus experiencing similar conditions to those found in the center of a growing tumor, MDA-MB-231 cells are able to survive and avoid apoptosis by elevating PLD activity. As previously reported, PLD signaling is critical for prevention of apoptosis [8, 55, 56]. Elevated PLD activity leads to the elevated expression of Myc [57], and activation of mTOR, mammalian target of rapamycin [58, 59]. In addition, it suppresses tumor suppressors such as p53 [60] and protein phosphatase 2A [61]. Thus, downstream targets of PLD have been identified in the suppression of apoptosis and the survival of cancer cells under serum stress.

While the role of PLD in survival of these cells has been examined, its role in the regulation of migration and invasion is less well understood. In other cell lines, it has been shown that PLD is involved in Erk signaling [45] and that Erk signaling is involved in cell migration through regulation of paxillin [42, 43]. Consistent with this, our results suggest that PLD activity is involved in migration of MDA-MB-231 cells through controlling Erk and paxillin. Previous studies done in MDA-MB-231 showed that serum removal is associated with loss of phosphoryated Erk. In addition, RUNX2 (Runt-related transcription factor 2) which stimulates cell motility of MDA-MB-231 cells, is reciprocally linked to activity of the MEK-Erk signaling pathway [51]. In accordance with this, our data imply that Erk phosphorylation is inhibited upon serum removal, which is characterized by elevated PLD activity, motility and invasiveness of MDA-MB-231 cells [8].

In our study, disruption of either PLD1 or PLD2 activity both caused decreased paxillin tyrosine phosphorylation, increased Erk phosphorylation and increased paxillin-Erk association, implying some functional redundancy for the two PLD isoforms. Such redundancy is not unexpected, as it has been seen in EGF receptor endocytosis [45] and cell proliferation in cells overexpressing a tyrosine kinase [62]. PLD2 is localized primarily to light membrane fractions containing many signaling molecules including the EGFR, unlike PLD1 which while found in light membrane fractions, has much broader cellular distribution [11, 63]. This would suggest that PLD2 is the major PLD isoform activated by mitogenic signals. However, PLD1 is known to be functionally associated with RalGTPase [16, 64] a downstream target of Ras, implying a role for PLD1 in mitogenic signaling. In addition, a negative correlation between PLD activity and Raf kinase activity, as measured by MEK (mitogen-activated protein kinase-kinase) phosphorylation, has been reported [65]. As MEK phosphorylates Erk 1/2, our finding that PLD activity inhibits Erk activity, as measured by Erk phosphorylation, is not surprising [66].

We showed that cells exposed to the stress of serum removal displayed a well characterized elevation of PLD activity. This led to a decrease in Erk phosphorylation, and presumably activity, and an increase in paxillin tyrosine phosphorylation. The increase in paxillin tyrosine phosphorylation is due to impaired Erk activity, as we were able to artificially reproduce this tyrosine phosphorylation by inhibiting Erk activity with U0126. Thus, elevated PLD activity, caused by serum removal, leads to the phosphorylation of paxillin, a protein known to be involved in regulating motility.

Paxillin regulates cell migration through controlling focal adhesion dynamics [67, 68]. As an adapter protein, paxillin recruits a number of signal transducers to focal adhesions. One signaling protein known to complex with paxillin is Erk [52]. Similar to what has been seen in

other cells, we found that paxillin can bind Erk in MDA-MB-231 cells. In addition, we showed that formation of this complex is regulated by serum levels through PLD activity. When serum levels are low, PLD activity is high, which inhibits the binding of Erk to paxillin.

Paxillin tyrosine phosphorylation leads to recruitment of a number of signaling molecules, thus provoking changes in cytoskeletal organization and regulating focal adhesion dynamics and cell migration. When stimulated with growth factors and cytokines, Focal adhesion kinase (FAK) can phosphorylate paxillin at two main sites (Tyr 31 and Tyr 118) [32]. Paxillin is known to bind to FAK and mediate FAK recruitment to focal adhesion complexes [33, 69].

In addition to binding Erk, paxillin is known to bind the tyrosine kinase FAK. FAK is also localized at focal adhesions, with paxillin being its primary target [68, 70]. Interestingly, Erk activity prevents paxillin from binding FAK as Erk phosphorylates FAK at Ser910 blocking the interaction of FAK with paxillin [43]. Thus, the observed decrease in paxillin tyrosine phosphorylation caused by increased Erk activity could be due to Erk disrupting the FAK-paxillin complex. Our experiments showed that under serum deprived conditions, impaired Erk activity allows association of FAK with paxillin and consequently, an increase in paxillin tyrosine phosphorylation. Previous studies have shown that adhesion-induced association of paxillin with FAK leads to paxillin phosphorylation of Tyr 31 and Tyr 118 [32, 33]. A major consequence of paxillin tyrosine phosphorylation is the generation of binding sites for SH2 domain-containing proteins. This would, of course, lead to remodeling of focal adhesions and changes in motility. However, the role of paxillin phosphorylation in modulating cellular motility is not well established and appears to be dependent upon cell type and context [37, 41]. Previous findings indicated that mTOR, mammalian target of rapamycin, may regulate phosphorylation of

focal adhesion proteins, including paxillin [71, 72] and cell motility and invasion [73]. In addition, phosphatidic acid, the metabolic product of PLD, is known to activate mTOR, [58, 59]. Thus, it is also possible that increased PLD activity under serum deprived conditions activates tyrosine phosphorylation of paxillin and consequently, cell motility, in part through increased production of PA and mTOR activation.

3.5 Conclusion

For MDA-MB-231 cells, our data suggest that tyrosine phosphorylation of paxillin is associated with increased motility in response to serum withdrawal. Specifically, the absence of serum promotes PLD activity. This activity, either directly or indirectly, inhibits Erk activity. The decreased Erk activity allows the association of paxillin with FAK, leading to the tyrosine phosphorylation of paxillin. This causes changes in focal adhesions, leading to increased motility (Fig. 12).

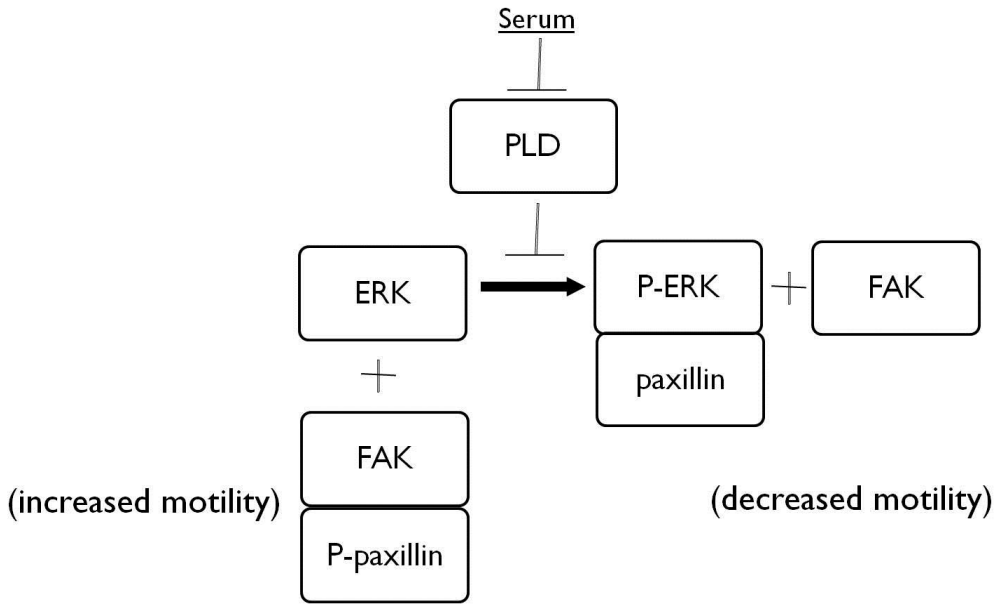

Figure 12. Model of role of PLD, Erk, FAK and paxillin in cell motility.
Increased motility of MDA-MB-231 cells after serum removal may be mediated by PLD, Erk and paxillin.

We have found that paxillin, Erk, PLD and FAK work coordinately to regulate cell motility in MDA-MB-231 cells, and therefore may play an important role in metastasis of cancer cells. Whether this coordination is present in other types of cancers characterized by elevated PLD activity (renal, gastric and colon), still remains to be revealed. In summary, our study suggests that elevated PLD activity is associated with low Erk activity and increased paxillin tyrosine phosphorylation, and constitutes a potentially novel target for inhibiting cancer cell motility and invasiveness.

3.6 References

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Chapter 4: Conclusion

4.1 Paxillin and Phospholipase D interact to regulate actin-based process in *Dictyostelium discoideum*

The actin cytoskeleton forms a membrane-associated network, whose proper regulation is essential for numerous processes. In this study we have focused primarily on elucidating the role of paxillin and PLD in coordinating the regulation of the actin cytoskeleton. It is through this function that these proteins exert the most significant impact on cell motility, differentiation, development, tissue morphogenesis, and pathologies such as tumor cell invasion. PLD activity has a profound impact on the structure and stability of cellular membranes, but also plays a role in regulating many critical cellular functions. It generates phosphatidic acid (PA), a multifunctional lipid second messenger that, as widely believed, is the most critical metabolite generated by PLD. PA alters membrane curvature, serves as a protein attachment site, activates selected enzymes or represents the starting material for the production of additional signaling lipids important for vesicle trafficking and cytoskeletal dynamics [1-5]. As a molecular scaffold, paxillin is important for the coordination of numerous signaling pathways. However, there are several instances in which a number of proteins must compete for a single binding domain or motif on the paxillin molecule [6]. Thus, it is important to identify the mechanisms by which the cell orchestrates these interactions in a spatiotemporal and context-specific manner.

We chose *Dictyostelium discoideum* as a model organism to study the role of paxillin and PLD in cellular activities that depend upon the actin cytoskeleton including cell differentiation, proliferation, adhesion, chemotaxis, endocytosis, exocytosis and multicellualar development. To

gain better insight into the roles of paxillin and PLD, and to investigate their potential interactions, we studied their *Dictyostelium discoideum* homologs, PaxB and PldB, respectively. As found previously in our lab, PaxB and PldB colocalize and co-immunoprecipitate, suggesting that they physically interact.

Evidence that the two proteins are functioning in the same pathways is found by examining the phenotypes of the *paxB*$^{-}$*/pldB*OE double mutants. Classical epistasis analysis can be used to determine whether two gene products function in the same pathway, and if so, their order of action. If the phenotypes associated with two mutations are different, then the phenotype of the double mutant dictates which gene product acts downstream of the other. For example, cells lacking *paxB* have decreased adhesion. Cells overexpressing *pldB* have normal adhesion. The double mutants, which lack *paxB* and overexpress *pldB*, have normal adhesion, just like the *pldB* overexpressing cells. Epistasis analysis says that since the *pldB* overexpressing cell phenotype is dominant, PldB must be acting downstream of PaxB. According to this logic, PldB acts downstream of PaxB in regulating adhesion and speed of chemotaxis to cAMP. In contrast, PldB acts upstream of PaxB in controlling directionality, chemotactic index, cAMP-induced F-actin polymerization, phagocytosis and exocytosis. Simple epistasis cannot explain the fact that the order of action of PaxB and PldB varies depending on the process measured. Thus, PaxB and PldB cannot be in a simple pathway where one protein activates the other.

To explain how PaxB and PldB are working together to regulate cytoskeletal functions, both their physical and functional interactions must be taken into account. Our observations provide evidence in favor of the hypothesis that PaxB and PldB form a complex. PLD catalyzes the hydrolysis of phosphatidylcholine, producing phosphatidic acid (PA), a second messenger [7]. PldB uses PaxB as an adaptor to be recruited to specific subcellular locations, where

production of PA can facilitate proper actin responses to environmental changes. This could trigger recruitment of other proteins, such as Phg2 and SadA by PaxB at focal sites, which in turn controls adhesion, motility and actin cytoskeleton organization [8, 9]. In *paxB*$^{-}$ cells, PldB is unable to properly localize, leading to improper actin reorganization. In *pldBOE* cells, an inordinate amount of PldB gets recruited to sites of action by PaxB, leading to hyperactive signaling and therefore cytoskeletal defects. These phenotypes are ameliorated in the *paxB*$^{-}$*/pldBOE* double mutant because even though there is an abundance of PldB, there is no PaxB to efficiently localize it. A relatively small percentage of the overproduced PldB is able to localize to its sites of action, allowing it to perform its signaling function and regulate the actin cytoskeleton.

4.2 Paxillin phosphorylation and complexing with Erk and FAK are regulated by PLD activity in MDA-MB-231 cells

Using *Dictyostelium discoideum* we have for the first time described a functional and physical interaction between paxillin and PLD. The fact that *Dictyostelium discoideum* has proven to be an excellent cellular model for studying the cytoskeleton and its regulation, prompted us to investigate whether a similar relationship between paxillin and phospholipase D exists in human breast cancer cells. For our study we used MDA-MB-231 human breast cancer cells which are highly aggressive and depend on PLD activity for survival [10, 11].

In response to the stress of serum withdrawal, MDA-MB-231 cells experience a rapid increase in PLD activity, triggering increased motility and invasiveness [12]. In addition to PLD, paxillin and Erk play vital roles in cell motility through regulation of focal adhesion dynamics.

We focused on elucidating the role of PLD activity on paxillin phosphorylation, Erk phosphorylation and paxillin-Erk association. Our experiments showed that inhibition of PLD activity, either by treatment with 1-Butanol or by dominant-negative mutation, leads to a decrease in paxillin tyrosine phosphorylation, an increase in Erk phosphorylation and enhanced paxillin-Erk association. In addition, we found that paxillin tyrosine phosphorylation depends upon Erk activity. Moreover, we showed that association of paxillin with FAK increases after serum removal suggesting that under serum deprived conditions, impaired Erk activity allows association of FAK with paxillin and consequently, an increase in paxillin tyrosine phosphorylation. This causes changes in focal adhesions, leading to increased motility.

Taken together, our data suggest that for MDA-MB-231 cells, tyrosine phosphorylation of paxillin is associated with increased motility in response to serum withdrawal. Specifically, the absence of serum promotes PLD activity. This activity, either directly or indirectly, inhibits Erk activity. The decreased Erk activity allows the association of paxillin with FAK and the tyrosine phosphorylation of paxillin leading to changes in focal adhesions and increased motility. Hence, Erk activity, governed by PLD activity, plays a role in cell motility by regulating tyrosine phosphorylation of paxillin. This study indicated that PLD, paxillin and Erk participate in the same signaling pathway in this breast cancer cell line, providing further insight into the role of these proteins in cancer and a better understanding of the clinical course of disease.

All in all, we used the social amoebae, *Dictyostelium discoideum*, as a model organism, and a highly invasive metastatic breast cancer cell model, MDA-MB-231, to bridge two existing fields of inquiry regarding processes that are based on reorganization of actin cytoskeleton. While it had been known that paxillin and PLD independently control the actin cytoskeleton we are the first to show that paxillin and PLD work together to regulate a number of actin-required

processes suggesting their functional interaction. Moreover, this research demonstrated that *Dictyostelium discoideum* can serve as an excellent model for studying signaling to the cytoskeleton and elucidating mammalian pathways.

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