A Novel Approach For The Identification of Cytoskeletal and Adhesion A-Kinase Anchoring Proteins

Laura Taylor Director

University of Vermont, Lauratdirector@gmail.com

Follow this and additional works at: http://scholarworks.uvm.edu/graddis

Part of the Medical Cell Biology Commons, and the Molecular Biology Commons

Recommended Citation

A NOVEL APPROACH FOR THE IDENTIFICATION OF CYTOSKELETAL
AND ADHESION A-KINASE ANCHORING PROTEINS

A Thesis Presented

by

Laura Taylor Director

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science,
Specializing in Cellular and Molecular Biology

October, 2014
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Science, specializing in Cellular and Molecular Biology.

Thesis Examination Committee:

____________________________________ Advisor
Alan K. Howe, Ph.D.

____________________________________
Jason Stumpff, Ph.D.

____________________________________ Chairperson
Bryan Ballif, Ph. D.

____________________________________ Dean, Graduate College
Cynthia J. Forehand, Ph.D.

June 13th, 2014
ABSTRACT

A-kinase anchoring proteins (AKAPs) are signaling scaffolds which provide spatial and temporal organization of signaling pathways in discrete subcellular compartments. Through tethering the cyclic-AMP dependent protein kinase A (PKA), AKAPs target PKA activity to distinct regions in the cell, bringing PKA in close proximity to its target proteins. This provides a high level of specificity and regulation of PKA and its role in mediating a number of biological processes, one of which is cell migration. Cell migration is a highly dynamic and fundamental process, when misregulated can lead to a number of pathologies. The process of cell migration requires integration and coordination of actin cytoskeletal dynamics, adhesion turnover, and contractility. The important role of PKA in regulating the cellular processes involved in cell migration has been extensively studied. Our lab has shown that PKA activity and spatial distribution through AKAPs are localized to the leading edge of migrating cells and are required for effective cell migration, yet the specific AKAPs responsible remain unknown.

Traditional methods for identifying AKAPs suffer from a number of limitations. Therefore the objective of the enclosed work is to establish and characterize a novel approach for the identification of cytoskeletal and adhesion-associated AKAPs. We show for the first time, an in vitro approach to identify cytoskeletal AKAPs which may be responsible for localizing PKA to the leading edge of migrating cells.
ACKNOWLEDGEMENTS

There have been a number of people whom I would like to thank for their continual support during my time at UVM, and who have aided in my personal and intellectual growth and development.

I would first and foremost like to thank my boss and mentor, Alan Howe, PhD. Without him I would not be half the person or scientist I am today. The training that I received from Alan, and everything I have learned during our time together has undoubtedly become an important part of who I am, and will pay countless dividends throughout my next endeavors. I cannot thank him enough for being supportive, flexible, and understanding as I made the difficult decision to apply to medical school. Above all, Alan has taught me how to tackle any problem I am faced with, and to always stay skeptical, think critically, and assume nothing. Whether in science, medicine, or any other area of life, I will carry the lessons I have learned from Alan throughout.

I would like the thank my fellow lab members, Andy McKenzie, Tamara Williams, Steph Hicks, Su Newberry, and Marissa Marzano for all their help along the way. I would especially like to thank Andy and Tamara for their advice and words of wisdom, both scientific and not. I cannot imagine a more collaborate and supportive environment to work in for the past two years, and I feel very fortunate to have had the opportunity to work with amazing individuals.

Additionally, I cannot go without thanking the Cellular and Molecular Biomedical Sciences Department. In particular, I would like to thank Erin Montgomery
for always taking the time to talk to me, check-in on me, and help me sort out any problem I had. Without her support, I would not have made it through this program. Also, within the CMB program, I would like to thank Stephen Everse, PhD., for all his time, guidance, and support throughout my time at UVM. Stephen epitomizes a true educator, and I am fortunate to have had him as a teacher and mentor.

I would also like to thank the members of my committee, Jason Stumpff, PhD., Gary Ward, PhD., and Bryan Ballif, PhD., for their guidance, time and patience. They have donated their time to give me expert advice, pushing my research and me to the intellectual boundaries.

I am fortunate to have many good friends but I want thank in particular my three best friends, Abby, Brian, and Elissa. From cooking dinner and walking my dog, to hours of phone calls, evening runs, and bottomless bags of sour patch kids, their help has been invaluable throughout this process. Their continual support and encouragement gave me the strength and motivation to carry on during the times I needed it most.

Last and certainly not least, I want to gratefully thank my parents. From a young age, they taught me the importance of education, to work hard, be passionate about what I do, and encouraged me to chase after my dreams. I owe all of my successes, past, present, and future, to them. They have been my biggest fans and supporters and I cannot thank them enough for providing me with everything I need to succeed.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ ii

LIST OF TABLES .................................................................................................................. vii

LIST OF FIGURES .............................................................................................................. viii

**CHAPTER 1: Literature Review** ......................................................................................... 1

1.1 Cell Migration .................................................................................................................. 1

1.1.1 Introduction to Cell Migration ..................................................................................... 1

1.1.2 Basic Mechanisms of Cell Migration .......................................................................... 1

1.1.3 The Cytoskeleton ......................................................................................................... 3

1.1.3.1 Actin and Actin Dynamics ...................................................................................... 3

1.1.3.2 Actin Binding Partners ......................................................................................... 5

1.1.3.3 Rho Family GTPases .......................................................................................... 6

1.1.3.4 Myosin II ............................................................................................................ 7

1.1.4 Cell Adhesion ............................................................................................................. 8

1.1.5 Importance of Cellular Adhesion ............................................................................... 9

1.1.6 Structure of Adhesions .............................................................................................. 9

1.1.7 Adhesion Dynamics .................................................................................................. 10

1.1.8 Focal Adhesion Components .................................................................................... 11

1.1.8.1 Scaffolding Proteins ......................................................................................... 11

1.1.8.2 Signaling Proteins ............................................................................................. 13

1.1.8.3 Integrins ............................................................................................................ 14

1.1.8.4 The Integrin Adhesome ...................................................................................... 17

1.2 cAMP/PKA Signaling ..................................................................................................... 19

1.2.1 Structure and Function of PKA .................................................................................. 19

1.2.2 Cellular Distribution of PKA .................................................................................... 20

1.2.3 Role of PKA in Cell Migration .................................................................................. 21

1.2.3.1 Negative Regulation .......................................................................................... 21

1.2.3.2 Positive Regulation ............................................................................................ 22

1.2.4 The Role of PKA in Cell Adhesion ........................................................................... 23

1.2.5 Substrates of PKA in Regulation of the Cytoskeleton, Adhesion, and Migration... 24

1.2.5.1 Actin .................................................................................................................. 25

1.2.5.2 Vasodilator-stimulated phosphoprotein (VASP) ................................................... 25

1.2.5.3 Myosin Light Chain (MLC) ................................................................................ 26

1.2.5.4 Filamin A ........................................................................................................... 27

1.2.5.5 Rho Family GTPases ......................................................................................... 28

1.3 A-Kinase Anchoring Proteins ......................................................................................... 30

1.3.1 The Importance of Scaffolding Proteins ................................................................... 30

1.3.2 Introduction to A-Kinase Anchoring Proteins .......................................................... 31

1.3.3 AKAP Structure and Function .................................................................................. 32

1.3.4 AKAP Nomenclature ............................................................................................... 32

1.3.5 Localization Signals .................................................................................................. 33

1.3.6 Mechanism of PKA Anchoring ............................................................................... 35

1.3.7 Type I vs. Type II Anchoring .................................................................................. 39

1.3.8 Noncanonical AKAPs ............................................................................................. 41

1.3.9 Evolution of AKAPs ................................................................................................. 42
### 3.1.7 List of candidate proteins selected from SKOV-3 cytoskeletal fraction and whole cell extract

Page 92

### 3.1.8 Development and Rationale of Algorithm

Page 98

### CHAPTER 4: Discussion

Page 104

### Comprehensive Bibliography

Page 118
LIST OF TABLES

Table                                  Page

CHAPTER 3: Results

TABLE 1: Identification of candidate proteins selected from SKOV-3 cytoskeletal fraction and whole cell extract ................................................................. 95
TABLE 2: Score for known A-kinase anchoring proteins using the developed algorithm ........................................... 102
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHAPTER 1: Literature Review</strong></td>
<td></td>
</tr>
<tr>
<td>Figure 1: Structural elements of a migrating cell</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2: AKAP signaling complexes localize to distinct subcellular regions to create focal points for signal transduction</td>
<td>34</td>
</tr>
<tr>
<td>Figure 3: Overview of structures of RII dimerization domain in complex with an amphipathic AKAP helix</td>
<td>35</td>
</tr>
<tr>
<td>Figure 4: Sequence alignment and conservation of hydrophilic and hydrophobic residues among known AKAPs</td>
<td>37</td>
</tr>
<tr>
<td>Figure 5: Details of the AKAP helix-RIIα dimerization domain polar contacts</td>
<td>39</td>
</tr>
<tr>
<td><strong>CHAPTER 3: Results</strong></td>
<td></td>
</tr>
<tr>
<td>Figure 1: Schematic for the application of biotin transfer coupled to RIIα method in SKOV-3 cells</td>
<td>70</td>
</tr>
<tr>
<td>Figure 2: Characterization and optimization of biotin transfer method</td>
<td>73</td>
</tr>
<tr>
<td>Figure 3: Ht31 but not Ht31P disrupts RIIα-AKAP79 interaction in SKOV-3 cells</td>
<td>79</td>
</tr>
<tr>
<td>Figure 4: Interaction between filamin A and RIIα in SKOV-3 cells</td>
<td>82</td>
</tr>
<tr>
<td>Figure 5: Cytoskeletal fraction from SKOV-3 lysate shows enrichment of cytoskeletal proteins</td>
<td>86</td>
</tr>
<tr>
<td>Figure 6: Coomassie staining of streptavidin pull-downs from SKOV-3 lysate submitted for mass spectrometry analysis</td>
<td>89</td>
</tr>
<tr>
<td>Figure 7: Development and rationale for algorithm</td>
<td>99</td>
</tr>
</tbody>
</table>
CHAPTER 1: Literature Review

1.1 Cell Migration

1.1.1 Introduction to Cell Migration

Cell migration is a fundamental process involved in many essential biological processes such as wound healing, embryogenesis, and the immune response. When left unregulated, aberrant cell migration can lead to autoimmune diseases and cancer cell metastasis (Vincente-Manzanares and Horowitz, 2011). Cell migration is a highly dynamic process, which involves the interplay between tension sensing, contractility, cytoskeletal dynamics, and adhesion turnover, all converging on directional cell movement. The involvement of cell migration in a number of fundamental biological processes underscores its importance and the need for tight spatiotemporal coordination and integration of many proteins and signaling pathways. While the field has significantly advanced our working knowledge of cell migration over the past few decades, furthering our understanding of the mechanisms underlying cell migration is critical.

1.1.2 Basic Mechanisms of Cell Migration

Currently, cell migration is viewed as a cyclical process (Ridley et al., 2003) and involves four steps: protrusion, adhesion to extracellular matrix, retraction, and translocation (Howe, 2004). Directional cell migration is initiated by an extracellular signal, which results in a dynamic reorganization of the actin cytoskeleton leading to protrusion of the cell (Parsons et al., 2010). These protrusive structures can be broad
and large lamellipodia, characterized by the branching of actin filaments, or are long, thin cables called filopodia. Protrusions make contact with the extracellular matrix, forming focal adhesions, stabilizing the protrusive structures (Zamir, 2001; Geiger 2011). Cell migration continues through the utilization of the contractile machinery, which among other things promotes disassembly of focal adhesions at the trailing edge of the cell, promoting directional migration (Figure 1) (Vincente-Manzanares et al., 2007).

**Figure 1: Structural elements of a migrating cell**
Directional cell migration requires cellular polarity, which means the molecular mechanisms at the leading edge (the front) and the trailing edge (the back) of the cell are different. Establishing and maintaining cell polarity is mediated by the integration of many proteins including Rho family GTPases, phosphoinositide 3-kinases (PI3Ks), actin polymerization, integrins, actin, microtubules, myosin II, and cAMP-dependent protein kinase A. While the molecular mechanisms that govern cell migration are generally consistent between cell types, the relative contributions of signals depend on the specific stimulus and cell type.

Cell migration is largely driven by changes in the cytoskeleton and interactions with the extracellular matrix (ECM) through complexes of structural and signaling proteins referred to as focal adhesions. This dynamic process is tightly regulated through a number of biochemical and mechanical signaling pathways.

1.1.3 The Cytoskeleton

The cytoskeleton is the cellular architecture and is responsible for establishing cell shape, providing mechanical strength, and cell motility. It is made up of three molecules, actin, microtubules, and intermediate filaments. While the role of actin and microtubules in cell migration has been well established, the contributions from intermediate filaments have not been defined.

1.1.3.1 Actin and Actin Dynamics

Actin filaments are comprised of monomeric globular, G-actin subunits which bind to one another through a process driven by ATP hydrolysis (Vincente-Manzanares
and Horowitz, 2011). Actin polymerization is the process by which ATP bound G-actin is hydrolyzed to ADP+P; and a bond is formed between actin subunits. The ADP+P; stabilize the subunits creating filamentous actin, F-actin, the stable filaments responsible for the cellular integrity. Actin filaments are polarized structures, displaying a fast “barbed” end, the site of new subunit incorporation, and a “pointed” end, the origin of the growing filament (Korn et al., 1987; Carlier et al., 2003). There are a number of different types of actin filaments, whose formation is regulated through the number of actin filaments and the available accessory proteins. The different morphologies give rise to different structures at distinct subcellular locations and can be characterized as lamellipodia, filopodia, or stress fibers (Mattila et al., 2008; Mejillano et al., 2004).

In lamellipodia, actin is arranged in large, broad, branched structures, the assembly of which is catalyzed by the ARP2/3 complex (Pollard and Borisy 2003; Mitchison and Cramer 1996). This complex binds to the sides of the growing actin filament, promoting the formation of a new filament, creating a branched network of actin filaments. Lamellipodia are followed closely with the lamellum, where the actin is more closely bundled (Ponti et al., 2004). This comprises the protrusions at the leading edge of cell, which in combination with contractile forces push the membrane forward resulting in directional migration.

In contrast, filopodia, are long, thin cable-like protrusions which initiate from the plasma membrane. They have been described as ‘antennae’ that cells use to probe their local environment (Mattila et al., 2008). Protrusion of filopodia is thought to occur
through filament tread milling, which involves attachment of actin subunits and elongation at the barbed end, while releasing actin from the pointed end (Glenney et al., 1981).

1.1.3.2 Actin Binding Partners

Building different morphologies of actin filaments, requires an extensive network of accessory proteins. These are intimately involved in not only the regulation of actin cytoskeletal dynamics, but are critical in tension sensing, mechanotransduction, and signal transduction. There are over 100 known actin binding partners, and while the purpose of this review is not to talk in detail about them, a few are of particular note. As mentioned before, the Arp2/3 complex is responsible for actin polymerization in the lamellipodia (Pollard and Cooper 2009). Activation of the Arp2/3 complex is localized by the Wiskott-Aldrich syndrome protein (WASP/WAVE) family members (Pollard and Borisy, 2003). Profilin binds actin monomers and prevents self-nucleation, while helping target the monomers to the barbed end of the growing filament (Ridley et al., 2003). The elongation of actin filaments is regulated by capping proteins such as coflin, which also aid in severing filaments and promoting actin dissociation (Ridley et al., 2003). There are a number of proteins, which help to crosslink and stabilize the actin network such as cortactin, filamin A, and α-actinin (Pollard and Cooper 2009). Filopodial structures have additional proteins, which are enriched such as the Ena/VASP proteins, which antagonize capping and branching and thus promote continuous elongation of filaments. Additionally, fascin helps to bundle actin filaments
and is thought to help generate the stiffness needed to propel the plasma membrane forward in filopodial-mediated protrusion (Pollard and Cooper 2009).

1.1.3.3. *Rho Family GTPases*

Rho family small guanosine triphosphate (GTP)-binding proteins (GTPases) comprise a family of molecular switches, which control many signaling pathways required for a number of cellular processes. GTPases are relatively small enzymes whose primary role is to hydrolyze the gamma phosphate from guanine-5-triphosphate (GTP) and are conformationally regulated by the binding of GTP and GDP. In their inactive form, they are bound to GDP, and are activated once GDP is exchanged with GTP. GTPase activity is mediated through a balance between guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) and is tightly regulated. GTPases have been implicated in a number of aspects of cell migration, including the control and formation of leading edge actin structures such as filopodia and lamellipodia, and in the coordination of adhesion structures.

The first report of Rho family GTPases regulating cell migration came in 1995, when Rho, Rac, and Cdc42 were identified (Nobes and Hall 1995). While all part of the same superfamily, Rho, Rac, and Cdc42 all have slightly different roles with respect to their regulation of cell migration. Rho primarily controls contractility-dependent processes including the formation or stress fibers (Brahmbhatt and Klemke 2003).

Both Rac and Cdc42 mediate actin polymerization through their interactions with WASP/WAVE family of Arp2/3 complex activators (Ridley and Cooper 2009).
Rac drives lamellipodial protrusion formation through activation of WAVE/Scar protein through binding of an intermediate protein, IRSp53 (Cory and Ridley 2002). This in turn regulates the activity of the Arp2/3 complex, controlling the formation of actin structures at the leading edge. It has been shown that Rac activation is localized to the leading edge of protruding and migrating cells (Aoki et al., 2004; Kraynov et al., 2000). Additionally, Rac affects the migratory machinery through its activation of several effector proteins such as PAKs, MLCK, RLC, LIMK, and stathmin (Vincente-Manzanares et al., 2007).

Cdc42 is the primary regulator among the Rho GTPases of cell polarity and is active towards the front of migrating cells (Itoh et al., 2002). Cdc42 binds WASP proteins, which stimulates Arp2/3 complex to induce branched actin polymerization. Cdc42 also exerts its effects on cell polarity through organization and localization of the microtubule-organizing center (MTOC) (Rodriguez et al., 2003). One downstream target of Cdc42 is PAK1, which itself can regulate Cdc42, creating a positive feedback loop (Ridley et al., 2003).

1.1.3.4 Myosin II

Myosins are a class of actin-binding molecules, which are often referred to as motor proteins (Aguilar-Cuenca et al., 2014). The globular head of the myosin binds actin and also has ATPase activity, which causes conformational movements which allow the myosin to slide along the actin filament, generating contractile forces (Warrick and Spudich, 1987). The affinity for actin changes, depending on the stage in ATP hydrolysis (Clarke and Spudich, 1977). The role of non-muscle myosin II (NMII)
has been well characterized as playing a central role in the cellular response to mechanical cues, because of its inherent ability to generate mechanical forces (Aguilar-Cuenca et al., 2014). Myosin II also cross-links the actin cytoskeletal and facilitates reorganization of lamellar actin through its interactions with formin and alpha-actinin (Choi et al., 2008). Myosin II motors have been shown to regulate focal adhesion morphology during the maturation process (Stricker et al., 2013). Myosin II activity is controlled by phosphorylation at several sites, the most important being Ser19 (Ikebe, 2008). A number of kinases known for their involved in migration and adhesion dynamics have been shown to be responsible for this phosphorylation. Additionally, cAMP-protein kinase A, Rho-associated kinase (ROCK) and integrins have been shown to regulate the activity of myosin II.

1.1.4 Cell Adhesion

Cellular migration requires stabilization of leading edge protrusions via interactions with their extracellular matrix. Cell-matrix adhesions are not simply static structure, but are highly dynamic and are under tight spatial and temporal control. Through this tight regulation, signaling proteins dictate the location, duration, and type of cell-matrix adhesion. Cell-matrix adhesions connect the cytoskeleton through the cytoplasmic domains of adhesions receptors, creating contact with the extracellular matrix, and allowing cells to create a stable and force-generating contact point. By using myosin II-mediated contractile forces, cells are able to translate the mechanical forces felt at adhesion sites into intracellular biochemical signals. This translational
process called mechanosensing (Schiller and Fassler, 2013). The mechanical tension at sites of adhesions, is responsible for promoting the maturation of adhesions (Balaban et al., 2001).

### 1.1.5 Importance of Cellular Adhesion

The deregulation of adhesion assembly and disassembly is common to many diseases. Of particular note is cancer, where the regulation of cell adhesion is critical to a cell’s ability to successfully detach and invade distal sites of metastasis. The ability of a cell to sense and respond to its local environment, migrate, and invade distal sites are dependent on the proper regulation of adhesion. Large scale genetic and proteomic studies have shown many adhesion proteins to be abnormally regulated during tumor progression (Hoffman et al., 2011).

### 1.1.6 Structure of Adhesions

Cell matrix adhesions were first described over 40 years ago (Curtis, 1964) however it took a number of years to elucidate their structure and function. Over the past few years, progress has been made in our understanding of the proteins involved at sites of cell-matrix adhesions, as well as the types of adhesive structures. More recently, focal adhesions have been isolated and shot-gun proteomics has been used to identify the constituents (Kuo et al., 2012; Zaidel-Bar and Geiger 2010; Schiller et al., 2011). Within the past four years, three-dimensional super-resolution fluorescence
microscopy has been used to understand the architecture of focal adhesions (Kanchanawong et al., 2010).

There are four classic types of adhesive structures, which can exist in a single cell at any one time: nascent adhesions, focal complexes, focal adhesions, and fibrillar adhesions (Puklin-Faucher and Sheetz, 2009; Zaidel-Bar and Geiger, 2010). The components of adhesions have been characterized over the past few years, and over 200 different molecules have been found to associate with adhesions (Zaidel-Bar et al., 2007) and proteins from a variety of families have been identified including cytoskeletal proteins, transmembrane receptors, and signal transduction molecules. The critical role adhesions play in diverse cell processes underscores their importance and the need for further characterization.

1.1.7 Adhesion Dynamics

Adhesion dynamics are tightly regulated and is a highly dynamic process involving continual assembly-disassembly. Adhesion of a protruding cell edge begins with the formation of a nascent adhesions in the lemmellipodium (Parsons et al., 2010). These are short lived and quickly turnover into focal complexes and eventually into focal adhesions which reside at the ends of large actin bundles (Gardel et al., 2010).
1.1.8 Focal Adhesion Components

As mentioned before, focal adhesions serve as the link between the actin cytoskeleton and the ECM, interpreting extracellular cues and converting them into intracellular signals. Their important function requires a complex array of molecules and recent proteomic and bioinformatics approaches have identified over 150 proteins associated with focal adhesions (Zaidel-Bar and Humphries 2007; Humphries et al., 2009). Central to focal adhesions are the major transmembrane receptors, integrins. The host of proteins at sites of focal adhesions come from a host of different molecular classes including cytoskeletal proteins, kinases, modulators of small GTPases, phosphatases, and other enzymes (Zamir and Geiger, 2001). Many of these proteins directly bind to actin or the cytoplasmic tails of integrins, while others transiently associate with adaptor/scaffolding proteins. Because of the diversity and breadth of protein-protein interactions at sites of focal adhesions, they have become a site for organization of multi-protein signaling complexes. The list of focal adhesion proteins is extensive and therefore this review will discuss a selected group of proteins drawn from each of the major families of proteins.

1.1.8.1 Scaffolding Proteins

Focal adhesions contain several proteins that function as signaling scaffolds for the components of focal adhesions. This provides a mechanism by which signaling
enzymes can associate with their substrates and leads to changes in cell morphology and behavior. In addition to p130Cas and Crk, paxillin is one of the best described scaffolding proteins at focal adhesions (Webb et al., 2004). Paxillin has a number of motifs that facilitate its binding to signaling proteins such as Src, FAK and PKL (Webb et al., 2004). It also contains C-terminal LIM domains which allows it to attach to the cell membrane and interact with the phosphatase PTP-PEST (Turner 2000). Recruitment to focal adhesion complexes is mediated by binding to the β1 and α4 integrin cytoplasmic tails (Liu et al., 2002).

Phosphorylation of paxillin is important for focal adhesion formation and maturation. For example, paxillin phosphorylation on Y31, Y119, or S273 results in robust protrusion and focal adhesion assembly in protrusions (Nayal et al., 2006, Zaidel-Bar et al., 2007). Additionally, in vivo data shows that the phosphorylation of paxillin leads to complex formation with FAK to regulate dynamics of nascent adhesions (Choi et al., 2011) and phosphorylation by FAK and Src lead to the creation of addition binding sites for adaptor proteins (Turner 2003). Paxillin has also been implicated to inhibit α4β1-dependent cell migration through its binding to α4 integrin tail (Goldfinger et al., 2003).

Paxillin acts as a critical component of focal adhesions through its activation of Rho family GTPases. Both Cdc42 and Rac co-localize with paxillin and stimulate the activity of p21-activated kinase (PAK) (Manser et al., 1998). Activated PAK in turns modulates the actin cytoskeleton into lamellipodia and filopodia formation (Delorme-Walker et al., 2011).
1.1.8.2 Signaling Proteins

Phosphorylation is one of the key signaling events that occur at focal adhesions, both at tyrosine and serine/threonine sites. Tyrosine phosphorylation at focal adhesions has been well characterized as providing binding sites for SH2-containing proteins thus regulating the activation of many signaling kinases and phosphatases (Webb et al., 2004). There are a number of tyrosine kinases such as focal-adhesion kinase (FAK), Src, Abl, and Csk as well as serine/threonine kinases such as PAK and PKC that are found in focal adhesions (Zamir and Geiger, 2001). While these all have important roles in regulating adhesion dynamics and cell behavior, FAK is one of the best characterized and therefore will be the focus of the subsequent section.

FAK is a tyrosine kinase which localizes to focal adhesions via its C-terminal focal adhesion targeting domain (FAT) (Hildebrand et al., 1993). It is centrally positioned for regulating focal adhesion dynamics through its association with talin, providing the link between integrin-mediated signal transduction and subsequent downstream pathways (Chen et al., 1995). It phosphorylates a number of targets involved in adhesion dynamics (Chen et al., 1995) and therefore has evolved as one of the central and most important regulators of signaling complexes at focal adhesions. Phosphorylation at Y397 has been shown to result in the recruitment of Src-family PTKs, which in turn causes Src-mediated phosphorylation and thus creating further binding sites for proteins such as Grb2 (Schlaepfer et al., 1999).
The requirement for FAK in cell migration has been demonstrated through experiments showing that fibroblasts from FAK-null mice exhibit a decreased rate of migration and an increase in the number of size of adhesions (Ilic et al., 1995). FAK’s role in adhesion turnover has been further characterized and recently it has been shown that FAK-Src signaling regulates adhesion disassembly through ERK and MLCK (Webb et al., 2004). Furthermore, FAK has been implicated in cancer cell invasion and studies with human tumor tissues and tumor-derived cell lines have shown FAK expression to be high (Cance et al., 1995). Furthermore, FAK recruitment and activation are associated with integrin clustering, and FAK complexes with proteins such as talin, have been shown to regulate focal adhesion turnover (Lawson et al., 2014).

FAK also plays a role in regulating adhesion dynamics through its activation of small GTPases by binding and phosphorylating their exchange factors (Webb et al., 2004). Given the role of small GTPases in regulating adhesions, further highlights the importance of FAK in focal adhesion dynamics.

1.1.8.3 Integrins

Integrins are one of the major transmembrane receptors central to adhesions. They play a structural role linking the actin cytoskeleton to the extracellular matrix (ECM) and mediate all cell-ECM interactions. Integrins also serve as a major site for transmission of mechanical and biochemical signals from the ECM to the inside of the
cell. Many cellular processes rely on signal cascades organized by proteins interacting with the cytoplasmic tail of integrins.

Integrins are comprised of non-covalently associated $\alpha$ and $\beta$-glycoprotein subunits, which pass the membrane through a single membrane helix (Lad et al., 2007). Combinations of the $\alpha$ and $\beta$-chains form the 24 different heterodimers, which contributes to the diversity of integrins and ultimately enabling cells to respond to different extracellular cues (Geiger and Zaidel-Bar, 2012). The integrin heterodimer is entirely dependent on the ECM ligand present surrounding the cell. Upon binding the extracellular ligand, a conformation change occurs allowing assembly of multi-protein complexes and thus initiating downstream signaling pathways. Because integrins contain no enzymatic activity, they rely on the recruitment of signaling enzymes for proper signal transduction.

The cytoplasmic tail has been shown to bind to at least 12 different adaptor proteins including tensin, filamin A, talin, plectin, paxillin, talin, and $\alpha$-actinin (Zaidel-Bar, 2007a). Further links to the actin cytoskeleton occur through second and third tier scaffolds such as, paxillin-actopaxin, kindlin-1-migfilin-VASP (Zaidel-Bar, 2007a). Maintaining the integrin-actin connection is critical to the function of adhesions, specifically with respect to mechanotransduction.

Integrin activation is the process defined by the switching of affinity for ECM, from that of low to that of high affinity state. In addition to the ECM itself, integrins can be regulated by a number of cytoplasmic proteins. The binding of talin has now been characterized as the final step in integrin activation (Calderwood et al., 2013) and
the binding of talin to integrins is a highly regulated process as well (Morse et al., 2014). Kindlin is another protein, which plays a role in integrin activation, while creating additional binding sites for other proteins involved in adhesion dynamics (Brahme and Calderwood, 2012; Brahme et al., 2013; Tu et al., 2003). The critical role of integrins requires their activity to be tightly spatial and temporally controlled. In addition to direct integrin activators, integrins have also been shown to be negative regulated by proteins binding to the cytoplasmic tails (Morse et al., 2014). Filamin A, is a large actin-cross linking protein is one of the most well characterized integrin inhibitors. Through competition for binding to the integrin β-tail with talin and kindlin-1, filamin A has been shown to suppress integrin activation (Kiema et al., 2006; Shifrin et al., 2009; Takala et al., 2008). Phosphorylation of filamin A has been shown to modulate the interaction with integrins, and thus their activity. Of particular note is regulation of integrin binding through phosphorylation at Ser2152 (Chen et al., 1995).

The multi-protein signaling complexes that form around integrins are responsible for transmitting signals from integrins and regulating the cytoskeletal dynamics involved in cell migration and adhesion. As mentioned before, there are tiers of proteins, which help connect the actin cytoskeleton to integrin cytoplasmic tails. Proteins such as talin and filamin A are particular important in this connection as they have been shown to induce conformational changes under tension, strain, and stretching (Ehrlicher et al., 2011; Chen et al., 2009; Nakamura et al., 2009; Nakamura et al., 2007). Furthermore, force applied across filamin actually causes release of the filaminA-associated Rho GTPase activating protein (FilGAP), which allows for another
level of regulation of the cytoskeleton (Ohta et al., 2006). Integrins are also able to affect cytoskeletal dynamics through regulation of Rho family GTPases although these signaling pathways are beyond the scope of this dissertation.

1.1.8.4 The Integrin Adhesome

Advances in technology over the past decade have allowed for the visualization of dynamic focal adhesion turnover and cytoskeletal dynamics. It is therefore widely appreciated that the proteins assembled at sites of integrins and focal adhesions are in constant flux. Currently, there are 156 components (Zaidel-Bar et al., 2007a,b) with an even larger number of direct protein-protein interactions. As the field begins to uncover the complexity and connectivity of the integrin adhesome, it has become clear that in order to provide both strong adhesions and dynamic turnover, the proteins present at any one time must change (Zaidel-Bar et al., 2007a). Due to the extensive network of protein-protein interactions, it is likely that a snap-shot of the cell at one time would only show proteins binding a fraction of their binding partners. This combined with differences in tissue and cell type expression, contribute to the diversity of signals which are transduced. Even more, proteins may interact with different binding partners within the same cell and even within the same adhesion, furthering the complexity of the adhesion networks that are possible.

The complex of proteins assembled at integrins and adhesions must be tightly regulated in order to exert a specific function. Regulation can occur through protein conformation, changes in temperature, pH or ion concentration, binding of proteins, or
post-translational modifications (Zaidel-Bar et al., 2007a). It is also likely that force acts to play a role in altering the profile of proteins involved in the integrin adhesome by causing conformational rearrangements in molecules exposing and hiding discrete binding sites. This has been shown recently in single-molecule stretch experiments of both talin and filamin A, known integrin binding partners (del Rio et al., 2009; Chen et al 2009).

As the extensive network of proteins, which make up the integrin adhesome unfolds, the mechanisms and regulation of these complexes become impossible to follow. What is clear however, is their importance in regulating important cellular processes including cell migration through numerous signaling pathways. One signaling pathway of particular importance is the cAMP dependent protein kinase A pathway, which the remainder of this work will focus on.

Signaling events are regulated through a variety of mechanisms but one of the most widely used is phosphorylation. Phosphorylation is the process in which a gamma phosphate is transferred from adenosine-triphosphate to its substrates in a mechanism called phosphorylation. Protein phosphorylation is a covalent, yet reversible, modification of proteins, which regulates many cellular functions. Almost every signaling pathway includes protein kinases and in commonly, protein phosphatases, which act to remove the phosphate group. Phosphorylation has diverse roles, and the presence of a phosphate group can affect enzymatic activity of proteins, induce conformational changes, target proteins for degradation, create docking sites for
subsequent proteins to bind, and mediate protein-protein interactions. Phosphorylation is one of the most widely used regulatory mechanisms in eukaryotic organisms.

1.2 cAMP/PKA Signaling

1.2.1 Structure and Function of PKA

The cAMP-dependent protein Kinase-A (PKA) is one of the most well characterized signaling enzymes in the cell, and whose role has been implicated in a number of cellular processes. In its inactive form, PKA is a heterotetrameric enzyme comprised of two regulatory (R) and two catalytic (C) subunits. There are two types of PKA holoenzyme, defined either as RI or RII. There are four types of R subunits, encoded by four genes (RIα, RIβ, RIIα, RIIβ) and only three genes encoding the C subunits (α, β, and γ). The regulatory subunits function to maintain holoenzyme integrity, facilitate N-terminal dimerization, provide a mechanism for A-kinase anchoring, and release the catalytic subunits upon binding of cAMP (Howe, 2004). Upon binding two molecules of cAMP to the regulatory subunits, a conformational change occurs releasing the catalytic subunits, which are then free to phosphorylate a number of downstream substrates.

PKA is a serine threonine kinase, which phosphorylates proteins at a conserved consensus site, RXXS/T (where X is any amino acid). PKA has a diversity of substrates, drawn from many functional classes including nuclear proteins, cytoplasmic,
mitochondrial, and cytoskeletal. Regulation of PKA is a well characterized mechanism through which activation of G-protein coupled receptors are coupled to the G-protein, \( G_{\alpha s} \), which activates downstream effectors such as adenylate cyclase to convert ATP to cAMP (Ross et al., 1977). PKA’s activity is dependent on the local concentrations of cAMP. As a second messenger itself, there is tight spatial control of cAMP and it’s availability is regulated through adenylyl cyclase and phosphodiesterases (PDEs). Additionally, specific PDE isoforms have been shown to be coupled to specific GPCRs, which allows for a tight level of regulation surrounding cAMP degradation (Benedetto et al., 2006). The conversion of cAMP to AMP by phosphodiesterases represents one of the feedback mechanisms responsible for down-regulation of PKA.

1.2.2 Cellular Distribution of PKA

Type I and type II R subunits have differential tissue distribution and even differential subcellular localization. While type I PKA is localized in the cytoplasm, the majority of type II PKA is localized to subcellular compartments, through binding to A-kinase anchoring proteins, which will be discussed in greater detail later. Additionally, the R subunits have different affinities for cAMP, which allows for different thresholds of activation. Because of the breadth and cellular distribution of PKA substrates, there is a large need for its activity to be regulated within the subcellular space. Both through anchoring and localized cAMP gradients, there is a dynamic level of regulation to PKA activity, allowing for the organization of spatial signaling cascades in response to specific stimuli.
1.2.3 Role of PKA in Cell Migration

The role of PKA in cell motility has been studied extensively and well characterized as both required for, and detrimental to cell migration (Edin et al., 2001; Ydrenius et al., 1997). PKA exerts both positive and negative effects to control adhesion, membrane protrusion and retraction, and cytoskeletal dynamics as many processes require its activity while others are inhibited (Howe, 2004). While the role of PKA in cell migration has been reviewed extensively (Howe, 2004), a few of the findings will be presented below.

1.2.3.1 Negative Regulation

PKA has been shown to negatively affect aspects of cell migration. One of the classic examples shows that PKA causes inhibition of α5β3-mediated endothelial cell migration (Kim et al., 2000). Similarly, Spina et al., reported that PKA inhibits leptin-induced migration of breast cancer cells (Spina et al., 2012). While PKA’s negative regulation of integrin-mediated cell migration has been well characterized, recently it has been shown that PKA may inhibit bladder cancer cell invasion through targeting of MAP4-dependent microtubule dynamics (Ou et al., 2014). Additionally, it was recently shown that increased levels of PKA activity, achieved through an increase in cAMP levels, inhibits migration and invasion of pancreatic ductal adenocarcinoma cells (Burdyga et al., 2013). Central to cell migration are actin cytoskeletal dynamics. PKA
has been found to inhibit the formation of actin stress fibers in endothelial cells (Liu et al., 2001). These observations represent a few mechanisms by which PKA is able to inhibit cell migration.

1.2.3.2 Positive Regulation

The requirement and positive effects for both, elevation of cAMP and activation of PKA, in cell migration have been well characterized for a number of cell types. It was found over a decade ago, that PKA was required for growth-factor stimulated migration of fibroblasts (Edin et al., 2001), and this finding became monumental in establishing PKA’s role in migration. Subsequently, PKA has been found to facilitate leading edge dynamics, specifically PKA activity is required for pseudopod stability and formation (Howe et al., 2005), and is required for the formation and attenuation of actin-induced membrane ruffles (Howe et al., 2008). Filopodial and lamellipodial formation, additional mechanisms of protrusion, were also shown to require PKA activity (Grieshaber et al., 2000; Plopper 2000). Most recently, PKA activity was found to be localized to the leading edge of migrating epithelial ovarian cancer cells and inhibition of activity prevented migration (McKenzie et al., 2011).

While the mechanism by which PKA contributes to cell migration has yet to be completely elucidated, it is important to point out that PKA has been shown to be important or necessary to many processes central to cell migration. For example, PKA has been shown to play a role in regulating a number of small GTPases intrinsic to actin cytoskeletal dynamics and cell migration such as Cdc42 (Feoktistov et al., 2000), Rac
(O’Connor et al., 2001), RhoA (Tkachenko et al., 2011; Chen et al., 2005), and Rap1 (Takahashi et al., 2013). Additionally, PKA activity has been shown to be required for cell migration through its regulation of $\alpha_4$-integrins (Lim et al., 2008; Goldfinger et al., 2003).

It has become increasingly clear that the PKA activity observed at the leading edge of cells does not simply have a negative or positive effect on cell migration. Instead, PKA activity is tightly spatially and temporally regulated such that it is able to exhibit an oscillating regulation over the mechanisms of cell migration.

1.2.4 The Role of PKA in Cell Adhesion

As mentioned prior, the reciprocal regulation between PKA activity and integrins is beginning to be defined (Lim et al., 2008, Goldfinger et al., 2003) thus providing evidence for the role of PKA in cell adhesion. PKA has been shown to be activated not only in response to integrin-mediated cell-ECM adhesion, but also by cellular detachment (Whittard et al., 2001; O’Connor et al., 2001; Howe et al., 2002). The activation and inactivation of PKA was actually shown to be both cell type and ECM-ligand specific (Whelan et al., 2003). This was shown when PKA activity was suppressed in endothelial cells plated on collagen I, thus engaging $\alpha_1\beta_1$ integrins, but not on laminin-1 (Whelan et al., 2003). Additionally, the dichotomous regulation of PKA was shown through two experiments which showed that adhesion through the $\beta_1$ integrin suppresses PKA activity during $\alpha_5\beta_3$-mediated endothelial cell migration yet activates PKA in carcinoma cells and fibroblasts (Howe et al., 2002; O’Connor et al., 2001).
The connection between PKA and cell-ECM adhesion is further supported by the reports of the reciprocal regulation between PKA activity and the \( \alpha_4 \) integrin (Goldfinger et al., 2003; Lim et al., 2004; Lim et al., 2008). Of particular note is the finding that the cytoplasmic tail of the \( \alpha_4 \) binds type I PKA in a mechanism distinctly different from canonical A-kinase anchoring interactions, which will be discussed in detail later (Lim et al., 2008). This localized PKA activity was actually shown further by the finding that the PKA-induced phosphorylation of the \( \alpha_4 \) cytoplasmic tail was completely localized to the leading edge of lamellipodia and absent from the rear of migrating cells (Goldfinger et al., 2003). This was followed up by the finding that this phosphorylation was necessary for \( \alpha_4\beta_1 \)-mediated migration (Goldfinger et al., 2003).

1.2.5 Substrates of PKA in Regulation of the Cytoskeleton, Adhesion, and Migration

The role of PKA in cell migration has been further defined by the identification of numerous substrates intimately involved in cytoskeletal dynamics, adhesion, and actomyosin contractility. These phosphorylation events have been shown in proteins borrowed from structural and signaling classes, representing the breadth of targets in which PKA exerts its effects to regulate migration. The list of PKA targets involved in migration is extensive and has been well reviewed before (Howe, 2004), therefore the subsequent section will cover a fraction of the relative targets in an effort to demonstrate PKA’s importance in regulating cell migration.
As mentioned previously, of particular note and certainly integral to migration and adhesion, the cytoplasmic tail of the $\alpha_4$ integrin, has been shown to be directly phosphorylated by PKA (Lim et al., 2004; Lim et al., 2008).

1.2.5.1 Actin

One of the most elegant examples of PKA’s role in regulating aspects of cell migration is exemplified through the direct phosphorylation of actin (Ohta et al., 1999). Furthermore it was shown that this phosphorylation led to the decreased ability of actin to polymerize (Ohta et al., 1999). While this mechanism has not been further defined, it does demonstrate a direct connection between PKA and the actin cytoskeleton. There are many ways other, which PKA regulates actin dynamics, which have been well characterized, and will be discussed subsequently.

1.2.5.2 Vasodilator-stimulated phosphoprotein (VASP)

The vasodilator-stimulated phosphoprotein (VASP) is a member of the cytoskeletal regulatory binding protein family Ena/VASP. These proteins contain several domains including an N-terminal EVH-1 domain, a C-terminal EVH-2 domain, and a central proline-rich domain (Howe et al., 2002). These domains enable VASP family proteins to localize to focal adhesions through binding to vinculin, zyxin, and actin itself (Reinhard et al., 2001; Krause et al., 2003; Grange et al., 2013). Additionally, VASP family proteins are thought to be required for filopodial formation.
as they directly and indirectly associate with actin-capping proteins and cross-link actin filaments (Lebrand et al., 2004; Breitsprecher et al., 2011; Pasic et al., 2008). Three phosphorylation sites on VASP have been linked to both PKA and PKG, although Ser-153 has been well characterized as the preferred site for PKA, and has been shown to correspond to a decreased ability to nucleate, bind and bundle actin filaments (Lambrechts et al., 2000). Furthermore it has been shown that the phosphorylation of VASP may disrupt its interactions with additional binding proteins, suggesting a further role for PKA in regulating the assembly of critical proteins involved in regulating adhesion dynamics (Lambrechts et al., 2000).

1.2.5.3 Myosin Light Chain (MLC)

Non-muscle myosin II is one of the critical regulators of the actin cytoskeleton during cell migration through myosin-contractility and is important for not only leading edge dynamics, but also retraction at the rear edge of the cell (Vincente and Horowitz, 2004). The phosphorylation of myosin through myosin light chain (MLC) regulates its activity, specifically the binding to F-actin and myosin-based contractility (Howe). The phosphorylation state of MLC is largely regulated through MLC kinase (MLCK) and MLC phosphatase (MLCP) activity, and PKA has been implicated in playing a role in this dynamic (Verin et al., 1998; Garcia et al., 1997). Although the role of PKA in regulating the aforementioned is complex, it nonetheless highlights the central role of PKA in cell migration through regulation of a protein central to actin dynamics.
1.2.5.4 Filamin A

Filamin A is an important regulator of actin cytoskeletal dynamics. Filamin A acts to promote orthogonal branching of the actin-cytoskeleton by binding, stabilizing, and bundling F-actin (Razinia et al., 2012). Filamin A is localized to both the protruding and retracting edge of migrating cells (Zhou et al., 2010), linking the cytoskeleton to the cell membrane and providing mechanical stability to cells. In addition to binding actin, filamin A has been shown to interact with over 90 diverse cellular proteins, allowing filamin A to act as a scaffolding protein and integrating multiple signaling pathways (Zhou et al., 2010).

Extensive work has been done to demonstrate the role of Filamin A in cell adhesion and migration (Kim et al., 2008). As eluded to before, Filamin A has been shown to localize to filopodia, lamellipodia, stress fibers, and focal adhesions (Campbell 2008). Additionally, filamin A-deficient melanoma cells (M2) exhibit decreased motility and decreased adhesion stability (Lynch et al., 2011; Cunningham et al., 1992; Baldassarre et al., 2009). Filamin A also regulates integrin-mediated lamellipodial formation and extension and cell spreading on collagen in a number of cell types (Kim et al., 2008, Byfield et al., 2009).

Filamin A has been shown to have over 90 binding partners, borrowed from many functional classes, yet a number of which are intimately involved in cytoskeletal dynamics and adhesion. Of particular interest is the binding to β-integrins, which as described earlier, are essential to both cell migration and adhesion (Lim et al., 2008). Filamin A was actually found to regulate cell spreading via the β1-integrin,
suggesting that filamin A may modulate β1 integrin activity directly and through the recruitment of additional proteins (Byfield et al., 2009; Kim et al., 2010). Filamin A has also been shown to bind to Rho Family GTPases and some of their regulators (Stossel et al., 2001), specifically Rac, Rho, Cdc42, and RalA (Baldassarre et al., 2009). Even more, it was shown that the interaction between Filamin A and RalA were required for RalA-induced filopodial formation (Ohta et al., 1999). Filamin A has also been shown to bind downstream effectors such as PAK1 (Vadlamudi et al., 2002) and ROCK (Ueda et al., 2003). While the list continues of filamin A associated binding partners, these partners alone provide strong evidence to support filamin’s role in cytoskeletal dynamics and adhesion.

Interestingly enough, filamin A has also been shown to be a phosphorylation target of PKA (Jay et al., 2000). Subsequent studies showed that this phosphorylation event occurs in filamin’s C-terminus at Ser2152 and Thr2336 (Jay et al., 2004). While the role of these phosphorylation sites has yet to be completely determined, it is thought to have some protection over filamin cleavage (Garcia et al., 2006).

1.2.5.5 Rho Family GTPases

The roles of Rho family small GTPases have been extensively studied and reviewed, and therefore a brief summary will be included below.

Recently, a role for PKA was identified in regulating protrusion-retraction cycles at the leading edge of cells. Specifically, the phosphorylation of RhoA on Ser188 by PKA was critical in controlling migration events at the leading edge of cells.
(Tkachenko et al., 2011). This evidence showed an alternative mechanism for PKA as it had previously been shown to negatively regulate RhoA through Rho-GDI (Qiao et al., 2008). Furthermore, over-expression of AKAP-Lbc, which will be discussed in more detail later, showed an increase in Rho-mediated stress fiber formation likely by coupling to PKA through the G-protein Ga12 (Diviani et al., 2001). Thus this mechanism perfectly exemplifies anchorage-mediated PKA regulation over cytoskeletal proteins.

PKA activity is required for both the activation of Rac and Cdc42 (O’Connor et al., 2001; Bachmann et al., 2013; Feoktistov et al., 2000). Previously, Rac activity has been observed to be localized to the leading edge structures in migrating or protruding cells (Aoki et al., 2004). Work from our lab has shown that in fact inhibition of PKA activity inhibited Rac activity through the regulation of Rac-GAP and Rac-GEF (Howe et al., 2005). Unlike RhoA however, Rac is not directly phosphorylated by PKA. However, it was recently found that Rac1 shows AKAP properties and showed a direct interaction with PKA type IIβ subunits (Bachmann et al., 2013). This provides evidence for the localization of PKA within protein complexes involved in cytoskeletal dynamics and cell migration.

The examples above were given do not fully encompass the multitude of PKA substrates however they were intended to give examples of a subset of targets that are intimately involved in actin cytoskeletal dynamics and adhesion. As the evidence for a role of PKA in cell migration is surmountable, an underlying mechanism is still to be elucidated. This mechanism is likely to be very complex, and tightly spatially and
temporally regulated. This spatial control is achieved through compartmentalization of PKA to distinct subcellular locations through a family of scaffolding proteins called A-kinase anchoring proteins (AKAPs). Thus anchoring allows spatial control of PKA signaling by regulating proximity to its specific substrates in discrete subcellular locations as well as providing temporal control through localizing PKA to small pockets of cAMP activity. Understanding which AKAPs are responsible for PKA anchoring during cell migration and adhesion is of critical importance to understanding the mechanism by which PKA is regulated during these fundamental processes.

1.3 A-Kinase Anchoring Proteins

1.3.1 The Importance of Scaffolding Proteins

Cells respond to and convert cues from the extracellular environment to intracellular effects through the use of signaling proteins. Many signaling proteins have broad substrate specificity, which requires a precise level of regulation to achieve efficacy and accuracy. Scaffolding proteins are one of the key mechanisms by which the cell coordinates and regulates signaling events. Classically, scaffolding proteins were defined as proteins which tether signaling enzymes, promoting their communication by proximity, while binding a number of other molecules acting as platforms for the assembly of signaling complexes (Alexa et al., 2010). More recently, regulatory roles for scaffolding proteins have been defined using basic mechanisms such as proximity, combinatorial binding partners, conformational fine-tuning of pre-
existing signaling cascades, and offer feedback and feed-forward regulation. One of the most appreciated aspects of scaffolding proteins is the ability to create tight spatial and temporal organization of signaling complexes, enabling the cell to not rely on simple passive diffusion, as a means of transmitting signaling events. One family of well characterized, yet diverse, scaffolding proteins are the A-kinase anchoring proteins (AKAPs).

1.3.2 Introduction to A-Kinase Anchoring Proteins

As mentioned before, PKA activity has been seen at the leading edge of migrating cells (McKenzie et al., 2011). AKAPs are responsible for the sequestration of PKA to these subcellular locations, organizing signaling complexes, and bringing PKA within close proximity to target proteins. Additionally, AKAPs are adaptors, which allow for the integration of a number of other signaling molecules, providing a mechanism for cross talk among diverse signaling pathways.

AKAPs provide a tight spatial and temporal control, which in turn provides enhanced specificity in signal transduction pathways. AKAPs do not only act to direct PKA in the cell, but they also couple PKA to its upstream and downstream regulators such as adenylyl cyclases and phosphodiesterases. These provide both positive and negative control while creating intracellular cAMP gradients culminating in stimulus-specific activation of PKA.

The remainder of this review will focus on the description and characterization of AKAPs, their mechanism of binding PKA, and their role within cellular dynamics.
Particularly, this review will focus on methods to identify and characterize new AKAPs.

1.3.3 AKAP Structure and Function

AKAPs are a structurally diverse, but functionally similar family of proteins that now includes over 50 family members (Tasken et al., 2010). While AKAPs share little primary sequence similarities, they all contain a highly conserved secondary structural element, which facilitates their binding to PKA. All AKAP family members contain three structural elements; a conserved helical PKA anchoring domain, binding sites for additional signaling molecules, and subcellular localization signal (Carnegie et al., 2004).

1.3.4 AKAP Nomenclature

Initially, AKAPs were named for their molecular weight identified by SDS gel electrophoresis. For example, AKAP79 can be found at 79 kDa in SDS-PAGE. However after finding that many AKAPs were fragments and smaller transcripts of larger genes, AKAPs were renamed using a sequential numbering system. The most recently identified AKAPs such as Ezrin, Rab32, and WAVE-1 have not been included in the numbering classification (Pidoux and Tasken, 2010).
1.3.5 Localization Signals

The ability for AKAPs to target PKA and other signaling molecules to distinct subcellular regions impinges on each AKAP’s individual targeting motif. AKAPs have been found to associate to different subcellular compartments, including the plasma membrane, mitochondria, nuclear membrane, and cytoskeleton (Figure 2). In some cases, several different AKAPs can be targeted to the same location. For example, both AKAP79/150 and Gravin, also known as AKAP250, are both targeted to the plasma membrane through phospholipid-binding sequences (Dell’Acqua et al., 1998; Grove and Bruchey 2001). Gravin however, is thought to contain an additional N-terminal myristoyl group as well (Grove and Bruchey 2001). AKAP18 is thought to target to the plasma membrane however this is through dual myristoyl and palmitoyl groups (Trotter et al., 1999). AKAP350 and pericentrin are targeted to the centrosome through a motif named the pericentrin-AKAP350 centrosomal targeting domain (PACT) (Diviani et al., 2000; Gillingham and Munro, 2000). At the mitochondria, D-AKAP1, Rab32, and WAVE1 are all localized, however while Rab32 and WAVE1 are targeted through the prenylation of cysteine residues at their C-termini, D-AKAP1 is localized through a more conventional mitochondrial targeting sequence (Huang et al., 1999; Alto et al., 2002; Danial et al., 2003). Although the subcellular location of many AKAPs has been identified, the details and specific targeting motifs have only been identified for a few molecules. The presence of multiple AKAPs within the same subcellular compartment suggests the need for multiple and distinct signaling complexes within one region of the
cell. This mechanism acts to provide tight signaling regulation by enabling PKA to phosphorylate a different panel of substrates within the same subcellular location.

In some cases, splice variants arising from the same gene are often targeted to different subcellular compartments. For example, AKAP18 is differentially spliced into three isoforms, alpha, beta, and gamma (Trotter et al., 1999; Fraser et al., 1998), and each isoform has distinct subcellular distribution. Additionally, AKAP350 can be spliced into shorter and longer isoforms (Trotter et al., 1999). The shorter isoforms are directed to synaptic membranes, binding to the NMDA receptor, where the longer isoforms are directed to the centrosome through the C-terminal PACT domain (Schmidt et al., 1999; Witczak et al., 1999).

Figure 2: AKAP Signaling Complexes Localize to Distinct Subcellular Regions to Create Focal Points for Signal Transduction
1.3.6 Mechanism of PKA Anchoring

One of the defining characteristics of AKAPs is the mechanism by which they bind PKA regulatory subunits. This binding occurs through a 14-18 amino acid sequence, which forms a highly conserved amphipathic alpha-helix (an alpha-helix with opposing polar and nonpolar faces) (Carr and Scott, 1992). The helix coils such that the hydrophobic residues of the helix contact the groove formed by the RII dimers (Figure 3) (Carr and Scott, 1992). AKAP-PKA interactions have been elucidated using AKAP derived peptides and the dimerization domains of PKA RIIα subunits.

![Figure 3: Overview of Structures of RII Dimerization Domain in Complex with an Amphipathic AKAP Helix](image)

The conserved amphipathic helix was first identified over 20 years ago through sequence comparisons between some of the first identified AKAPs, Ht31, Map2, Ht21,
and P150. While sequence comparison revealed little overall homology, all RIIα binding regions revealed a conserved glutamic acid at position 3. Comparing the polar and nonpolar residues in these regions, showed a strong alignment of types of hydrophilic and hydrophobic residue types (Figure 4). Furthermore, when using a helical wheel projection for each of the sequences, hydrophilic and hydrophobic amino acids became grouped on opposing faces of the helix. No other sequences in the proteins could be similarly aligned, suggesting the functional significance of these regions within each of these proteins (Carr et al., 1991). Mutagenesis of amino acid residues within the putative RIIα binding region of Ht31 disrupted the secondary structure of the helix and abolished PKA binding, highlighting the importance of the helical structure (Carr et al., 1991). Furthermore, introduction of proline residues outside of the putative PKA binding region had no effect on binding. The amphipathic helix motif is conserved throughout the AKAP family with the exception of pericentrin (insert refs). NMR structural analysis confirmed the helical structure of the AKAP RIIα binding domain when in complex with the RIIα dimer (Newlon et al., 2001).
Of equal importance in PKA anchoring is the requirement of PKA regulatory subunit dimerization. The AKAP binding region was identified in the N-terminus of the RIIα subunit. Preliminary experiments in which RII mutants lacking the first 14, 30, or 35 residues resulted in the inability to bind the well characterized AKAP, MAP2 (Scott et al., 1990). Further characterization revealed that disrupting RII dimerization resulted in abolished binding to MAP2, showing the requirement for RIIα dimerization in PKA anchoring. It is now known that residues 1-44 of RIIα are highly conserved and that dimerization is maintained within this region (Scott et al., 1987; Dell’Acqua et al., 1998; Newlon et al., 1999). Triple resonance, three-dimensional nuclear magnetic resonance techniques further characterized the highly conserved residues 1-44 of RIIα revealing the dimerization structure. This analysis in conjunction with crystal structures...
of the RIIα dimerization domain showed the formation of a four-helix bundle with two protomers forming an antiparallel conformation with their N-termini (Gold et al., 2006; Newlon et al., 1999; Newlon et al., 2001; Kinderman et al., 2006). This well-ordered four-helix structure is maintained through extensive hydrophobic interactions (Figure 5) (Newlon et al., 1999). These hydrophobic residues are also responsible for creating the extended hydrophobic groove of the RIIα dimer interface, necessary for AKAP binding. Mutations in hydrophobic residues in either the RIIα dimer or within the AKAP RIIα binding domain diminish or abolish binding, suggesting the importance of the hydrophobic residues for maintaining PKA anchoring (Miki and Eddy, 1999). Specifically, mutagenesis studies identified isoleucines at position 3 and 5 within the RIIα dimerization domain that are important for AKAP binding (Hausken et al., 1994; Hausken and Scott, 1996).
1.3.7 Type I vs. Type II Anchoring

While the majority of AKAPs identified to date bind PKA type II, type I binding has been well established and characterized. Additionally, dual-specificity AKAPs (D-AKAPs) are able to bind both type I and type II regulatory subunits (Huang et al., 1997; Huang et al., 1999). Many dual-specificity AKAPs, while able to bind both types of subunits, often bind RI subunits with 10-100-fold lower affinity than RII...
(Welch et al., 2010). For example, binding assays of D-AKAP1 with all four isoforms of PKA revealed binding to RIIα and RIIβ at $K_D = 0.5\text{nM}$ compared to a $K_D = 185\text{nM}$ for RIα and RIβ (Stokka et al., 2006). Rate constants for AKAP79 binding to all four regulatory subunits were also identified revealing higher affinity binding to RIIα and RIIβ, indicating this is not unique to D-AKAP1 (Stokka et al., 2006).

NMR structural analysis of the RIα dimerization domain reveals some subtle structural differences between RIα and RIIα within the N-terminus. The structure of the RIα dimerization domain (D/D) is similar to that of RIIα, forming the four-helix bundle with the major difference existing at the extreme N terminus. Within the RIIα dimer, the extreme N-terminus is extended (Newlon et al., 1999; Newlon et al., 2000), where in RIα it forms a helical structure (Banky et al., 2000). This helical structure partially occludes the AKAP binding surface and is therefore responsible for the observed differences in AKAP binding among RIα and RIIα (Banky et al., 2003). Further characterization of the RIα extreme N-terminus shows this region to contain more charged residues than that of RIIα (Banky et al., 2000) disrupting the hydrophobic groove of the AKAP binding surface. The presence of these charged residues within the AKAP binding region provides evidence for an alternative RIα AKAP binding mechanism, which involves stabilization through acidic and basic residues with the addition of an electrostatic component (Burns et al., 2003). Solution structures of the D/D of RIα showed the formation of a deep cleft maintained by disulfide bonds in contrast to the hydrophobic groove formed by the D/D of RIIα (Banky et al., 2003; Sarma et al., 2010).
It was recently shown that dual-specificity AKAPs contain an additional PKA binding element, which is located outside of the conserved amphipathic helix (Jarnaess et al., 2008). In vitro binding experiments with the dual-specific AKAP ezrin, identified this region as the RI specifier region upstream of the canonical helix (Jarnaess et al., 2008). Sequence alignments of other known dual-specificity AKAPs including D-AKAP1, D-AKAP2, PAP7, and Merlin revealed the presence of this conserved region. Furthermore, classical RII AKAPs such as AKAP-Lbc and AKAP79 do not contain this region, suggesting the specificity for RI binding (Jarnaess et al., 2008).

1.3.8 Noncanonical AKAPs

Not all AKAPs bind to the dimerization domain through an amphipathic helix (Skroblin et al., 2010). There are a few noncanonical AKAPs which have been identified, however few are known likely due to the fact that they are missed in screens. Pericentrin is the best characterized noncanonical AKAP, whose binding to PKA RIIα is facilitated through a non-helical, 100 amino acid, leucine-rich region (Diviani et al., 2000). Additionally, the α4 integrin cytoplasmic domain, which is not predicted to form an α-helix, interacts with type I PKA in a manner that is not disrupted by Ht31 disruptor peptides (Lim et al., 2007). This suggests a noncanonical mechanism of PKA anchoring. It is possible that this noncanonical binding may provide an additional level of PKA regulation and the potential for many AKAPs to be uncovered.
1.3.9 Evolution of AKAPs

The majority of the currently identified AKAPs have been found from human or rat origin (Scott et al., 2013). However, orthologs of a number of mammalian AKAPs were also found in lower vertebrates such as Xenopus (Isoldi et al., 2010; Klingbeil et al., 2001; Park et al., 2007; Semenova et al., 2009) and Danio rerio (Goehring et al., 2007; Reynolds et al., 2007). AKAPs have also been identified in invertebrates such as the nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster (Angelo and Rubin, 1998). AKAPs have even been identified in the unicellular green algae, Chlamydomonas reinhardtii (Gaillard et al., 2001). There is little known about AKAPs in invertebrates, and it is most likely that there are a larger number of AKAPs expressed in vertebrates. Thus giving rise to their role in the regulation of large signaling processes required for specialized cell functions and cell-cell communications in complex multicellular organisms. The current knowledge shows the mechanism of PKA anchoring is similar across species, suggesting the highly conserved and indispensable role for AKAPs.

1.3.10 AKAPs as Organizers of Multivalent Complexes

While the most appreciated and biologically significant role of AKAPs is their interaction with PKA, an additional feature of these molecules is their ability to bind a number of other signaling enzymes. Thereby allowing AKAPs to form multivalent signaling complexes. In many cases, AKAPs bind enzymes with opposing actions such as kinases and phosphatases, defining their role as regulators of both signal transduction
and signal termination. Two of the most well characterized examples of this are AKAP79 and AKAP220, as they bind to PKA and either calcium-calmodulin-dependent phosphatase (PP2B, also known as calcineurin) (Klauck et al., 1996) or protein phosphatase 1 (Schillace et al., 1999) respectively. Furthermore, AKAP79 has a capacity to bind a number of other proteins involved in signaling, which allows for the formation of customized and localized signaling complexes to the plasma membrane in a number of different cell types (Carr et al., 1992). Targeting enzymes to the cell periphery therefore positions them in a way, which enables them to respond to pools of intracellular second messengers such as cAMP, calcium, and phospholipids. In fact, AKAP79 has been shown to be one of the anchoring proteins, which targets PKA to β-adrenergic receptors to play a role in their phosphorylation-mediated down-regulation (Fraser et al., 2000). AKAP79 has also been shown to physically associate with adenylyl cyclase 5 terminating cAMP synthesis upon phosphorylation (Bauman et al., 2006).

Multiple signaling pathways can be integrated via a single AKAP complex. Interestingly, AKAP79/150 is also able to coordinates protein kinase C mediated signaling events (Navedo et al., 2008). These findings suggest the flexible and dynamic role AKAPs and their ability to be involved in a number of signaling events, allowing for crosstalk and large coordination of multivalent complexes.

Another aspect of AKAPs is their ability to anchor enzymes to selected substrates. This therefore facilitates signal transduction through the dual anchoring of both enzymes and specific substrates within distinct subcellular regions. The functional
implication of which is rapid and highly regulated signal transduction. Instead of relying on diffusion of proteins involved in signaling cascades, AKAPs create pockets of signaling complexes. Phosphorylation of proteins in one of the universal means of intracellular communication and one in which requires a high level of regulation. Although the physiological roles of anchored PKA phosphorylation events have been elucidated in a number of cell types, the organization and operation of individual protein complexes within AKAP complexes has yet to be defined. Even more, the functional significance of effects on substrate phosphorylation when PKA anchoring is disrupted has yet to be elucidated.

1.3.11 Regulation of Signaling Complexes/Context Dependent Complexes

As AKAPs are continually characterized, and their binding partners elucidated, it has become obvious that a single anchoring protein is able to interact with only a small subset of proteins at any given time. However, there exists great potential in the ability to organize different enzyme combinations and therefore the repertoire of signals that are processed through a single AKAP. This complexity can be expanded through context specific regulation, either at specific compartments within the cell or through tissue-specific cues.

The WAVE family of proteins (WAVE1, 2, 3) coordinate different signaling complexes at both the compartment and tissue-specific level. For example, in neurons, growth-factors stimulate WAVE1 bound Rac to cause actin reorganization (Miki et al., 1998). However in hepatocytes, WAVE1 is involved in the regulation of PKA
phosphorylation of BCL2, contributing to apoptosis (Danial et al., 2003). Within the same cell, there is differential targeting of AKAP isoforms to distinct subcellular locations. This allows for the regulated distribution of PKA to discrete pockets of substrates. For example, yotaio can be associated with the plasma membrane, targeting PKA to channels and receptors such as the NMDA receptor (Westphal et al., 1999) while the longer splice variants are targeted to the Golgi apparatus (Shanks et al., 2002). These vastly different mechanisms for single AKAPs within different cellular contexts allows for the expansive roles of AKAPs and implicates their importance in the coordination of signaling.

The recruitment and release of binding partners can also be determined by covalent modifications, thereby modulating not only the composition of signaling complexes but also the activity of anchored enzymes. Phosphorylation has been shown to regulate both the binding to and release of proteins from AKAPs (Carnegie et al., 2004; Carlisle-Michel et al., 2004; Diviani et al., 2000; Kapiloff et al., 1999). Specifically, it has been shown that phosphorylation of PDE4D3 increases its affinity for the anchoring protein mAKAP (Carlisle-Michel et al., 2004) while PKA mediated phosphorylation of AKAP-Lbc disrupts its association with PKD (Carnegie et al., 2004).

1.3.12 Disruptor Peptides as a Tool for Understanding Anchoring Function

In order to study the functional roles of AKAPs, peptides that disrupt the PKA-AKAP interaction have been developed. These have been widely used in the field and
have additionally served as important tools in the study of PKA’s functional implications in cellular processes. All of the peptides developed comprise the PKA binding domain of AKAPs, the 14-18 stretch of amino acids forming the amphipathic helix. The first anchoring disruptor to be characterized was a 24 amino acid peptide from Ht31 (now called AKAP-Lbc) which lies between residues 494-507 (Carr et al., 1991; Carr et al., 1992). Further characterization of the Ht31 peptide showed this 24 amino acid stretch is sufficient to disrupt both PKA type I and type II anchoring (Herberg et al., 2000). This peptide binds RIIα with low nanomolar affinity ($K_D=2.2$ nM) (Newlon et al., 2001). Second-generation disruptors were later developed in order to distinguish between RI and RII AKAP interactions (Burns-Hamuro et al., 2003; Alto et al., 2002; Carlson et al., 2006; Gold et al., 2006).

1.3.12.1 Type I Specific Disruptor: RIAD

A peptide derived from D-AKAP2, a known dual-specific AKAP, was used as a template to design a specific RIIα disruptor (Burns-Hamuro et al., 2002). Truncations and amino acid substitutions were made to identify critical residues and minimal length requirements for bindings and regulatory subunit binding affinity was assessed by SPOT synthesis (Burns-Hamuro et al., 2002). Using both bioinformatics and a peptide array screening, this peptide was further characterized and a higher affinity binding peptide called RIAD (RI anchoring disruptor) was developed which has 1000-fold higher selectivity for type I PKA than type II PKA (Carlson et al., 2006).
1.3.12.2 Type II Specific Disruptors: AKAP-IS and sAKAP-IS

Just as a specific type I disruptor was created, a specific type II disruptor was also created. Through a combination of bioinformatics, screening of peptide arrays, and RII overlay assays, a high-affinity RII binding peptide was created, called AKAP-in silico (AKAP-IS) (Alto et al., 2003). In vitro binding assays showed that the AKAP-IS had subnanomolar affinity for RII and was more effective at displacing RII from AKAP79 than the Ht31 peptide (Alto et al., 2003). Furthermore, a scrambled form of this peptide was shown to have no affect the subcellular distribution of PKA in cells (Alto 2003). The AKAP-IS peptide was later optimized by analysis of 340 AKAP-IS peptide derivatives where each residue in the sequence was replaced by all 20 amino acids. RI and RII binding was assessed using RIα-P$^{32}$ and RIIα-P$^{32}$ overlays (Gold et al., 2006). The resulting peptide, named SuperAKAP-IS, contained amino acid substitutions which both increased the RII binding affinity and decreased the RI binding affinity. This peptide, exhibits a 4-fold higher affinity for RIIα and 12.5-fold reduced affinity for RIα compared to the AKAP-IS (Gold et al., 2006).

Modifications such as affinity tags, fluorescent dyes, or cell-penetrating tags have been made to these peptides to expand their experimental usability. While these peptides have been widely used experimentally, they do have their limitations. The global disruption of PKA anchoring within the cellular context liberates PKA, creating excessive amounts of free PKA in the cytoplasm, which can result in irregular phosphorylation of substrates. Additionally, the modifications of these peptides can
result in the uneven distribution within the cell, leading to their enrichment in some subcellular locations.

1.4 AKAPs at the Cytoskeleton

1.4.1 Introduction of AKAPs at the Cytoskeleton

As mentioned earlier, the actin cytoskeleton is essential for a number of biological processes. Our lab and others have shown that PKA is essential for cell migration and that PKA is critical for regulating actin cytoskeletal dynamics (McKenzie et al., 2011; Howe et al., 2004; Rivard et al., 2009). Due to the breadth of its targets and their subcellular localization, the need for focusing and directing PKA to specific cellular compartments is essential. Our lab has shown that PKA is spatially regulated during cell migration, and more specifically is enriched in protrusive leading edge structures.

There are a number of cytoskeletal proteins that have been shown to be substrates for PKA. Additionally, the phosphorylation of which has been shown to be essential for regulating their function, particularly in the role of cell migration. Many hallmarks of cell migration and cytoskeletal dynamics have been shown to require PKA activity (Rac, Cdc42, microfilament assembly), while others are inhibited by PKA (Rho, p21 activated kinase, VASP). Integrin dependent endothelial cell migration is both positively and negatively regulated by PKA. These observations implicate the critical role for PKA in regulating cell migration.
The combination of complexity of cytoskeletal dynamics, diversity and breadth of PKA’s targets, and PKA’s role in regulating cell migration/actin cytoskeletal dynamics underscores the importance of PKA’s localization in the aforementioned. It is clear that PKA is neither a positive nor a negative regulator, but rather cytoskeletal dynamics/cell migration requires a balance of PKA activity tightly spatially and temporally regulated. Given the function of AKAPs as scaffolding proteins for multiple signaling enzymes and as anchors for PKA, it is likely that there are AKAPs within the cytoskeleton/adhesions responsible for anchoring PKA to sites where it can regulate cytoskeletal dynamics/migration.

1.4.2 Cytoskeletal AKAPs

There are several AKAPs to date which have been identified as being involved in actin cytoskeleton dynamics including, AKAP-Lbc/AKAP13 (Diviani et al., 2006; Cavin et al., 2014; Klussmann et al., 2001), gravin (Gelman et al., 1998), ezrin (Dransfield et al., 1997; Bosanquet 2014), and the WASp and verprolin homology protein-1 (WAVE1) (Yamazaki et al., 2005; Takenawa 2005; Takenawa and Miki 2001).

1.4.2.1 Gravin

Gravin, also known as AKAP12, was originally identified in a screening of endothelial cell expression library with serum from a myasthenia gravis patient
(Gordon et al., 1992). A few years later, it was shown to be a RIIα specific AKAP which could also bind to PKC, beta2 adrenergic receptor, PDE4D, and Ca^{2+}/calmodulin (Nauert et al., 1997; Fan et al., 2001; Malbon et al., 2004; Willoubuy et al., 2006). It is now well appreciated that Gravin plays a role in cellular adhesion, migration, cytoskeletal dynamics, and maintenance of the cytoskeletal architecture (Akakura and Gelman 2012). The C-terminal domain of Gravin is required for targeting PKA to the cell periphery (Yan et al., 2009). Gravin has been described as attenuating and inhibiting chemotaxis and cell invasion (Busch et al., 2008; Gelman et al., 2000) and likely through differential activation of both PKA and PKC (Su et al., 2013). Gravin has also been shown to be intimately involved in FAK-mediated signaling (Gelman) and playing a role in FAK-mediated adhesion and motility pathways (Su et al., 2013). Even more, Gravin has been shown not only to bind to the actin cytoskeleton (Gelman et al., 2012) but also act as regulator of its architecture, regulating the formation and maintenance of stress fibers (Lin et al., 2000). Taken together, these observations demonstrate the critical role Gravin plays in actin cytoskeleton dynamics during cell migration.

1.4.2.2 WASP family verprolin homologous protein 1 (WAVE1)

WAVE1 is one of the Wiskott-Aldrich syndrome protein family members (Takenawa and Miki 2001). WAVE1 directly binds to actin and is intimately involved in actin dynamics, both through Rac-1 mediated actin reorganization (Miki et al., 1998) and by coupling Rho GTPases to the Arp2/3 complex (Higgs et al., 1999; Machesky
and Gould 1999). Recently, WAVE1 was identified as an AKAP and of particular note is the finding that pools of WAVE1 localize from focal adhesions to sites of actin reorganization upon PDGF treatment (Westphal et al., 2000). Furthermore, Westphal et al., showed that upon treatment with PDGF, PKA also localizes to sites of actin reorganization (Westphal et al., 2000). A unique feature about the PKA-WAVE1 interaction is that actin may compete for the RII binding site (Westphal et al., 2000).

The highly dynamic WAVE1-mediated complex formation is a possible mechanism used by the cell to both regulate the activity and location of PKA and coordinate actin reorganization thus cytoskeletal dynamics following Rac activation. Not only does WAVE1 bind PKA RII subunits, but it also has the ability to bind the c-Abl tyrosine kinase, forming a multi-signaling enzyme complex (Westphal et al., 2000). This dual binding ability of WAVE1 is particularly interesting given that PKA has been shown to phosphorylate VASP and cause its uncoupling from Abl (Howe et al., 2002) suggesting PKA plays a central role in the regulation of complexes at sites of WAVE1 mediated actin reorganization.

1.4.2.3 Ezrin

Ezrin is a member of a superfamily of cytoskeletal associated proteins including merlin, radixin, moesin, and talin (Algrain et al., 1989; Bretscher et al., 2002). These proteins share homology in their N-terminus which has been implicated in their membrane associations (Arpin et al., 1994). Ezrin is part of a smaller subfamily of proteins, the ERM family, including radixin and moesin, which share homology across
the sequence (Salto et al., 2012). Experiments have shown the binding of PKA RII to Ezrin to disrupted using the Ht31 anchoring disruptor peptide (Dransfield et al., 1997). These results, taken with further characterization of the PKA binding domain on Ezrin, have identified it as a bonafide AKAP (Dransfield et al., 1997). Of particular interest is that the C-terminal domain of ezrin has been implicated in the association with actin filaments (Hanzel et al., 1991). Therefore it is likely that Ezrin plays a role in anchoring PKA to the actin cytoskeletal, possibly providing an avenue for which PKA could regulate actin dynamics.

1.4.2.4 AKAP-Lbc (AKAP13)

AKAP-Lbc is a unique cytoskeletal associated AKAP as it not only anchors PKA but it has the ability to modulate the Rho GTPase (Klussman et al., 2001). In vitro experiments identified AKAP-Lbc as a guanine nucleotide exchange factor (GEF) with a specificity towards Rho and not Rac or Cdc42, implicating its ability to regulate stress fiber and focal adhesion formation (Diviani et al., 2001). Furthermore, the Rho-GEF activity of AKAP-Lbc was found to be bi-directionally regulated via G-protein Gα12 activation and inactivation through PKA-induced phosphorylation and 14-3-3 protein binding (Diviani et al., 2004; O’Connor et al., 2012). Taken together this highlights a mechanism by which PKA and Rho signaling pathways are integrated through scaffolding via AKAP-Lbc. Given what is known about Rho’s regulation of the actin cytoskeleton, this provides a likely mechanism for PKA’s regulation over cytoskeletal dynamics. Further experiments demonstrated a role for AKAP-Lbc in establishing and maintaining PKA gradients (Paulucci-Holthauzen et al., 2009). This in conjunction
with the well-established role of compartmentalized PKA activity in cell migration implicates AKAP-Lbc’s intimate involvement in regulating cell migration.

The multiple identified actin-associated AKAPs highlights the importance for both PKA activity and the regulation of that activity at sites of actin reorganization and thus processes requiring cytoskeletal dynamics. This taken together with the myriad of known PKA substrates involved in actin cytoskeletal dynamics, adhesion, and migration, support the critical role of PKA activity in regulating such processes.

1.5 Methods for Detecting A-Kinase Anchoring Proteins

The design of specific anchoring disruptor peptides has advanced the field’s understanding of A-kinase anchoring and ability to identify novel AKAPs. However, before the inception of these peptides, AKAPs were identified using more traditional methods used, all of which had their limitations.

1.5.1 Identification of the First AKAP

The first described AKAP was microtubule associated protein 2 (MAP2) and was found rather serendipitously as a molecule that co-purified with the regulatory subunit of PKA on cAMP-agarose affinity columns (Theurkauf and Vallee, 1981). Detailed study of PKA anchoring was achieved after the observation that many AKAPs retained their ability to bind RII after transfer to nitrocellulose (Keryer et al., 1993).
1.5.2 RII Overlay

This observation prompted the development of the RII overlay technique where proteins are separated by SDS-PAGE electrophoresis, transferred to nitrocellulose, and subsequently incubated with $^{32}$P-labeled RII probe (Carr and Scott, 1992). This technique, called a far-western or RII-overlay, is essentially a modified western blot, and has been widely used by many laboratories for the detection of AKAPs. Extending this technique further, Rubin and colleagues used RII to screen cDNA expression libraries and since eight AKAPs cDNAs have been identified and characterized with this method (Ludvig et al., 1990). These methods have their limitations however. Due to the strong denaturing conditions used in SDS-PAGE, which causes protein misfolding, a number of AKAPs often lose their ability to bind RII, resulting in missed interactions.

1.5.3 Band-Shift

These limitations allowed for use of band-shift analysis as a means of identifying AKAPs. This technique examines protein-protein interactions under non-denaturing conditions and takes advantage of the differential motility that protein complexes exhibit compared to their individual components (Carr and Scott, 1992). This led to the demonstration that AKAPs bind to the RII subunit, regardless of the association with the catalytic subunit (Carr and Scott, 1992). Not unlike the far-western, this technique has its limitations. One of which is the requirement of high concentrations of protein for detection and the disruption of equilibrium conditions.
during electrophoresis. The combination of these techniques with the use of disruptor peptides such as Ht31, became standard within the field.

1.5.4 Immunoprecipitation

Co-immunoprecipitations (co-IPs) and cross-linking IPs have been classically used as a method for identifying protein-protein interactions and are often used for characterizing PKA-AKAP interactions. Co-IPs are often used as a way to affinity purify a protein and characterize its associated binding partners. However, this approach often misses weak, less abundant and transient interactions. Additionally, co-IP data is commonly misinterpreted as proof of direct protein-protein interactions, and is rarely followed up with the correct experiments to prove such a conclusion. Another limitation to co-IPs is the stringency of the conditions under which the experiment is taking place, which can often disrupt native protein interactions, causing them to be undetected. Inherent in these experiments is the nature of the antibody-target interaction, which itself is subject to harsh conditions as well as the potential, and reality, of non-specific binding.

Chemical crosslinking and crosslinking IPs by contrast, were implemented as a way to improve upon the traditional co-IP. Due to the formation of covalent bonds, they are often more suitable for capturing transient and low-affinity interactions. However, this method is highly non-specific and often results in the aggregation of multi-protein complexes, which present solubility problems in downstream applications.
1.5.5 Bioinformatics

Within the past decade, bioinformatics has allowed for significant progress in the field of AKAP identification and characterization. Bioinformatic screens in conjunction with peptide overlays and traditional methods have allowed for large screens leading to the identification of novel AKAPs (Hundsrucker et al., 2010). This approach has its limitations however, one of which is the high number of false positives detected simply through searching for proteins containing the AKAP consensus sequence. Additionally, the presence of the canonical AKAP sequence alone does not sufficiently provide indication of an AKAP, but instead is suggestive and further structural considerations must to given to the sequence. More specifically, as described previously, the consensus sequence often forms an amphipathic alpha-helix motif to mediate PKA-AKAP binding. Searching for a simple consensus sequence search will not take this structural requirement into consideration, therefore generating many false positives.

1.5.6 Anchoring Disruptor Peptides

The generation of both RIAD and sAKAP (is) have allowed for the ability to distinguish between type I and type II anchoring. However, these peptides have their limitations as they do not allow for the contributions from individual AKPAs to be elucidated within the cellular context. These difficulties were the impetus for developing a structure-based phage selection strategy, which creates RII subunit fragments with selective binding to individual AKAPs, therefore enabling the examination of individual AKAPs (Gold et al., 2013).
Given the limitations and antiquity of the aforementioned techniques, a need to develop new methods with higher selectivity, specificity, and applicability within the cellular context has been well established. This thesis aims to optimize and characterize a new method for detecting AKAPs. Using a combination of both biochemical techniques and large-scale proteomics, this method focuses on a novel technique to identify putative AKAPs. This body of work describes first the application of this technique in whole cell extract, and later extends the application to cellular fractionations enriched with cytoskeletal and adhesion proteins to identify AKAPs involved intimately in cytoskeletal dynamics, adhesion, and cell migration.
CHAPTER 2: Materials and Methods

2.1 Cell Culture

SKOV-3 and HEK293 cells were obtained from American Type Culture Collection and maintained in antibiotic-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS). All cells for lysis were grown in 10cm dishes coated with 10µg/µL human fibronectin at 37°C in a humidified incubator containing 5% CO₂.

2.2 Antibodies and Other Reagents

Primary antibodies were obtained commercially from Millipore (Actin C4, filamin A), BD Transduction Laboratories (AKAP79, Ezrin, Lamin A/C, paxillin clone349,), Cell Signaling (GAPDH), Santa Cruz Antibodies (PKA RIα, AKAP79) and Sigma (Tubulin DM1α). DMSO was acquired from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated secondary antibodies were from Calbiochem, with the exception of horseradish peroxidase-conjugated streptavidin, which was from Thermo Fisher Scientific. Human fibronectin was acquired from BD Biosciences (Bedford, MA). Sulfo-SBED Biotin Label Transfer Reagent was purchased from Pierce (Rockford, IL). Dynabeads® Myone™ Streptavidin T1 beads, Dynabeads® Protein G, and DynaMag™ were acquired from Life Technologies (Thermo Fischer Scientific). Pharmacological inhibition of PKA anchoring was achieved using Ht31 (aka AKAP-Lbc) from Sigma-Genosis, a peptide comprising the PKA R-subunit binding domain. Ht31 acts as a competitive inhibitor of the interaction between AKAPs and both RIα
and RIα PKA subunits (Gold). The Ht31 and Ht31p synthetic peptides contained the sequences LIEEAASRIVDAVEQVK and LIEEAASRPVDAPVEQVK respectively, were diluted in MMQ H2O and stored at -20°C. IPTG acquired from Thermo Fisher Scientific (Waltham MA). Duolink® PLA reagents were obtained from Sigma (St. Louis, MO).

2.3 RIIα Purification

BL21 (DE5) PLysS bacteria containing pET28(b)-RIIα(WT) plasmid were inoculated into 5mL Terrific broth containing 1x kanamycin and 34µL chloramphenicol (from 147x stock). Inoculations were placed at 37°C shaking for 6-8 hours and subsequently back inoculated into a 25mL culture of Terrific broth containing 1x kanamycin and 170µL chloramphenicol (from 147x stock). Culture was let shake at 37°C shaking overnight. The following day, culture was back inoculated into a 1L culture of terrific broth with 1x kanamycin and 6.8mL chloramphenicol (from 147x stock) and put at 37°C shaking until OD600=0.6-0.8 (1mL of culture was removed before induction to be used for subsequent immunoblotting and coomassie staining).

Induction of bacteria: IPTG was added to a final concentration of 1mM in culture and let shake for 4h at 37°C. 1mL of culture was removed at each 1h interval to be used for subsequent immunoblotting and coomassie staining. Cells were pelleted at 4,000xg for 10’at 4°C. Supernatant was removed and saved. 40mL cold lysis buffer (recipe described subsequently) was added to pellet was resuspended. Cell suspension was sonicated 5x10sec, with a 15sec interval between each sonication. Culture was then
spun down at 18,000xg for 15’ at 4°C. Supernatant was removed and put on ice, pellet was discarded.

Batch Binding: In large tube, lysis buffer and cAMP agarose beads were mixed (5:1 v/v) (Sigma) and gently resuspended. Beads were spun at 500xg for 5’ at 4°C (or until beads collect at bottom of tube). Supernatant was removed and this repeated two times to thoroughly wash the beads. Lysate added to washed cAMP beads and mixed end over end for 16h at 4°C.

Column Purification: Fresh lysis and wash buffers were made prior to start of column preparation. cAMP lysate mixture was spun at 500xg for 5’ at 4°C to collect beads at bottom of tube. Supernatant was removed and saved on ice, 100µL was removed for SDS-PAGE confirmation. 10mL lysis buffer added to beads and beads were resuspended gently. Slurry was poured into a column and beads were let settle for 1h at room temperature. Lysis buffer was run out into a small beaker, 100µL removed for SDS-PAGE confirmation. Beads washed with 5-10 column volumes of lysis buffer, lysis buffer+1M NaCl, 10mM MOPS, lysis buffer, 100µL removed at each wash step for SDS-PAGE confirmation. Beads eluted with 2 column volumes 100mM cAMP diluted in lysis buffer. Eluents were collected in 0.5mL fractions, and 10µL was removed from each for SDS-PAGE confirmation. Fractions were frozen at -80°C until SDS-PAGE was run to confirm purity of prep. 10µL of each fraction was mixed with equal volumes of 2x Laemmli sample buffer and run on 10% SDS-PAGE gel. Gel was stained with coomassie-silver stain (recipe listed subsequently) for 15’ followed by destaining in MMQ H₂O for 1h. Purity of samples was assessed and highest purity
elution fractions were pooled for dialysis. Pooled RIIα was dialyzed in 10mM MOPS, 100mM NaCl, for 1h at room temperature, buffer changed 2x.

2.4 Conjugating RII to Sulfo-SBED

Sulfo-SBED multifunctional cross-linker (Pierce, Thermo Fisher Scientific) was dissolved in 22µL DMSO. 5-20µg purified RIIα was conjugated to 5µL dissolved Sulfo-SBED and incubated for 40’ at room temperature and protected from light. Sample was passed over a desalting column (Pierce) and frozen at -80°C in between uses.

2.5 Biotin Transfer

Lysate was “pre-cleared” as follows: 50-100µg SKOV-3 protein extract was incubated with 2:1 v/v streptavidin magnetic beads for 1h at room temperature. Beads were pelleted on magnet and supernatant was removed and placed into new ice cold microcentrifuge tube. 100µM Ht31 or Ht31P was added to lysate and incubated rocking end over end for 1h at room temperature. To lysate, RIIα-Sulfo SBED, 1:200 w/w, covered with foil, and incubated for 45’ at room temperature rocking end over end. Foil was removed and samples were exposed to UV light (365nm) for 30’ at room temperature, mixing and spinning down every 7-8’ to ensure samples were thoroughly mixed. DTT was added to a final concentration of 100mM and samples were heated at 50°C for 30’. Streptavidin magnetic beads were added to samples (after buffer exchange in RIPA buffer), 1:2 v/v, and samples were incubated at 1h at room temperature rocking end over end. Beads were pelleted on magnet, supernatant was
saved and put on ice. To beads, 1mL RIPA buffer was added, vortexed, and placed on ice for 1’. Beads were pelleted on magnet for 1’, removed wash and repeated for a total of 4 washes. After last wash, all remaining buffer was removed and 40µL 1x Laemmli sample buffer was added, beads were resuspended and spun down, and boiled for 10’. Beads were pelleted on magnet for 2’ and samples were loaded directly onto 10% SDS-PAGE gel.

2.6 Cytoskeletal Preparation

10cm dishes were coated with 10µg/mL human fibronectin at 37°C for 1h. Dishes washed 3x with sterile 1xPBS prior to plating cells. SKOV-3 cells were plated and let grow to sub-confluence (~90%) prior to lysing.

Media was removed from cells and cells were washed gently with Dulbecco’s complete 1x PBS. 1.5mL cytoskeleton stabilizing buffer (recipe described below) was added to side of dish, rocked gently, and incubated for 4’ on ice. Dish rocked gently side to side and contents were pooled, and collected from bottom of dish using a P1000 pipette man, fraction labeled “cytoplasmic fraction”. Dish washed gently with 2mL cytoskeleton stabilizing buffer and wash discarded. 250µL RIPA buffer containing protease and phosphatase inhibitors was added to dish, and rocked back and forth to cover entire surface area of dish. Using a rubber police-man, dish was scraped very gently (minimal mechanical shearing), and lysate pooled at bottom of dish. Lysate removed carefully and spun at 100xg for 5’ at 4°C. Supernatant removed and transferred to new ice-cold tube and labeled “cytoskeletal fraction”. 200µL 1x Laemmli sample buffer was added to pellet (“nuclear fraction”) and heated at 75°C for 3-4h.
2.7 In-gel Digestion and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Extracted biotin-tagged proteins were separated by 10% SDS-PAGE and stained with coomassie blue. Following sufficient destain (50mM NH₄HCO₃ and 50% acetonitrile (MeCN), the no RIIα lane, and +/- Ht31 peptide lanes were each cut into 10 equivalently sized pieces. Each gel slice was cut up further into 1mmx1mm cubes and washed with HPLC grade H₂O. Pieces were incubated with 700µL desain solution (50mM ammonium bicarbonate and 50% MeCN) for 30min at 37°C. Destain was removed and pieces were subjected to dehydration by adding 100µL 100% MeCN for 20’. Gel pieces were further dried in a speed vacuum for 5’. Proteins were digested into peptides using sequencing grade modified trypsin at a concentration of 12ng/µL in 50mM ammonium bicarbonate at 37°C overnight (16-18h). The following day, digests were centrifuged at 12000xg for 1’ and supernatant was transferred to a 0.6mL tube. 50µL extract solution A (described below) was added to samples for 1h at room temperature. Samples were centrifuged at 12000xg for 15’ and supernatant was transferred to a 0.6mL tube (same tube as before). 50µL extract solution B (described below) was added to samples for 1h at room temperature. Samples were centrifuged at 12000xg for 15’ and supernatant was transferred to a 0.6mL tube (same tube as before). 50µL 100% MeCN was added to tubes for 5-10’ at room temperature. Samples were centrifuged at 12000xg for 15’ and supernatant was transferred to a 0.6mL tube (same tube as before). Samples in 0.6mL tubes were dried in a speed vacuum for 3-4h and stored at -80°C with proteomics facility.
2.8 Immunoprecipitations

For AKAP79 immunoprecipitations, SKOV-3 whole cell extract was first treated as biotin transfer (previously described). Subsequently 100µg of protein was incubated with 1.4µg anti-AKAP79 (sc-10764) antibody for 2h at 4°C followed by 1h at room temperature. Immunocomplexes were incubated with protein G-magnetic beads for 1h at room temperature, washed four times with RIPA lysis buffer, resuspended and boiled in 40µL of 1x Laemmli sample buffer for 10’.

For RIIα immunoprecipitations, SKOV-3 or HEK293 100µg whole cell extract was incubated with 1.4µg anti-RIIα (sc-908) antibody for 2h at 4°C. Immunocomplexes were incubated with protein G-magnetic beads for 1h at 4°C, washed four times with RIPA lysis buffer, resuspended and boiled in 50µL of 1x Laemmli sample buffer for 10’.

2.9 Western Blotting

For preparation of whole cell extract, cells were washed twice with ice-cold complete Dulbecco’s phosphate buffered saline, then lysed in RIPA lysis buffer (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8.0) containing protease and phosphatase inhibitors or m-RIPA (0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1.0% NP-40, 50mM Tris, pH 8.0). After 10’ on ice, lysates were scraped into ice-cold microcentrifuge tubes and centrifuged at 14,000 rpm (in an Eppendorf microcentrifuge) for 10’ at 4°C. Protein concentration of the supernatant lysate was determined by bicinechonic acid assay (Pierce). For direct immunoblotting, aliquots of lysate were mixed with 5x
Laemmli sample buffer and boiled for 10’ before loading on 10% or 7.5% SDS-PAGE gels. Samples were separated with SDS-PAGE followed by transfer to nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk powder in TBS 0.01% Tween-20 for 1h at room temperature. Membranes were incubated with primary antibody overnight at 4°C. Following incubation with primary, membranes were washed in TBS with 0.01% Tween-20 (TBS-T) 5x 5’and incubated with HRP-conjugated secondary antibody diluted in 5% nonfat dry milk in TBS-T, 1:1250 for 15’ at RT. Detection was performed using chemiluminescence (Pierce).

2.10 Streptavidin-HRP (sA-HRP) Blotting

For detection of biotinylated proteins using sA-HRP, a protocol was optimized from Kyle Roux, Ph.D. at The University of South Dakota. Following transfer of SDS-PAGE to nitrocellulose, membrane was incubated in BSA blocking buffer (recipe as described below) for 20’-30’ shaking at room temperature. sA-HRP was added at 1:40,000 in 10mL BSA blocking buffer and incubated for 40’ at room temperature shaking. Membrane washed 3-4x vigorously with 1xPBS over sink, followed by 5’ incubation with ABS blocking buffer (recipe described below), and 5’ incubation with ABS+150mM NaCl shaking at room temperature. Membrane washed 3-4 times vigorously with 1xPBS over sink. Membrane washed with 2x5’ 1xPBS+250mM NaCl, followed by 2x5’ 1xPBS. Detection was performed using chemiluminescence (Pierce).
2.11 Immunofluorescence

For visualization of ezrin, paxillin, FAK, RIIα, and lamin A/C, SKOV-3 cells were plated on 10µg/mL fibronectin and were fixed in 3.7% formaldehyde in TBS for 10’, permeabilized for 10’ in TBS containing 0.25% triton X-100, and blocked with TBS containing 3% BSA for 1h at room temperature. Cells were incubated with anti-paxillin (1:500, BD Transduction), rabbit anti-RIIα (1:200, Santa Cruz Biotechnology), mouse anti-ezrin (1:200, BD Transduction), mouse anti-lamin A/C (1:200, BD Transduction), or rabbit anti-FAK (sc-558) overnight at 4°C in a humidity chamber. The following day, cells were treated with Proximity Ligation Assay probes according to manufacturer’s instructions.

For visualization of filamin A, SKOV-3 cells were plated on 10µg/mL fibronectin and were fixed in pre-chilled anhydrous MeOH. Cells were incubated in -20°C for 20’. Cells were then treated with 3.7% formaldehyde in TBS for 10’ at RT, blocked in PBS containing 1.5% BSA for 1h at room temperature. Cells were incubated with mouse anti-filamin A (1:400, Millipore) overnight at 4°C. The following day, cells were treated with Proximity Ligation Assay probes according to manufacturer’s instructions.

2.12 In Situ Proximity Ligation Assay

Protein interactions in SKOV-3 cells were studied using an In situ Proximity Ligation Assay Kit (Duolink®) from Sigma. Cells were plated on 10µg/mL fibronectin coated coverslips and let adhere overnight. Cells were fixed, permeabilized, and incubated with primary antibodies as described in immunofluorescence assays. The
Proximity Ligation assay was used as recommended by the manufacturer. In short, cells were incubated with secondary antibodies with attached nucleotides, including both anti-rabbit and anti-mouse coupled to PLUS and MINUS nucleotides respectively. If nucleotides were close (less than 30-40nm), and after ligation, a circular DNA strand formed. After amplification of the DNA circle and hybridization of fluorescently (563nm) labeled complimentary oligonucleotide probes, protein interactions were visualized as red dots.

2.13 Coomassie Staining

Following electrophoresis, SDS-PAGE gels were stained in 0.006% Coomassie Brilliant Blue R-250 (Bio-Rad) in 10% acetic acid for 1h. Destain solution (50mM NH₄HCO₃ and 50% acetonitrile) was used for destaining for 12h, changing destain periodically.

2.14 Lysis Buffers and Other Buffers

All buffers stored at 4°C unless otherwise indicated. Cytoskeleton Stabilizing Buffer contained 10mM MES pH 6.1, 138mM KCl, 3mM MgCl₂, 2mM EGTA, 0.32M sucrose, and 0.5% triton X-100. RIPA (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8.0) and modified RIPA buffer (0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1.0% NP-40, 50mM Tris, pH 8.0) were used as indicated. RIIα purification lysis buffer contained 10mM MOPS (pH 6.9), 100mM NaCl, 1mM DTT, protease and phosphatase inhibitors. BSA blocking buffer contained 1xPBS with 1% bovine serum albumin and 0.2% (w/v)
Triton X-100. ABS blocking buffer contained 1xPBS with 10% adult bovine serum, 1% (w/v) Triton X-100, 150mM NaCl. Extract solution A (for mass spectrometry) contained 5% formic acid. Extract solution B (for mass spectrometry) contained 5% formic acid 50% MeCN.
CHAPTER 3: Results

3.1 Results

3.1.1 Model for application of the biotin transfer method

There is surmounting evidence from our lab and others, demonstrating discrete pockets of PKA activity at the leading edge of cells. Additionally, our lab has shown that disruption of the AKAP-PKA interaction, with the use of anchoring disrupting peptides, leads to a decreased ability of SKOV-3 cells to migrate (Mckenzie et al., 2011). Therefore, we sought to generate a method for searching for and identifying leading edge AKAPs, which may be responsible for type II anchoring of PKA, thus responsible for the observed PKA activity. With the limitations and advantages of classically used techniques, we created a method based on the use of purified PKA RIIα and the use of a commercially available sulfo-SBED biotin transfer reagent (Fig. 1).

Because biotin is relatively uncommon in vivo, and it is amenable to selective isolation, it became an obvious choice for the modification on which we chose to focus.

Sulfo-SBED is an abbreviation for Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azidobenzamido)-hexanoamido) ethyl-1,3’-dithioprprionate. It is a heterobifunctional chemical crosslinker, with an amine-reactive NHS ester group at one end, capable of binding to any lysine group. On the other arm, there is a UV light-activatable azide group, which crosslinks non-specifically to any protein side chains. What makes this method distinguishable from other chemical crosslinkers, are the biotin group and cleavable disulfide bond. Together, these groups lend the ability to
Figure 1: Schematic for application of the biotin transfer reagent coupled to RIIα in SKOV-3 cells
**Figure 1: Schematic for the application of biotin transfer coupled to RIIα method in SKOV-3 cells.** RIIα was purified from BL21 PLysS (DE3) bacteria expressing pET28(b)-RIIα(WT) and coupled to SULFO-SBED. Whole cell extract from SKOV-3 cells plated on 10µg/mL fibronectin was incubated with RIIα-sulfo SBED. Prior to incubation with RIIα, lysate is incubated with 50µM Ht31 or Ht31P for 1h. UV-activatable azide group was crosslinked to putative AKAPs binding to RIIα with UV-light (365nm). 100mM DTT was used to reduce the disulfide bond, completing the transfer from RIIα to its binding partners. Streptavidin beads were used to selectively isolate biotinylated proteins.
crosslink RIIα to interacting partners, and transfer the biotin affinity tag onto binding partners. Selective pulldown of the biotin adduct using streptavidin-beads, allows the isolation of binding partners and subsequent identification. This is a powerful in vitro method for protein interaction discovery and has been used successful in the characterization of novel protein interactions.

For our application, we wanted to enrich for PKA type II interactions with AKAPs, and therefore made use of the ability to purify the PKA RIIα subunit. Additionally, we anticipated treatment of lysate with our Ht31 disruptor peptide, would result in disrupted PKA-AKAP interactions, preventing the transfer of the biotin adduct. Based on the previous data, and the lab’s interest in cell migration, it is conceivable to think the PKA activity is spatially regulated through an AKAP intimately involved in cytoskeletal dynamics.

3.1.2 Characterization and optimization of the biotin transfer method

Because of the novelty of the technique, there were a number of optimizations and characterizations that were necessary upfront. To determine if the sulfo-SBED reagent worked with our application, purified RIIα from BL21, was coupled according to the manufacturer’s directions. RIIα coupled and uncoupled to the sulfo-SBED were analyzed by SDS-PAGE and blotting for biotin with streptavidin-HRP (Fig. 2A). As expected, the uncoupled RIIα showed no presence of biotin, however the coupled RIIα
Figure 2: Characterization and Optimization of Biotin Transfer Method
Figure 2: Characterization and Optimization of Biotin Transfer Method. (A) Purified RIIα either coupled to Sulfo-SBED or not, was analyzed by SDS-PAGE. Streptavidin-HRP was used to detect the presence of biotin groups and detection of multiple bands shows RIIα is not 100% pure. (B) SKOV-3 cells were plated on 10µg/mL and lysed in m-RIPA buffer. Biotin transfer method was done in 100µg whole cell extract while control lane did not receive RIIα-sulfo SBED. Streptavidin pull-down was done and following washes with RIPA buffer, biotinylated proteins were eluted and separated by SDS-PAGE. Pull-downs were immunoblotted with α-mouse AKAP79 antibody and whole cell extract was included for control. (C) Biotin transfer method in 100µg whole cell extract from SKOV-3 cells with titrated amounts of RIIα (1:100, 1:200, 1:1000, 1:2000 w/w RIIα:extract). A no RIIα control lane was used additionally. Streptavidin pull-down was done and following washes with RIPA buffer, biotinylated proteins were eluted and separated by SDS-PAGE. Pull-downs were immunoblotted with α-mouse AKAP79 antibody and α-mouse tubulin antibody and whole cell extract was included as a control. (D) Biotin transfer method in 100µg whole cell extract from SKOV-3 cells with titrated amounts of streptavidin beads used in pull-down (25µL, 50µL, 100µL). A no RIIα control lane was used additionally. Streptavidin pull-down was done and following washes with RIPA buffer, biotinylated proteins were eluted and separated by SDS-PAGE. Pull-downs were immunoblotted with α-mouse AKAP79 antibody and α-rabbit RIIα (sc-908) antibody and whole cell extract was included as a control.
had multiple detectable bands, suggesting the coupling of sulfo-SBED to other proteins. This suggested that the purity of the RIIα had been compromised. Given the specificity of Ht31 for PKA-AKAP interactions, the purity of the RIIα was less concerning although still provides substantial evidence for further optimization of the technique.

To determine if the biotin was successfully being transferred to a known AKAP, we searched for the presence of the biotin group on a well characterized AKAP in the presence or absence of the RIIα-Sulfo SBED. AKAP79 has been well described as a canonical AKAP which binds to PKA RIIα through the conserved amphipathic helix (Scott). As expected, the pull down with streptavidin beads revealed the biotin group on AKAP79 only when lysate was treated with RIIα-sulfo SBED and not with purified uncoupled RIIα (Fig. 2B). This supported the hypothesis that AKAP79 can only be pulled down if the RIIα had successfully transferred a biotin group. This provided strong evidence of the method working with our target interest group of proteins. Additionally, to be sure we were detecting AKAP79 as a function of RIIα and it was not alternatively selectively pulled-down by interacting with the beads, we examined whether or not we could detect it in the absence of RIIα-sulfo SBED. The absence of AKAP79 with no RIIα, suggested that it was in fact selectively pulled-down as a result of biotin transfer from RIIα (Fig. 2B).

The conditions used in previously described experiments were based on initial experiments done a number of years prior. As these conditions were not previously optimized, there were justifiable grounds to do so. To examine the amount of RIIα needed, the ratio of RIIα to lysate (w/w) was titrated. Additionally, there were some
initial observations of non-specific proteins present in the pull-downs, suggesting possibly the RIIα was in great excess and therefore non-selectively binding and transferring biotin to a number of proteins in the cell. In order to reduce the signal to noise ratio of specific RIIα binding partners to non-specific partners, RIIα amounts were titrated in WCE (Fig. 2C). Pull-downs followed up with immunoblotting of both AKAP79 and tubulin, specific and non-specific PKA binding partners respectively, were used to assess biotin incorporation. The amount of AKAP79 selectively pulled down dropped proportionally as a function of amount of RIIα as expected (Fig. 2C). By contrast, no tubulin was detected as a function of streptavidin pull-down, suggesting no biotin incorporation onto non-specific proteins. Additional immunoblotting of other proteins not expected to bind RIIα, showed similar results as tubulin (data not shown).

In order to determine the optimal amount of streptavidin-beads to use in the pull down, a similar titration was done varying the amounts of the beads relative to the lysate. Consistent with previous results, we detected no AKAP79 in the no RIIα control lane. Unexpectedly, we did not see an increase in the amount of AKAP79 selectively pulled-down as a function of increasing the amount of beads (Fig. 2D).

3.1.3 Ht31 disrupts the AKAP79-PKA interaction in SKOV-3 cells

The Ht31 peptide has been well characterized as disrupting the PKA-AKAP interaction. It has been widely used as a mechanism to study PKA anchoring (Scott et al., 2013). Taken from a known AKAP, AKAP-Lbc, Ht31 contains the PKA R subunit-binding domain, the canonical amphipathic helix. We therefore we sought out to
determine whether or not this peptide would disrupt RIIα-sulfo SBED interaction in lysates, and thus lead to a decrease in the amount of biotin transferred to known AKAPs. Using varying concentrations of Ht31, or the control peptide, Ht31P, we saw a decrease in AKAP79 in our pull-downs in the presence of the disruptor peptide, but not with the control peptide (Fig. 3A). Quantification of this shows over a two-fold reduction is relative amounts of AKAP79 selectively pulled-down using streptavidin beads (Fig. 3B). While 50μM showed the most significant decrease in amount of AKAP79, there were concerns about the control peptide also disrupting the AKAP-PKA interaction (Fig. 3A). While non-AKAPs were found in the pull-down, Ht31 did not appear to have an effect on the amount of those proteins detected (data not shown). These data suggested that Ht31 was in fact specifically disrupting the PKA-AKAP interaction.

3.1.4 Interaction between Filamin A and RIIα in SKOV-3 cells

The search for leading edge AKAPs began a few years ago in our lab, and the biotin transfer method had been previously applied to both cell body and pseudopods (purified leading edge structures). Mass spectrometry data from the initial experiments provided strong evidence for some potential and novel AKAPs that may be responsible for the observed PKA activity at the leading edge. From this data set, the protein identified with the most significant p-value, was Filamin A (FLNa), an actin-binding protein intimately involved in actin cytoskeletal dynamics (Stossel et al., 2001;
Nakamura et al., 2007). Showing both a strong reduction as a function of Ht31, and strong biological relevance,
Figure 3: Ht31 but not Ht31P disrupts RIIα-AKAP79 interaction in SKOV-3 cells
**Figure 3: Ht31 but not Ht31P disrupts RIIα-AKAP79 interaction in SKOV-3 cells.**

(A) Biotin transfer method in 100µg whole cell extract from SKOV-3 cells treated with titrated amounts of Ht31 or Ht31P (10µM, 25µM, 50µM) for 1h. Streptavidin pull-downs were done and following washes with RIPA buffer, biotinylated proteins were eluted and separated by SDS-PAGE. Pull-downs were immunoblotted with α-mouse AKAP79 antibody and whole cell extract as a control. (B) Relative densitometry is depicted in the black bar graph.
we attempted to characterize filamin A as a potential AKAP. Given filamin’s role in mechanotransduction, myriad of over ninety binding partners, and dynamic regulation over cytoskeletal dynamics, this was a very exciting finding.

Some of the initial sA pull down and co-immunoprecipitation experiments showed strong evidence of an interaction between RIIα and FLNa. Even more, an in silico approach identified a region in the N-terminus whose sequence has the potential of forming an amphipathic helix (data not shown). This supported our hypothesis that FLNa is a cytoskeletal AKAP. Therefore, to carry our investigation further, we sought to confirm this interaction using traditional biochemical methods. Immunoprecipitations for both RIIα and IgG, as a control, were performed in both HEK293 and SKOV-3 cells under varying conditions. Many results showed filamin A present only with RIIα and not with IgG (Fig. 4A), supporting our hypothesis and further suggesting an interaction between RIIα and FLNa. However, when immunoprecipitations were repeated, different results were observed, showing filamin A present in both the RIIα and IgG lanes, suggesting filamin A may not be specifically interacting with RIIα (Fig. 4B). FLNa binding partners is comprised of 24 immunoglobulin-like domains, which have been described as inherently sticky, it is plausible to believe that the observed interaction between filamin A and RIIα is non-specific. AKAP79 is as a well described and canonical AKAP, therefore we were able to use it as our positive control. Our data showed AKAP79 present only in the presence of RIIα and not with IgG, supporting the ability of this technique to confirm AKAPs.
Figure 4: Interaction between Filamin A and RIIα in SKOV-3 cells
Figure 4: Interaction between Filamin A and RIIα in SKOV-3 cells. (A) Untransfected SKOV-3 cells were cultured on 10µg/mL fibronectin and lysed in RIPA lysis buffer. 100µg of whole cell extract was treated for immunoprecipitation for PKA RIIα or rabbit IgG as a control. Immunoprecipitations were separated by SDS-PAGE and immunoblotted with antibodies against filamin A (FlnA), PKA RIIα, and AKAP79 as indicated. The positions of the molecular weight markers are indicated. (B) Immunofluorescence detection of proximity ligation assay signal for filamin A/ RIIα interaction in SKOV-3 cells after plating and fixing on 10µg/mL fibronectin. Paxillin/FAK and ezrin/ RIIα were used as positive controls and FAK/Lamin A/C interaction was used for negative control. Individual events of red PLA signal were used as reading output. DAPI was used to stain nuclei (blue). Images were taken at a magnification of 20x.
Given that the observed results varied substantially and we were unable to resolve the inconsistencies in the data, we sought to confirm this interaction using a non-traditional approach. Proximity ligation assay was used to analyze the interaction between RIIα and FLNa in SKOV-3 cells plated on 10µg/mL fibronectin. The interaction between focal adhesion kinase (FAK) and paxillin (pxn) has been extensively characterized and served as a positive control (Hildebrand et al., 1993). Additionally, we used ezrin and RIIα as a positive control for AKAP-PKA interaction as this interaction has been well described (Dransfield et al., 1997). FLNa and RIIα showed a distinct pattern similar to what was observed in both positive controls (Fig. 4C). These data further supported our hypothesis that filamin A and RIIα interact and that filamin A may be a potential novel AKAP.

3.1.5 Cytoskeletal fraction from SKOV-3 lysate shows enrichment of cytoskeletal proteins

While the data confirming the RIIα FLNa interaction were inconsistent, the RIIα AKAP79 data were strongly suggestive of the potential of using the biotin transfer method as a successful method to identify AKAPs. Although the data was inconclusive of filamin A being the potential AKAP responsible for leading edge PKA activity, the question remained the same. We therefore sought out to repeat the biotin transfer method, with a few modifications. Filamin A was originally found in protein extracts isolated from pseudopods. However, pseudopodial preparations suffer from several limitations, not at least of which is the recovery of very little protein.
We sought out to selectively isolate the cytoskeletal fraction, to enrich for potential cytoskeletal AKAPs. Borrowing methods from the literature (Avnur and Geiger, 1981; Kuo et al., 2012), we fractionated lysate from SKOV-3 and isolated the nuclei away from the cytoplasmic and cytoskeletal contents (Fig. 5A). The isolation of focal adhesions has been well described (Kuo et al., 2012), and confirmation of their isolation technique was confirmed using western blotting. We therefore chose protein targets for immunoblotting, which should remain in distinct subcellular fractions in order to confirm our method. Actin remained in the cytoskeletal fraction, after cytoplasmic contents were collected; suggesting our cytoskeletal stabilizing buffer (see Materials and Methods) was working as expected. While there was still actin remaining in the nuclear fraction, this was likely due to the contamination from un-lysed whole cells remaining (Fig 5B). Paxillin, a known focal adhesion protein, showed enrichment in the cytoskeletal fraction compared to the nuclear fraction (Fig. 5B). Additionally, GAPDH, a predominant cytoplasmic protein appeared to be enriched in the cytoplasmic contents (Fig. 5B). Lamin A/C was used a marker for the nucleus, and showed significant enrichment in the nuclear fraction, suggesting we were retaining nuclear integrity during the cytoskeletal isolation (Fig. 5B). Taken together, these data suggested we had a successful method for isolation of the cytoskeletal fraction. This method however had little optimization, and further improvement could allow for a
Figure 5: Cytoskeletal fraction from SKOV-3 lysate shows enrichment of cytoskeletal proteins
Figure 5: Cytoskeletal fraction from SKOV-3 lysate shows enrichment of cytoskeletal proteins. (A) Schematic of method used for cell fractionation and cytoskeleton isolation. SKOV-3 cells were plated on 10µg/mL fibronectin and let adhere for at least 24 h and cells were grown to ~90% confluence prior to lysis. Cytoskeletal stabilizing buffer was added to cells to puncture the cell, removing cytoplasmic contents. Cytoskeletal fraction is solubilized in RIPA and in-tact nuclei are separated from cytoskeletal fraction by centrifugation. (B) 15µg of protein from each fraction were separated by SDS-PAGE and immunoblotted with antibodies against Lamin A/C, GAPDH, Paxillin, and Actin as indicated.
greater isolation of the cytoskeleton from cytoplasmic and nuclear fractions. The greatest challenge with this preparation is the amount of protein recovered from in the cytoskeletal fraction. Consistently, protein yields were around 40µg from a 10cm dish of confluent SKOV-3 cells.

3.1.6 Coomassie staining of streptavidin pull-downs from SKOV-3 lysate submitted for mass spectrometry analysis

Both previous data and initial optimization of the biotin transfer method suggested this was in fact a successful technique to identify AKAPs. We therefore wanted to first apply this method to whole-cell extracts from SKOV-3. Due to the technical difficulties involved with the cytoskeletal fractionation, we wanted to establish a working system before proceeding to the application of the biotin transfer in the cytoskeletal preparations.

To be sure that we were non-specifically pulling down proteins independently of RIIα, we included a streptavidin (sA) pull down from whole cell extract excluding RIIα. Streptavidin pull downs from whole cell extract were done in 150µg of protein in the absence and presence of Ht31. While this amount much lower than those typically used for mass spec analysis, observation of proteins via coomassie staining and advice from the proteomics facility encouraged us to proceed using the pull-downs from 150µg (Fig. 6A). Three lanes were cut out of the gel, and subsequently cut into smaller slices (see materials and methods). A tryptic digest was done following the instructions provided by the proteomics facility and samples were submitted for analysis.
Figure 6: Coomassie staining of streptavidin pull-downs from SKOV-3 lysate submitted for mass spectrometry analysis
Figure 6: Coomassie staining of streptavidin pull-downs from SKOV-3 lysate submitted for mass spectrometry analysis. (A) Whole cell extract from SKOV-3 cells plated on 10µg/mL fibronectin were lysed in m-RIPA buffer. Streptavidin pull-downs were performed in 150µg of whole cell lysates treated with RIIα-sulfo SBED for 45’ followed by treatment with or without 50µM Ht31 disruptor peptide for 1h. A streptavidin pull-down in lysates not treated with RIIα was included as a negative control. Pull-downs washed in RIPA buffer and eluent was separated by SDS-PAGE. Samples were run on 10% gel and stained with coomassie true blue. 10µL of pull-down supernatants was separated by SDS-PAGE and run alongside pull-down. (B) Cytoskeletal fractions from SKOV-3 cells plated on 10µg/mL fibronectin were isolated from cell extracts and pooled together. Streptavidin pull-downs were performed in 1000µg of cytoskeletal lysates treated with RIIα-sulfo SBED for 45’ followed by treatment with or 50µM Ht31 or 50µM Ht31P for 1h. Pull-downs washed in RIPA buffer and eluent was separated by SDS-PAGE. Samples were run on 10% gel and stained with coomassie true blue. Gels were subsequently sliced, trypsinized, and submitted for mass spectrometry analysis.
After receiving the data from the facility, we were able to identify AKAP79, as well as other known AKAPs (data not shown). Both AKAP79 as well as other known A-kinase anchoring proteins, showed a decrease in number of peptides in the presence of Ht31, supporting our hypothesis. Furthermore, after examining the dataset more closely, we noticed that as expected, Ht31 had no observed effect on another family of proteins, the annexins. These observations showed specific disruption of the PKA-AKAP interactions. This suggested the method was working within our application, and provided evidence to proceed using the cytoskeletal preparations. Concerned about missed proteins due to low abundance and to achieve levels of bound proteins sufficient for mass spectrometry, we scaled-up the amount of input from 150µg to 1000µg. Based on the data from the no RIIα control, we decided to omit this from our second pull down in an effort to save materials. Pooling together a number of cytoskeletal preparations, we performed streptavidin pull-downs in the presence of either Ht31 or the control peptide Ht31³ (Fig. 6B). Coomassie staining showed both a decrease in total number of bands and intensity of bands observed in the presence of the Ht31 peptide compared to the Ht31³ control peptide (Fig. 6B). Lanes were sliced and tryptic digests were done as previously described for whole cell extract. Based on recommendations from the proteomics facility, an additional alkylation and reduction step was done on samples from cytoskeletal preparations. Peptides were extracted and subjected to liquid chromatography tandem mass spectrometry in a linear ion trap mass spectrometer.
3.1.7 List of candidate proteins selected from SKOV-3 cytoskeletal fraction and whole cell extract

Mass spectra were analyzed using SEQUEST and Mascot and a concatenated forward and reverse human NCI protein database approach (Elias and Gygi, 2007). Our initial data analysis involved filtering of top SEQUEST peptide matches to around 1% false discovery rate (described in materials and methods). In our whole cell extract database, this method identified 3366 peptides and 3364 peptides in the absence or presence of Ht31 respectively (data not shown). In the cytoskeletal fractions, we identified 1636 and 1371 peptides in the presence of either Ht31P or Ht31 respectively. This provided a very high stringency and confidence for protein identification. These experiments were intended to act as a preliminary screen for AKAPs, which subsequent biochemical methods would be required for confirmation of any potential hits. With this in mind, we therefore decided to lower the stringency of which our proteins were identified, to create a more comprehensive list of potential AKAPs. Following the “high confidence” analysis, we lowered the cross-correlation scores (x-corr), which facilitated SEQUEST peptide matches to >5% false discovery rate (see materials and methods) and created a “low confidence” dataset (data not shown). In our whole cell extract, this method identified 31273 and 33301 peptides in the absence and presence of Ht31 respectively, while in the cytoskeletal fractions the method identified 30653 and 26915 proteins in the presence of Ht31P or Ht31 respectively. While the strength of the mass spectra assignments was compromised slightly, the dataset generated is much greater. This allowed us to identify known AKAPs, including AKAP79 which had been
previously confirmed by immunoblotting (Fig. 2 and 3). Given the size of these datasets, we needed a way to narrow down the proteins and select a list of candidate proteins that are potential AKAPs.

Although Ht31 should ideally greatly disrupt PKA-AKAP interactions, there was still evidence of AKAPs by mass spectrometry identification. Using total number of peptides identified for a protein as an initial rubric, we sought to determine the peptide fold-decrease from Ht31\(^{\text{p}}\) to Ht31 for each protein (see materials and methods). In order to trim the dataset further, the fold decreases or “displacement factor” were sub-divided into four categories; \(x=100\) \(x \geq 50\), \(50>x>0\), \(x=0\), with \(x=100\) representing those proteins found exclusively in the presence of the Ht31\(^{\text{p}}\) control peptide. Simultaneously, proteins were analyzed using PANTHER Gene Ontology and sorted by protein class. Comparing proteins with a “displacement factor” between 50 and 100 to the gene ontology protein class, a list of candidate proteins was generated subjectively (Table 1). While this protein list was chosen relatively arbitrarily, particular attention was paid to protein class and included only those proteins labeled by PANTHER as “cytoskeletal”, “cell adhesion/cell junction”, or “signaling”. Additional proteins were included if they had a significant “displacement factor” and were known to interact with cytoskeletal proteins (eg. filamin A-interacting protein 1). The number of peptides identified was included in this list to allow for another level of stringency, as proteins with 1 or lower peptides identified were removed from the list. The datasets were scanned and searched for known A-kinase anchoring proteins. In some cases, the identification of these varied between the datasets. This provided internal positive
controls for what the data should represent for known AKAPs. A number of proteins identified from the “cytoskeletal fraction” were known DNA binding proteins, nuclear proteins, and a number of cytoplasmic proteins. The presence of these clearly suggests the need for improvement and optimization in the fractionation technique.

The list of candidate proteins was of particular interest as one of the proteins which showed the highest “displacement factor” was in fact filamin A. Additionally, there were a number of canonical focal adhesion and cytoskeletal proteins which showed both a large number of identified peptides and a large “displacement factor”. Of particular interest is talin-1, which was previously identified from the initial screen (Howe and Baldor unpublished data) in addition to well characterized cytoskeletal AKAPs such as ezrin. To this point, we have established a list of potential AKAPs which may be responsible for the spatial regulation of PKA activity during cell migration and adhesion. Additionally, these data suggest that we have a method to selectively isolate PKA RIIα binding partners from SKOV-3 cells.
<table>
<thead>
<tr>
<th>IPI Number</th>
<th>Candidate Protein</th>
<th>Displacement Factor</th>
<th>PANTHER Protein Class</th>
<th>Mass Spec &quot;Strength&quot;</th>
<th># of Peptides in HT31P</th>
<th>Dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPO0297210.4</td>
<td>Isoform 1 of Filamin-A-interacting protein 1</td>
<td>98.36065574</td>
<td>Other</td>
<td>Low</td>
<td>61</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0642180.1</td>
<td>Isoform 3 of Filamin-A-interacting protein 1</td>
<td>98.21428571</td>
<td>Other</td>
<td>Low</td>
<td>56</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0745798.1</td>
<td>Isoform 2 of Filamin-A-interacting protein 1</td>
<td>98.11320750</td>
<td>Other</td>
<td>Low</td>
<td>53</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0298994.1</td>
<td>Talin-1</td>
<td>98.05825243</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>103</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0456723.1</td>
<td>Isoform 5 of Ral GTPase-activating protein subunit alpha-1</td>
<td>97.91666666</td>
<td>Other</td>
<td>Low</td>
<td>48</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0472044.2</td>
<td>Isoform 1 of Ral GTPase-activating protein subunit alpha-1</td>
<td>97.87234043</td>
<td>Signaling</td>
<td>Low</td>
<td>47</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0742521.2</td>
<td>Isoform 4 of Ral GTPase-activating protein subunit alpha-1</td>
<td>97.87234043</td>
<td>Other</td>
<td>Low</td>
<td>47</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0307545.2</td>
<td>Tensin-1</td>
<td>97.61904762</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>42</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0940816.2</td>
<td>Isoform 3 of Rho guanine nucleotide exchange factor 2</td>
<td>97.56097561</td>
<td>Other</td>
<td>Low</td>
<td>41</td>
<td>WCE-Low</td>
</tr>
<tr>
<td>IPO0434580.2</td>
<td>Isoform 1 of Myomesin-1</td>
<td>97.5</td>
<td>Adhesion</td>
<td>Low</td>
<td>40</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0479390.2</td>
<td>Isoform 2 of Myomesin-1</td>
<td>97.5</td>
<td>Adhesion</td>
<td>Low</td>
<td>40</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0979519.2</td>
<td>Isoform 2 of Rho guanine nucleotide exchange factor 10</td>
<td>97.43589744</td>
<td>Signaling</td>
<td>Low</td>
<td>39</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0006854.1</td>
<td>Ral GTPase-activating protein nGAP</td>
<td>97.36842105</td>
<td>Signaling</td>
<td>Low</td>
<td>38</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0298994.1</td>
<td>Talin-1</td>
<td>97.22222222</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>36</td>
<td>WCE-High</td>
</tr>
<tr>
<td>IPO0141318.4</td>
<td>Cytoskeleton-associated protein 4</td>
<td>96.66666666</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>30</td>
<td>Cyto-Low</td>
</tr>
<tr>
<td>IPO0333541.1</td>
<td>Isoform 1 of Filamin-A</td>
<td>83.33333333</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>12</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0215914.1</td>
<td>ADP-ribosylation factor 1</td>
<td>75</td>
<td>Signaling</td>
<td>High</td>
<td>4</td>
<td>WCE-High</td>
</tr>
<tr>
<td>IPO0215917.1</td>
<td>ADP-ribosylation factor 3</td>
<td>75</td>
<td>Signaling</td>
<td>High</td>
<td>4</td>
<td>WCE-High</td>
</tr>
<tr>
<td>IPO0021263.1</td>
<td>14-3-3 protein zeta/delta</td>
<td>66.66666666</td>
<td>Other</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0967485.1</td>
<td>Ankyrin isoform b</td>
<td>66.66666666</td>
<td>N/A</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0759532.1</td>
<td>Isoform 1 of Ankyrin</td>
<td>66.66666666</td>
<td>Other</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0292953.1</td>
<td>Isoform 2 of Ankyrin</td>
<td>66.66666666</td>
<td>Other</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0965169.1</td>
<td>Isoform 3 of Ankyrin</td>
<td>66.66666666</td>
<td>Other</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0024992.1</td>
<td>Plakin7</td>
<td>66.66666666</td>
<td>Adhesion</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0027222.1</td>
<td>Prohibitin-2</td>
<td>66.66666666</td>
<td>Other</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0413344.1</td>
<td>Cofilin-2</td>
<td>66.66666666</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
</tr>
<tr>
<td>IPO0028931.1</td>
<td>Desmin-2</td>
<td>60</td>
<td>Junction</td>
<td>High</td>
<td>5</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0012011.1</td>
<td>Cofilin-1</td>
<td>60</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>5</td>
<td>WCE-High</td>
</tr>
<tr>
<td>IPO0215914.1</td>
<td>ADP-ribosylation factor 1</td>
<td>50</td>
<td>Signaling</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0215917.1</td>
<td>ADP-ribosylation factor 3</td>
<td>50</td>
<td>Signaling</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0930263.1</td>
<td>eDNA FLI5099, highly similar to ADP-ribosylation factor 1</td>
<td>50</td>
<td>Signaling</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0027547.1</td>
<td>Dermcidin</td>
<td>50</td>
<td>N/A</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
</tr>
<tr>
<td>IPO0005159.1</td>
<td>Actin-related protein 2</td>
<td>50</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>2</td>
<td>WCE-High</td>
</tr>
<tr>
<td>ID</td>
<td>Protein Name</td>
<td>Source</td>
<td>Location</td>
<td>Expression</td>
<td>Significance</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>-------------------------</td>
<td>----------</td>
<td>------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>PI00969616</td>
<td>Desmoplakin la</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>11</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00005690</td>
<td>F-actin-capping protein subunit alpha-1</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00216691</td>
<td>Profilin-1</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00013933</td>
<td>Isoform DI of Desmoplakin</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>13</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00033541</td>
<td>Isoform 1 of Filamin-A</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>51</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00030250</td>
<td>Isoform 2 of Filamin-A</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>51</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00219688</td>
<td>Isoform 1 of Plakophilin-2</td>
<td>Junction</td>
<td>High</td>
<td>4</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00005264</td>
<td>Isoform 2 of Plakophilin-2</td>
<td>Junction</td>
<td>High</td>
<td>4</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00039802</td>
<td>Isoform 3 of Plectin</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>64</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00922115</td>
<td>cDNA FLJ7321, moderately similar to Moesin</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>26</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00219365</td>
<td>Moesin</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>65</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00033541</td>
<td>Isoform 1 of Filamin-A</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>161</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00302592</td>
<td>Isoform 2 of Filamin-A</td>
<td>Cytoskeletal</td>
<td>Low</td>
<td>161</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00418471</td>
<td>Vimentin</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>46</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00552858</td>
<td>Filamin A, alpha</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>8</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00872684</td>
<td>cDNA FLJ514141, highly similar to Ezrin</td>
<td>Cytoskeletal</td>
<td>High</td>
<td>13</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00554811</td>
<td>Actin-related protein 2/3 complex</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00925052</td>
<td>Actin-related protein 2/3 complex</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>3</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00215920</td>
<td>ADP-ribosylation factor 6</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00215920</td>
<td>ADP-ribosylation factor 6</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00012011</td>
<td>Cofilin-1</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00843975</td>
<td>Ezrin</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>1</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00219365</td>
<td>Moesin</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00010800</td>
<td>Nestin</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>2</td>
<td>Cyto-High</td>
<td></td>
</tr>
<tr>
<td>PI00301346</td>
<td>DNA-directed RNA polymerase III</td>
<td>Unique to Hist1P</td>
<td>Low</td>
<td>59</td>
<td>Cyto-Low</td>
<td></td>
</tr>
<tr>
<td>PI00477324</td>
<td>Isoform 2 of Filamin A-interacting protein 1-like</td>
<td>Unique to Hist1P</td>
<td>Other</td>
<td>60</td>
<td>Cyto-Low</td>
<td></td>
</tr>
<tr>
<td>PI00219365</td>
<td>Moesin</td>
<td>Unique to Hist1P</td>
<td>Low</td>
<td>38</td>
<td>Cyto-Low</td>
<td></td>
</tr>
<tr>
<td>PI00215918</td>
<td>ADP-ribosylation factor 4</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00215919</td>
<td>ADP-ribosylation factor 5</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00910783</td>
<td>cDNA FLJ1215, highly similar to ADP-ribosylation factor 5</td>
<td>Unique to Hist1P</td>
<td>Signaling</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00099515</td>
<td>cDNA FLJ1495, highly similar to ADP-ribosylation factor 5</td>
<td>Unique to Hist1P</td>
<td>Signaling</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00219399</td>
<td>Talin-2</td>
<td>Unique to Hist1P</td>
<td>High</td>
<td>3</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00022228</td>
<td>Vinculin</td>
<td>Unique to Hist1P</td>
<td>Other</td>
<td>5</td>
<td>WCE-High</td>
<td></td>
</tr>
<tr>
<td>PI00867677</td>
<td>Isoform 2 of A-kinase anchor protein 12</td>
<td>Unique to Hist1P</td>
<td>Other</td>
<td>48</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00940222</td>
<td>Isoform 3 of A-kinase anchor protein 12</td>
<td>Unique to Hist1P</td>
<td>Other</td>
<td>48</td>
<td>WCE-Low</td>
<td></td>
</tr>
<tr>
<td>PI00926625</td>
<td>Zyxin</td>
<td>Unique to Hist1P</td>
<td>Low</td>
<td>10</td>
<td>Cyto-Low</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Identification of candidate proteins selected from SKOV-3 cytoskeletal fraction and whole cell extract
Table 1: Identification of candidate proteins selected from SKOV-3 cytoskeletal fraction and whole cell extract. Subjectively selected proteins chosen from complete dataset from mass spectra analysis, as described in materials and methods, for both whole cell extract and cytoskeletal fraction. Dataset indicates which subsequent table protein can be found. IPI number represents accession numbers from the International Protein Index. Displacement factor represents the fold change as a function of Ht31; calculated as the difference in number of peptides between Ht31P sample and Ht31 relative to number of peptides in Ht31P sample as identified by the mass spectrometer. Displacement factor was binned according to materials and methods, x≥50 (green), 50>x>0 (orange), 100 indicates those unique to Ht31P (blue). Mass spec strength correlates to the filters and xcorr scores used to filter the protein datasets. PANTHER protein class refers to the gene ontology as indicated by the PANTHER database.
3.1.8 Development and Rationale of Algorithm

Based on the criteria included in our selected list, we sought to determine a less subjective approach to selectively identify potential AKAPs from our large dataset. We therefore generated an algorithm which uses the criteria from table 1, and creates a score for each of the potential candidates (Fig. 7). Using the same algorithm, we scored known AKAPs to establish a range of numerical values in which we would expect potential AKAPs to lie.

The criteria included in the algorithm are as follows: “displacement factor”, biological relevance, mass spec strength, total number of peptides in Ht31P, AKAP consensus site, and helical projection of the AKAP consensus site. Of these criteria, some are more important and therefore carry more weight in the algorithm. Given that the Ht31 peptide should displace all canonical PKA-AKAP interactions, the most weight was given to those proteins which showed the greatest decrease in number of peptides in the presence or absence of the Ht31 inhibitor peptide. This is referred to as the “displacement factor”, which represents the difference in number of peptides found from the Ht31P to Ht31 treatments, divided by the total found in the Ht31P treatment, and was given the most weight in the algorithm. Biological relevance was determined based on PANTHER Gene Ontology information, assigning numeric values to candidate proteins found in the following PANTHER protein classes: cytoskeleton, signaling, junction, and adhesion. Biological relevance was given the second highest priority in the algorithm. Both mass spectrometry strength and total number of peptides found in the Ht31P experimental lane were given equal priority. Mass spectrometry
Figure 7: Development and Rationale of Algorithm to

A

\[ 3A + 2B + C + D + (ExF) = \text{Score} \]

B

A: Displacement Factor
   90-100=6
   50-89=4
   0-49=2
B: Biological Relevance
   Cytoskeleton=6
   Adhesion=6
   Junction=2
   Signaling=2
C: Mass Spectrometry Strength
   “High”=2
   “Low”=1
D: Total Number of Peptides in Ht31° Treatment
   >50=6
   20-50=4
   10-20=3
   3-10=2
   2-3=1
E: Identification of AKAP Consensus Site
   “Yes”=3
   “No”=1
F: Predicted Helix Structure of Consensus Site
   “Yes”=2
   “No”=1
Figure 7: Development and Rationale of Algorithm. (A) Algorithm used to assign a numerical score to candidate AKAPs. Certain criteria given higher weight are indicated by the numerical coefficient. (B) List of criteria included in the algorithm. Numerical values assigned as indicated. Displacement factor represents the difference in total number of peptides found in the Ht31 compared to Ht31P treatments, divided by the total number of peptides found in the Ht31P treatment. Biological relevance was determined using the protein classes as assigned by PANTHER Gene Ontology database. Mass spectrometry strength either indicated as high or low, was determined by x-correlation values and peptide confidence as determined by the Thermo Proteome Discoverer software. AKAP consensus site used in search was the following: (AVLISE) XX (AVLIF) (AVLI) XX (AVLI) (AVLIF) XX (AVLISE) where X represents any amino acid. Predicted helix structure determined using software through The University of Virginia.
strength was either considered “High” and assigned a numerical value of 2, or “Low” and assigned a numerical value of 1. “High” and “Low” refer to the strength of the cross-correlation scores and peptide confidence as described previously. Finally, the identification of an AKAP consensus site and the likelihood that site forms a canonical helix were included in the algorithm. The identification of an AKAP consensus site was done using an in-silico approach in conjunction with Benjamin King. The helical wheel structure was predicted using a helical wheel projection application found through the University of Virginia. Helical wheel structure was assigned a value of 2 for “yes” and 1 for “no” based on the likelihood the AKAP consensus site would form a helix. AKAP consensus site was assigned a numerical value of 3 if a potential site was identified and 1 if there was no site. A value was still assigned even if no AKAP consensus site was identified because of the potential the candidate protein could be a non-canonical AKAP, binding PKA through an alternative mechanism. The following AKAP consensus site was used in the search: (AVLISE) XX (AVLIF) (AVLI) XX (AVLI) (AVLIF) XX (AVLISE) where X represents any amino acid.

We applied the algorithm described above and looked at some known A-kinase anchoring proteins, some of which are also known to be involved in cytoskeletal and adhesion dynamics (Table 2). Gravin, WAVE1, AKAP-Lbc, and ezrin, are known cytoskeletal-associated proteins which have also been confirmed as AKAPs. AKAP 5 (AKAP79) and AKAP11 were used to generate values for other known AKAPs, that are not cytoskeletal or adhesion proteins. These gave us a numerical range for where we would expect to see other potential cytoskeletal and adhesion associated AKAPs.
<table>
<thead>
<tr>
<th>Candidate Protein</th>
<th>Displacement Factor</th>
<th>Biological Relevance</th>
<th>Mass Spec Strength</th>
<th># of Peptides</th>
<th>AKAP Consensus Site</th>
<th>Helical Wheel?</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravin/AKAP12</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>AKAP Lbd/AKAP 13</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>WAVE1</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Ezrin</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>A-kinase anchor protein 5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>A-kinase anchor protein 11</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 2: Score for Known A-Kinase Anchor Proteins Using Developed Algorithm
Table 2: Score for known A-kinase anchoring proteins using the developed algorithm. Using the algorithm as described in figure 7, known AKAPs were scored. Gravin (AKAP12), ezrin, WAVE1, and AKAP-Lbc (AKAP13) are known cytoskeletal and adhesion associated proteins. AKAP 5 (AKAP79) and AKAP11 are other known AKAPs which have not been described as involved with cytoskeletal and adhesion dynamics.
CHAPTER 4: Discussion

A-kinase anchoring proteins (AKAPs) are scaffolding proteins which contribute to cAMP signaling pathways through tethering of the cAMP dependent kinase A (PKA). AKAPs target PKA to discrete subcellular compartments, as well as simultaneously binding multiple other proteins to create large and integrated signaling complexes. In this way, AKAPs tightly coordinate and highly localize PKA with its target substrates as well as its effectors, to allow for a tight regulation of PKA-mediated signaling events distributed around the cell. AKAPs provide temporal and spatial regulation and specificity and therefore are essential to mediating a number of cellular effects among many diverse biological processes, one of which is cell migration.

Cell migration plays a crucial role in many aspects of cellular biology and subsequently healthy physiology and misregulation can lead to many diseases, including cancer metastasis. Successful cell migration requires the organization and coordination of many complex processes the least of which are is integrin activation, cytoskeletal rearrangements, and turnover of adhesive structures (Howe, 2011). The cell’s migration machinery must therefore be tightly regulated by signaling proteins which can efficiently convert upstream stimuli into diverse and multiple downstream effects. Additionally, complexity of cell motility requires its regulation be controlled by a multi-faceted enzyme which is able to integrate multiple pathways with migration dynamics. The role of the cAMP-dependent kinase A is perhaps the best example of such a regulator. PKA has been well studied as a regulator of cell migration, exerting both positive and negative effects (Howe, 2004), and has a number of substrates
involved in cytoskeletal organization, adhesion dynamics, and in general cell migration (Howe, 2004). Given the important role of PKA in cell migration, the goal of the enclosed work was to identify potential AKAPs which may be responsible for regulating the pools of PKA activity involved in cell migration.

Work in our lab has demonstrated the enrichment of discrete pockets of PKA activity in the leading edge of migrating cells (Mckenzie et al., 2011; Howe et al., 2002) and this activity is both required for migration and focal adhesion dynamics (McKenzie unpublished data). This data is corroborated by observations that disrupting PKA anchoring inhibits the cell’s ability to form leading edge structures, retain proper focal adhesion dynamics, and migrate effectively. These data underscore the importance of AKAPs in the processes of cell migration and provide molecular insight into important aspects of cell migration. While these and other data established the complex, dynamic, and important role for PKA in cell migration, PKA exerts many other effects within the cellular context. Therefore, there is a need for localizing and specifying PKA activity to sites of cytoskeletal and adhesion dynamics to allow PKA to exert its effects on any number of its adhesion and migration-associated substrates (Howe, 2004). The goal of this body of work focused on identifying migration and adhesion-associated AKAPs. Considering the adhesion and migration machinery largely impinges on cytoskeletal dynamics and integrin activation, we chose to enrich for the proteins involved in these processes with the hope of identifying potentially novel AKAPs.
Dissecting the molecular pathway and regulation of PKA activity within the leading edge remains to be the focus of our lab, one aspect of which is identifying cytoskeletal AKAPs. The question of which AKAPs are responsible for localized leading edge PKA activity during cell migration remains to be answered. This body of work presents a novel method for the identification of potential AKAPs \textit{in vitro}. While there have been reports of large screens to identify novel AKAPs, this work represents the first report of a search for cytoskeletal-specific AKAPs which may be responsible for leading edge PKA activity. Furthermore, this study provides sufficient evidence for further exploration of cytoskeletal proteins not yet identified as AKAPs, and whose contribution to cell migration have yet to be elucidated. Not only did this study propose potentially novel AKAPs, but it also provides evidence for further exploration of known AKAPs, whose contributions to cell migration have yet to be understood.

Several AKAPs have been identified as interacting with the cytoskeleton and adhesive structures. For example, gravin/AKAP12/SSeCKS, ezrin/radixin/moesin family, AKAP-Lbc/AKAP13, and Wiskott-Aldrich syndrome/verpolin protein family members 1 and 2 (WAVE1 and 2). These reports however focus mostly on loss-of-function effects broadly within the cellular context and do not examine the specific effects on PKA signaling with respect to cell migration. Indeed, the functional consequence of disrupting PKA anchoring and have yet to be elucidated. In fact, these studies focus on the phenotypic effect of loss of PKA activity and fail to identify the discrete targets which are effected by the loss both PKA anchoring and activity.
As part of their role, AKAPs are responsible for assembling and integrating large signaling complexes, recruiting multiple binding partners, and anchoring PKA in proximity to its targets (Scott et al., 2013). One hypothesis that arises is that PKA could regulate migration and adhesion through the direct phosphorylation of key proteins involved in these processes, and in fact there have been observations showing direct PKA phosphorylation of adhesion and migratory proteins including but not limited to VASP, α4 integrin, and filamin A (Dopler and Storz, 2013; Yeo et al., 2011; Howe, 2004). However, the effects on these PKA substrates as a result of disrupting the PKA-AKAP interaction have yet to be identified. These observations would provide an important insight into the potential mechanism by which PKA regulates migration through anchoring.

This body of work identified exciting and potential novel cytoskeletal-AKAPs, whose role in PKA anchoring and cell migration has yet to be elucidated. Not only can AKAPs contribute to cell processes through anchoring of PKA, but they can contribute independent of anchoring function. In fact, a number of proteins with a defined cellular role have been subsequently identified as AKAPs (Diviani et al., 2000; Klussman et al., 2011; Alto et al., 2002; Lim et al., 2008). This provides evidence that a number of AKAPs potentially involved in cell migration have yet to be identified. Additionally, this provides ground for further characterizing and delineating their contribution to cell migration, defining the potential molecular targets of PKA, and the effects on those targets as a result of disrupting PKA anchoring.
Much of the work done in examining the role of PKA anchoring with respect to cell migration has been done by global inhibition of anchoring, through knockdown or knockout models. Therefore making it difficult to delineate the contributions of specific AKAPs. A better understanding of these specific contributions could be substantially advanced through selective disruption of PKA-anchoring interaction. Deletion mutants within the dimerization domain of RIIα, Δ2-5, have been extensively used and well characterized (Kinderman et al., 2006). This study provides an elegant platform for further characterization of specific AKAPs and their role in cell migration.

Considering the differential roles for the previously identified cytoskeletal AKAPs, the extensive list of cytoskeletal associated PKA substrates, and the diversity of both type I and II PKA anchoring, it is likely that there are multiple AKAPs which contribute to the regulation of PKA activity during cell migration. Furthermore, it is now clear that multiple AKAPs are even localized to the same subcellular compartment (Colledge and Scott, 1999; Edwards and Scott, 1999). It would be prudent to assume that while there is a potential for multiple, yet distinct AKAPs to play a role, there are likely significant overlapping targets and functions. Additionally, given the differential role of both PKA activation and regulation in cell migration and cell adhesion, it is likely that distinct AKAPs are involved in these processes.

With that in mind, we have identified a number of proteins, which are of particular interest and warrant further investigation. Filamin A was identified both in this screen as well as in prior investigations. As an actin-crosslinker and mechanosensitive protein (Razinia et al., 2012), filamin A is very enticing as a potential
AKAP involved in cytoskeletal dynamics and cell migration. While this was a formal hypothesis, attempts at characterization of the filamin-RIIα interactions were technically challenging. Evidence from both biochemical and in silico data has strongly shown the potential of filamin A as an AKAP, with the exciting identification of an AKAP-consensus site (Hundsrucker et al., 2010) forming the classic amphipathic helix (data not shown). However structure information that was released during the time of this investigation showed this sequence to be involved in substantial protein secondary structure. More specifically, the potential AKAP sequence is deeply embedded in a β-barrel of one of the 24 immunoglobulin domains that comprise filamin A, making the likelihood of forming the canonical helix strongly unlikely. However, this does not exclude the formal possibility of conformational rearrangement of the protein, leading to the sequence releasing from its β-barrel and thus forming a helix to facilitate PKA binding. Given filamins mechano-properties, a hypothesis that arises from these data is that force and tension create strain across filamin A, causing conformational rearrangements, and lead to the exposure of cryptic binding sites. Thus as a function of tension, there is differential binding of proteins to filamin A (Chen et al., 2009; Rognoni et al., 2014; Rognoni et al., 2012). In fact, it was shown through the use of some very elegant biophysical techniques that applying mechanical force across filamin A can expose the integrin binding site on IgFLNa21 (Ruskamo et al., 2012; Pentikainen and Ylanne, 2009). These forces are of physiologically importance since filamin A is subjected to forces from the cytoskeletal network of actin filaments, as well as the extracellular forces conveyed through integrins.
Consistent with the hypothesis that filamin A may be an AKAP, it is plausible to assume that PKA binding could be regulated through a similar mechanism. This would provide a beautiful integration of tension to PKA-mediated signaling events and one, which is tightly controlled. Given the lab’s focus on mechanotransduction of PKA activity at the leading edge of migrating cells, filamin A would be an ideal candidate for contributing in this process.

Although identifying filamin A as a mechanosensitive AKAP involved in cell migration would contribute significantly to the fields of cell migration and mechanobiology, there are other potential proteins which are of similar excitement. Using a largely subjective approach to screen the mass spectrometry data, this study identified a number of proteins of biological relevance and showed disruption with the Ht31 peptide. While this is a good starting point for further investigations, it is not unlikely that a number of proteins were missed using this screen. There have been reports of “noncanonical AKAPs” including pericentrin and α4 integrin whose RIIBD is distinct from the amphipathic helix highly conserved across known AKAPs (Diviani et al., 2000; Lim et al., 2008). Furthermore, these reports have shown that 50uM Ht31 does not disrupt the PKA-AKAP interaction, which provides evidence that our screen could be missing other such noncanonical AKAPs. Additionally, our lab has shown that PKA activity is localized to the leading edge of neuronal cells, a pool of which is resistant to disruption suggesting further evidence of PKA localization through noncanonical AKAP mechanisms (Rivard et al., 2009).
Given the large and likely possibility of both false positives and false negatives resulting in potentially missed proteins, there is obvious need for improvement and optimization of this method. The biotin transfer method was intended as a preliminary screen, the list of identified proteins was not to be directly published, but instead provided primary evidence to warrant further biochemical methods for confirmation. Therefore although there was some optimization prior to the pull-downs, errors in experimental design could be tolerated due to the more stringent nature of the analysis with the dataset. For example, the observed contaminations in our purified RIIα sample were likely subjected to the same coupling to sulfo-SBED, allowing proteins other than RIIα to be capable of biotin transfer. However, the specificity of Ht31 for PKA-RIIα interactions provides an intrinsic way of distinguishing canonical AKAPs from other non-specific proteins containing a biotin group transferred from either RIIα or other contaminants.

The list of candidate proteins contained in this thesis was as previously mentioned, subjectively chosen. The extensive list of proteins identified by mass spectrometry provides evidence for further optimization. The PANTHER gene ontology database provides an excellent way to group proteins by class, and should be expanded upon further as a way of eliminating proteins. An algorithm was developed (Fig. 7) as a way of objectively screening the list of hits for potential candidates. This takes into account the criteria that were used in the subjective screen, and by adding a numerical coefficient, we were able to weight different criteria more heavily than others. While this is a general first attempt at an algorithm, a better algorithm will need
to be developed. Each of the proteins were scanned to determine if any contained the canonical AKAP consensus site. While the presence of a site is included in our algorithm this does not exclude the fact that there may be others AKAPs which bind PKA through an alternative and non-canonical method of binding, which has been seen for other proteins (Howe, Waterman). Using this algorithm and applying it to known AKAPs, we have generated a numerical range of scores which we can use to compare to those scores generated from the list of potential candidate proteins.

Within the list, talin-1 stood out as potentially interesting not only because it had a substantial effect from the Ht31, but also because it was previously identified in the original dataset. An in silico search for the AKAP consensus site (AVLISE)XX(AVLIF)(AVLI)XX(AVLI)(AVLIF)XX(AVLISE) indicated a possible site in the c-terminus, which also showed the formation of an amphipathic helix. Talin is of particular interest because of its involvement in integrin activation and its role as a scaffolding protein in focal adhesions, providing a link from integrins to the actin cytoskeleton (Kanchanawong et al., 2010). Talin-1 was also found in a recent screen for AKAPs (Hundsrucker et al., 2010). Taken together, this provides substantial evidence to characterize the interaction with RIIα using traditional biochemical methods. A number of cytoskeletal and adhesion-associated proteins were identified as having a substantial effect from the Ht31, providing good support of our hypothesis.

Not only did we create a list of potentially uncharacterized and novel AKAPs, other known AKAPs were identified. There is merit in their identification beyond acting solely as a positive control. The role of AKAPs in cell migration has been only
conceptually linked yet the contributions and regulations from specific AKAPs on cell
migration is not yet understood. Therefore, their identification within our mass
spectrometry data provides evidence for further elucidating their role in cell migration.

Ezrin and moesin, members of the ERM family, have been implicated as cytoskeletal-
associated proteins however their role in cell migration has only begun to be explored.
The mechanisms by which ezrin affect migration and adhesion still remains unclear.
Additionally, gravin (AKAP12) has only minimally been described with respect to cell
migration (Akakaura and Gelman, 2012). Gravin has been shown to play diverse
functions from cytoskeletal rearrangements (Gelman, 2010) to direct involvement with
the beta2 adrenergic receptor however whether or not these functions are regulated by
PKA has yet to be described.

Of the known cytoskeletal AKAPs, AKAP-Lbc (AKAP13) has perhaps been
best understood with respect to cell migration. Recently, AKAP-Lbc was found to be
responsible for playing a substantial role in formation of PKA activity gradients
(O’Connor et al., 2012). Additionally, AKAP-Lbc was identified as a RhoA regulator,
specifically as a guanine nucleotide exchange factor (Diviani et al., 2006). While these
observations begin to delineate the role of AKAP-Lbc in migration, they focus on this
role with respect to the guanine exchange factor function of AKAP-Lbc instead of its
function as a PKA anchoring protein. WAVE1 was one of the first cytoskeletal
associated AKAPs and functions as a scaffolding protein to couple Rho GTPases to the
Arp2/3 complex, regulating actin polymerization (Diviani et al., 2006). WAVE1 has
been found to play a critical role in cell migration through actin reorganization.
However, similar to ezrin and gravin, the contribution to cell migration through PKA anchoring has yet to be defined. Taken together, this establishes the need to define cytoskeletal AKAPs responsible for regulating PKA activity and contributing to cell migration.

While this study was successful in identifying potential cytoskeletal AKAPs, there are a number of experiments needed to confirm and characterize any of the candidates as a bonafide AKAP. As mentioned earlier, there are in silico approaches that although they have their limitations, can provide both a quick and inexpensive way to search for AKAP consensus sites. Traditional biochemical approaches should be taken which can serve to confirm the binding to RIIα. Co-immunoprecipitations, when including the proper controls, can be powerful mechanisms to assess binding. Not only can Ht31 and Ht31\(^p\) be used within this context, but evoking the use of the RII deletion mutant (Δ2-5) and other deletion mutants, can help to characterize interactions while mapping particular regions of binding. In vitro binding experiments can allow for the confirmation of protein-protein interactions, eliminating any problematic experimental conditions, and further elucidating whether or not the interaction is direct or indirect. Given the integrity and accessibility of purified proteins, these are relatively inexpensive.

Beyond confirming the interaction, there are a number of additional experiments that would be provide functional significance to any potential candidate. Given the labs interest in PKA’s role in regulating cell migration and adhesion dynamics, another facet to identifying new cytoskeletal AKAPs is to identify the discrete targets of PKA whose
phosphorylation is regulated through AKAP binding. Identifying these PKA substrates can be done through a number of biochemical approaches such as performing phosphoproteomics on samples treated with PKA inhibitors and effectors as well as general immunofluorescence. Cytoskeletal and focal adhesion proteins can be enriched using similar methods as presented in this text. Mutagenesis studies could be used to follow up potential candidates to confirm the precise role of PKA phosphorylation. Considering the canonical role AKAPs play in targeting PKA and organizing signaling complexes, determining the discrete PKA targets effected by disruption of PKA anchoring would be provide important insight into the molecular mechanisms of PKA-mediated cell migration.

After potential cytoskeletal or adhesion-associated AKAPs are identified, and characterized, further experiments would be required to determine a functional role in cell migration. A number of approaches could be taken at this juncture. Given the lab’s expertise with the FRET-based PKA activity reporter, the contribution of identified AKAPs within our cell migration model could be examined in this context. Deletion mutants in candidate proteins would be particularly interesting, given they would provide insight into the effects of specifically disrupting PKA anchoring compared to broad scale anchoring disruptors (Ht31, sAKAP-is, RIAD). Migration assays, such as the lab’s well used ‘donut’ assay and wound healing assays would be additional studies to examine the specific role in cell migration.

A parallel project in the lab in collaboration with Neil Sarkar, Ph.D., is looking at identifying potential PKA phosphorylation sites in adhesion and integrin associated
proteins, using an *in-silico* approach. This approach may provide evidence of groups of proteins which contain a PKA phosphorylation site, clustered around a single protein, either through direct or indirect binding. Given that many known AKAPs are scaffolding proteins, we hope to combine the data generated with Neil, with our candidate protein list and look for any proteins which overlap. Any protein from our candidate list which also interacts with a number of proteins containing PKA phosphorylation sites, provides strong evidence as a potential AKAP. A preliminary screen using Talin-1, identified a number of interacting partners which also contain a known PKA phosphorylation site. This provides strong evidence that talin-1 may in fact be the AKAP responsible for anchoring PKA and placing it within close proximity to its target substrates.

We and others have shown the critical role for PKA in cell migration and adhesion. Our laboratory has additionally established the importance of PKA anchoring for migration however; the specific AKAPs responsible have yet to be identified. AKAPs, as scaffolding proteins, are able to bind both kinases and phosphatases, and an interesting hypothesis is that AKAPs may function as central regulators of the activity of signaling complexes in space and time. Specifically, this dual binding ability could enable AKAPs to regulate the phosphorylation of PKA substrates, providing transient pockets of PKA activity. Identifying the specific cytoskeletal AKAPs involved in leading edge PKA activity, associated PKA substrates, and the functional consequences of disrupting PKA-AKAP interactions is essential to understanding the role of PKA in cell migration and is an ongoing investigation. Given that misregulation of cell
migration often leads to cancer and cancer metastasis, understanding the contributions of the PKA-AKAP interaction can provide insight into a potentially interesting mechanism for the development of targeted therapeutics.


