THE REGULATION OF CELLULAR LOCALIZATION OF BOTH

ACTIVE AND INACTIVE ERK1/2

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DEDICATION

I would like to thank my mother Marybeth Goad without her love and support throughout the many paths I traveled I would not enjoy my current successes. I would also like to thank my step father Eugene Pavlinski who has always offered steady and reliable advice. I would like to thank Melanie Cobb my mentor, for her understanding, support and most of all for her patience. I could not have completed my work without the advice, constructs, and protocols I received from of Yuh-Min Chook. Colleen Vanderbilt, Steve Stippec, Kathy McGlynn , Michael Lawrence, Mustafa Yazicioglu, are fellow members of the Cobb laboratory that provided advice, reagents, and critical assistance to me and this project and I would like to express my gratitude to them as well as all the other members of the Cobb laboratory. The support of my graduate committee and all of the members of the UT Southwestern family have been and continue to be an invaluable resource and a unique academic community in which I take immense pride.

# THE REGULATION OF CELLULAR LOCALIZATION OF BOTH ACTIVE AND INACTIVE ERK1/2

by

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DISSERTATION

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The University of Texas Southwestern Medical Center at Dallas, 2008

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A key question concerning the regulation and activity attributed to the extracellular signal regulated kinase1/2 (ERK1/2) cascade is how the cellular response to different ligands, cellular environment, and other cellular signals is generated. Part of the answer to the question is the ligand and context-specific spatial control of ERK1/2 in the cell. By confining activity of ERK1/2 to localized regions, cells modulate signaling output. To gain insights into the spatial control of ERK1/2 I examined nuclear import of ERK2 mutants, the interactions of ERK2 with karyopherins β1 and β2, and explored the roles of cytoskeletal elements, motor proteins and scaffolding complexes in the localization of ERK1/2.

Mutation or dysregulation of the ERK1/2 signaling cascade has been identified in a host of diseases from cancer to type II diabetes (Hill *et al.*, 2002). The ERK1/2 proteins have well defined regions involved in protein-protein interactions. We mutated ERK2 in these regions and tested the nuclear import of the mutants. The rationale for this series of experiments was to interfere with known docking motifs on ERK2 to determine residues essential for nuclear translocation. By varying the import conditions it was possible to identify ERK2 residues that impacted nuclear import in different contexts, in particular upon activation by phosphorylation and in the presence of energy. Mutation of certain residues only affected import of phosphorylated ERK2 in the presence of energy. From these data we hypothesize there are at least two mechanisms for nuclear entry of phosphorylated ERK1/2.

Since our mutational analysis of ERK2 demonstrated an energy-dependent means of ERK2 nuclear import, we examined the potential roles of karyopherins in this process. Solution binding assays showed binding of both active and inactive ERK2 to the karyopherins β1 and β2. Both forms of ERK2 were released from the karyopherins in the presence of RanGTP. Based on these and other studies, we suggest that multiple karyopherins are involved in the energy-dependent transport of active ERK1/2.

In addition to their interactions with upstream activators and downstream ligands, ERK1/2 interact with scaffolds and other regulatory proteins, such as kinase suppressor of Ras 1 (KSR1) and phosphoprotein enriched in astrocytes of 15 kDa (PEA15), a protein overexpressed in type II diabetes. In this study I mutated two serine residues on PEA15. Phosphorylation of these residues is known to affect ERK1/2 binding and interaction.

Knock down of PEA15 by RNAi (RNA interference) caused an increase in cellular motility.

Using a variety of cell types and immunofluorescence microscopy I was able to show the heterogeneity of the endogenous total ERK1/2 pools and the active ERK1/2 pools within the cell. Additionally, I showed distinct control of nuclear localization of endogenous ERK1/2 following treatment of cells with microtubule and actin filament destabilizing drugs.

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2D-PAGE – two dimensional polyacrylamide gel electrophoresis

Arp1 – actin related protein 1

ATTC – American type culture collection

ATP – adenosine -5'- triphosphate

Boss – Bride of sevenless

BSA – bovine serum albumin

CamKII – calmodulin-dependent kinase II

CCD – charge-coupled device

CD – common docking motif

Cdc – cell division cycle protein

CNK – connector enhancer of KSR

DAPI – 4',6'-diamidino-2-phenylindole

DED – death effector domain

DIC – differential interference contrast microscopy

DIM7 – *Drosophila* importin 7

DMSO – dimethyl sulfoxide

DTT – dithiothreitol

EGF – epidermal growth factor

EGTA – ethylene glycol tetraacetic acid

ERK – extracellular signal-regulated kinase

FADD – Fas-associated protein with death domain

FBS – fetal bovine serum

GEF – guanine nucleotide exchange factor

GFP – green fluorescent protein

GST – glutathione S-transferase

GDP – guanosine -5'-diphosphate

GTP – guanosine- 5' – triphosphate

HEAT – huntingtin, elongation factor 3, 'A' subunit PP2A, TOR

IPTG – isopropyl-β-D-thiogalactopyranoside

JNK – c-Jun N-terminal kinase (also known as SAPK – stress-activated protein kinase)

KSR1 – kinase suppressor of Ras

KSS1 – kinase suppressor of SST2 (super sensitivity)

LPA – lysophosphotidic acid

MAP2 – microtubule-associated protein

MAPK – mitogen-activated protein kinase

MAP2K – MAPK kinase

MAP3K – MAPK kinase kinase or MAP2K kinase

MBP – myelin basic protein

MEK – MAPK / ERK kinase

MEKK – MEK kinase

MKP – MAP kinase phosphatase

MNK – MAPK interacting kinase

MP1 – MEK partner 1

MSK – mitogen-and stress-activated kinase

NES – nuclear export sequence

NLS – nuclear localization sequence

NPC – nuclear pore complex

NTA – nitrilotriacetic acid

Nup - nucleoporin

p38 – MAP kinase also known as stress-activated protein kinase

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PEA15 – phosphoprotein enriched in astrocytes of 15 kDa (also known as PED)

pERK – phosphorylated ERK

PFA – para-formaldehyde

PKC – protein kinase C

PMA – phorbol 12- myristate 13 acetate

PMSF – phenylmethylsulfonyl fluoride

PP2A – phosphoprotein phosphatase 2A

RanBP – Ran binding protein

RCC1 – regulator of chromosome condensation 1

RIM – Raf interacting motif

RNAi – RNA interference

RSK – ribosomal S6 kinase, also known as p90

RTK – receptor tyrosine kinase

RT-PCR – reverse transcriptase PCR

SDS – Sodium dodecylsulfate

Sev - sevenless

SREBP – sterol response element binding protein

Ste5p – Sterile 5p MAPK scaffolding protein

TBS – tris-buffered saline

TBST – tris-buffered saline plus Tween 20

TNFα – tumor necrosis factor α

TNFR1 – TNF receptor

TOR – Target of rapamycin

Tpr – Translocated promoter region

WGA – wheat germ agglutinin

# CHAPTER ONE

# DISSERTATION OVERVIEW

## External signals and the cellular response

### *External signals*

Eukaryotic cells are intricate biological machines that sense and respond to cues from their external environment. External signals may consist of molecules generated by the autocrine, paracrine, or endocrine systems. Additionally cellular receptors respond to environmental conditions such as pH, nutrients, foreign bodies or other cells. Cellular responses to external stimuli may include changes in motility, proliferation, metabolism, or may cause initiation of apoptotic programs.

### *Mitogen-activated protein kinases (MAPKs)*

Many types of signaling cascades are used by the cell to move information carried by the signaling molecule on the cell surface to the targets within the cell. The MAPKs are a family of enzymes that lie in pathways consisting of a cascade of protein kinases and their regulatory proteins, which transduce signals generated at the cell membrane to target proteins throughout the cell. The classic MAPK cascade consists of at least three protein kinases acting in series known as the MAP3K (MAPK kinase kinase), MAP2K (MAPK kinase), and the MAPK. Receptors interact with one or several molecules that lead to activation of the MAP3K. The MAP3K in turn phosphorylates the MAP2K. The activated MAP2K activates the MAPK by phosphorylation. Finally, the active MAPK phosphorylates its target molecules. The phosphorylation of MAPK

targets results in the regulation or activation of enzymes or other proteins involved in a wide variety of cellular processes.

*The extracellular signal regulated kinase (ERK) cascade*

ERK1/2 are members of the MAPK family of proteins and are known to interact with a number of scaffolds, ligands, and cytoskeletal elements. Using a variety of cell types and immunofluorescence microscopy I was able to show the heterogeneity of the endogenous ERK1/2 pools within the cell. I also visualized activation and distinct control of nuclear localization of endogenous ERK1/2 following treatment of cells with microtubule destabilizing drugs. This pool of ERK1/2 may dissociate from proteins associated with the cytoskeleton that serve to regulate the activation and function of ERK1/2.

*Phosphoprotein enriched in astrocytes of 15 kDa (PEA15)*

PEA15 is a multifunctional protein that acts as an ERK1/2 modulator and scaffolding protein. It has been shown that PEA15 exerts regulatory control by sequestering a pool of ERK1/2 in the cytoplasm. By anchoring ERK1/2 in the cytoplasm PEA15 acts to limit the availability of ERK1/2 to phosphorylate transcription factors and other nuclear proteins. My project focused on phosphorylation of PEA15 which may regulate ERK1/2 binding and on phenotypes in cells in which PEA15 has been knocked down.

*Energy and nuclear import*

We previously reported that ERK1/2 can interact directly with the FxF motifs found in nucleoporins, and that an energy regeneration system was not necessary for the nuclear import of ERK1/2 by this mechanism. Using import reconstitution, we showed that there is also an energy-dependent system of nuclear import of activated ERK2, which is phosphorylated on residues T183 and Y185 (pERK2), that operates independently of the facilitated mechanism used for unphosphorylated ERK2. That led us to consider that either karyopherins or molecular motors may be involved in the selective nuclear uptake of ERK1/2. Both karyopherins and motor proteins require energy to move molecules in the cell. Molecular motors could be involved in transporting distant pools of active ERK1/2 to the nuclear pore complex, thereby increasing the local concentration of ERK1/2 at the pore and effecting nuclear translocation via a concentration gradient. A study that used fluorescence recovery after photo-bleaching in conjunction with expression of green fluorescent protein fused to ERK2 showed an immobilized pool of ERK2 within the nucleoplasm (Costa *et al.*, 2006). Once imported into the nucleoplasm, transcription factors and other proteins may be involved in sequestering active ERK1/2 thereby maintaining a local gradient at the nuclear pore complex. Using binding assays, I showed that ERK2 has the ability to bind at least two karyopherins that could be involved in its active transport. Thus I performed experiments to determine the role of karyopherins and cytoskeletal elements in the energy-dependent nuclear entry of ERK1/2.

# CHAPTER TWO

# MAPKs

## ERK1/2, classic MAPK proteins

ERK1 and ERK2 are members of the MAPK family of protein kinases and act to regulate cellular functions such as proliferation and motility. Mutation or dysregulation of MAPK pathways has been implicated in a diverse group of disease processes including but not limited to cancer, diabetes, polycystic kidney disease, and cardio-facio-cutaneous syndrome (Lawrence *et al.*, 2008b; Rodriguez-Viciana *et al.*, 2006). The MAPKs function as part of dynamic system at the nexus of a myriad of cellular signals and outputs. Due to the number and breadth of cellular processes regulated by MAPK signaling, a key question we raise is how do the MAPKs integrate a constant and diverse set of cellular inputs into defined cellular outputs?

### *MAPKs*

Ray and Sturgill *et al*. searched for hormonally regulated protein kinases that phosphorylated microtubule-associated protein 2 (MAP2). The MAP2 kinase activity, which was stimulated by insulin and other growth factors, also showed activity towards the ribosomal S6 kinase II (RSK) (Anderson *et al.*, 1990; Sturgill *et al.*, 1988) . In 1990 and 1991 Boulton *et al*. purified a MAP2 and RSK kinase and isolated cDNAs encoding the family members ERK1, ERK2, and ERK3, the first evidence for more than one such enzyme (Boulton and Cobb, 1991; Boulton *et al.*, 1991a; Boulton *et al.*, 1990). The sequences of these family members revealed similarity to a yeast kinase named kinase

suppressor of SST2 (KSS1), leading to the conclusion that these protein kinases, subsequently named MAPKs, exist in all eukaryotes (Courchesne *et al.*, 1989; Elion *et al.*, 1990) . Over the next few years several groups working with yeasts and mammalian cell lines identified other members of the MAPK family (Brewster *et al.*, 1993; Derijard *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Lee *et al.*, 1993; Rouse *et al.*, 1994) Along with the discovery of new MAPK family members came the realization that the MAPK family consists of a diverse set of proteins regulating distinct cellular signaling pathways.

MAPKs are serine / threonine protein kinases present in all eukaryotic organisms from the yeast *Saccharomyces cerevisiae* to metazoan organisms including humans. MAPK family members include ERK1/2, ERK3α, ERK3β, the c-Jun N-terminal kinases / stress activated protein kinases (JNK / SAPK), JNK1, JNK2, and JNK3, p38α, p38β, p38γ, p38δ, ERK5, and ERK7 (Pearson *et al.*, 2001). ERK1 is a 43 kDa protein while ERK 2 is a 41 kDa protein (Raman *et al.*, 2007). Three modules have been extensively studied. In addition to the ERK1/2 module both the JNK and p38 kinase modules have served as model MAPK systems (Lawrence *et al.*, 2008b; Raman *et al.*, 2007; Yoon and Seger, 2006). MAPK modules transduce their signals via a cascade of kinases: MAP3Ks (MAPK kinase kinase) phosphorylate MAP2Ks (MAPK kinase), which in turn activate MAPKs.

In the canonical MAPK signaling pathway, external signals activate cell surface receptors which recruit adaptor and activator molecules that stimulate the MAPK module. Figure 2-1 presents the paradigm of the canonical pathway initiated by receptor tyrosine kinases (RTK). Phosphatases can either act on the members of the MAP kinase module

as regulatory factors by dephosphorylation of the cascade kinases leading to their inactivation, or as antagonists to negative regulators of MAPK cascades (McKay and Morrison, 2007).

MAPK family members share several characteristics, in spite of diverse actions. Table 2-1 illustrates the family of mammalian MAPKs. The MAPKs are activated by phosphorylation of both a threonine and a tyrosine residue that flank a single variable residue. Phosphorylation of these residues causes a conformational change in the MAPK that makes the catalytic site available to MAPK substrates.

ERK1/2 are among the most frequently engaged signaling pathways with over 140 reported substrates (Yoon and Seger, 2006). Some of the well validated targets of the ERK1/2 pathway include RSK, mitogen and stress activated kinase (MSK), and MAPK-interacting kinase (MNK), as well as transcription factors like Elk1, c-Fos, and c-Myc (Raman *et al.*, 2007; Zhang *et al.*, 1994).

*Structure of ERK1/2*

ERK2 consists of two major folding domains defined as the N-terminal domain and the C-terminal domain (Figure 2-2) (Zhang *et al.*, 1994). The active site of ERK2 is formed at the interface of the two domains (Zhang *et al.*, 1994). Genetic analysis of protein kinases twenty years ago led to the identification of 12 conserved sequence elements known as sub-domains that form the catalytic core and stabilize the kinase fold. A region of 32 residues known as the MAP kinase insert is located within the C-terminal domain. This insert is only found in MAPKs, cyclin dependent kinases, and glycogen synthase kinase 3. Lysine 52 is the essential catalytic residue that binds the α and β

phosphoryl groups of ATP. As with other protein kinases mutation of K52 results in an ERK2 mutant with very little kinase activity. ERK1/2 are activated upon phosphorylation, by the MAP2Ks MAPK / ERK kinase 1/2 (MEK1/2), on both T183 and Y185 (residue numbers of rat ERK2) (Anderson *et al.*, 1990). Dephosphorylation of either residue inactivates the kinases (Ahn *et al.*, 1991; Anderson *et al.*, 1990). This nonprocessive phosphorylation of ERK1/2 by MEK leads to an activation threshold, requiring the accumulation of ERK1/2 phosphorylated on tyrosine before phosphorylation of threonine can occur to activate the proteins (Ferrell and Bhatt, 1997; Robbins and Cobb, 1992). Mutation of either T183 or Y185 inactivates ERK2 showing both sites must be phosphorylated for full activation of the molecule. Activation of ERK2 by MEK1/2 causes both global and local changes to the ERK2 conformation (Zhang *et al.*, 1994). ERK2 was the first kinase with both the inactive and active structures solved.

*Tissue expression*

The ERK1/2 proteins are ubiquitously expressed in all tissues. The highest expression levels for ERK1 were found in nerve tissue, intestine and placental tissues while ERK2 showed high expression levels in the forebrain, muscle, thymus and heart tissues (Boulton *et al.*, 1991b). The concentration of ERK1/*2* in cultured mammalian cells has been estimated to be 100-500 nM. Although ERK1/2 share 84% sequence identity, the proteins have discrete functions during development. ERK 1 knockout mice are both viable and fertile, with a minor T-cell defect and increased physical activity in open field tests (Selcher *et al.*, 2001). ERK2 knockout mice produced an early

embryonic lethal phenotype with major defects in placental development (Hatano *et al.*, 2003; Saba-El-Leil *et al.*, 2003).

*Raf and MEK1/2, upstream activators of ERK1/2*

Mark and Rapp cloned v-raf in 1984, using a replication defective retrovirus to identify the transforming gene and named the gene v-Raf (Mark and Rapp, 1984). The non-oncogenic form of the protein in mice is known as c-Raf (Kyriakis *et al.*, 1992). There are three isoforms of the Raf protein known as A-Raf, B-Raf, and c-Raf or Raf-1 (McCubrey *et al.*, 2007). Small G-proteins of the Ras family activate Raf proteins by binding to an N-terminal Ras binding domain and causing conformational changes in Raf that lead to its phosphorylation (Rapp *et al.*, 2006). There are at least thirteen known regulatory phosphorylation sites on c-Raf. Dephosphorylated of S621 causes the 14-3-3 protein, which stabilizes its autoinhibitory conformation, to dissociate from Raf. After release by 14-3-3, Raf is activated by the phosphorylation of S338, S339, Y340 and Y341 (McCubrey *et al.*, 2007). The MAP3Ks Mos and Tpl2 also regulate ERK1/2 in a few well defined cases (Raman *et al.*, 2007).

In the ERK1/2 cascade Raf activates the MAP2Ks MEK1/2 by phosphorylation. MEK1/2 like other MAP2Ks are dual specificity protein kinases with the ability to phosphorylate serine, threonine and tyrosine residues. Ahn *et al*. demonstrated MEK activity in 1991, by combining fractions containing inactive ERK1/2 from unstimulated Swiss 3T3 cells with fractions containing MEK from cells that had been stimulated with growth factor and screening for increased activity toward myelin basic protein (MBP), an ERK1/2 substrate (Ahn *et al.*, 1991; Seger *et al.*, 1991). Subsequently MEK1 was

purified and cDNAs encoding MEK1 and MEK2 were isolated. MEKs share very high sequence identity except for a variable insert near the C-terminus (Lewis *et al.*, 1998). MEK1/2 contain a canonical nuclear export sequence (NES). Overexpression of MEK1 can anchor ERK1/2 in the cytoplasm (Rubinfeld *et al.*, 1999; Torii *et al.*, 2004). It has been suggested that *Mycobacterium leprae,* the pathogen responsible for leprosy, highjacks the ERK pathway by circumnavigating the MEK requirement and recruiting protein kinase Cε (PKCε) and leukocyte-specific protein tyrosine kinase to activate ERK (Noon and Lloyd, 2005; Tapinos and Rambukkana, 2005). Anthrax lethal factor targets the MAPK cascade by cleaving the N-terminus of MEK1/2, thereby eliminating the docking site for ERK1/2 (Vitale *et al.*, 1998). Constitutively active forms of MEK1 can transform cells and cause tumors in either transgenic mouse models or regenerated human skin models, as originally shown by Mansour *et al*. (Mansour *et al.*, 1994).

*Ligands of ERK1/2*

ERK1/2 have been shown to interact with and regulate a surprisingly large number of substrates. ERK1/2 activate several downstream protein kinases, as well as other targets in the cytoplasm, nucleus (Pearson *et al.*, 2001). The protein-protein interactions of ERK1/2 and ligands are mediated by at least two separate docking motifs that bind to regions of the ERK1/2 molecule. A significant portion of ERK1/2 interacts with elements of the cytoskeleton; Reszka *et al*. estimated one-third to half of the total ERK1/2 is associated with the cytoskeleton (Reszka *et al.*, 1995).

The common docking (CD) region is a feature found in the MAPK family, composed of acidic and hydrophobic residues that are on the surface of the MAPK and

exposed to the solvent. In rat ERK2 this region is defined as encompassing residues L311-A325 (Tanoue *et al.*, 2000). Mutation of a key residue, D319 (residue numbers of rat ERK2) within at this region is responsible for the sevenmaker gain-of-function phenotype in *Drosophila melanogaster*. In *Drosophila* the sevenless (sev) gene encodes a RTK required for determination of the cell fate in the eye. Activation of this RTK starts a classic ERK pathway activation cascade progressing through son of sevenless, to Dras1/ Ras, Raf, Dsor1/MEK, and Rolled / ERK. The dominant sevenless mutation was discovered during a genetic screen that selected for mutants that had an activated sev pathway in the absence of bride of sevenless (Boss), the activating ligand for the pathway. Sequence analysis of the mutant fly ERK showed a G to A transition causing the change of aspartic acid 334 to asparagine; this corresponds to the 319 position in rat ERK2 (Brunner *et al.*, 1994). Further study by Bott *et al*. showed this mutation causes reduced interaction with regulatory dual specificity phosphatases (Bott *et al.*, 1994). A yeast two-hybrid screen of ERK2 mutants showed mutation of the D316 and D319 residues caused the loss of interaction with MNK1, MAPK phosphatase 3 (MKP3) and RSK (Robinson *et al.*, 2002a). The same study showed that these mutations had a weak effect on binding of the ERK activating proteins MEK1/2 in spite of the fact that MEK1/2 are known to interact at this site. The CD motif interacts with proteins that carry the D motif which has been described as $(R/K)_{2-3}$-$X_{1-6}$-$\Phi_A$-X-$\Phi_B$. Extensive interaction tests demonstrated the significance of D motif-mediated interactions (Chang *et al.*, 2002; Tanoue *et al.*, 2000). Structural studies of the interaction of peptides derived from MKP3, a hematopoietic tyrosine phosphatase, and ERK2 demonstrated exposure of

the phosphorylation sites in the activation loop to solvent (Liu *et al.*, 2006; Zhou *et al.*, 2006).

Arvind *et al*. reported a novel point mutation within the CD motif of ERK2 in the human squamous cell cancer line HSC6. They found that ERK2 in some of these cells migrated faster than wild type ERK2. Upon examination of this phenomenon, they showed that HSC6 cells contain two distinct alleles of the ERK2 gene, a wild type allele, and an allele producing the protein with the mutation of E322K (E320 in rat ERK2). The authors mutated E322 to K and expressed the protein in HeLa cells. Western analysis of the HeLa cell lysates displayed the same migration patterns for the mutant ERK2 as in HSC6 cells (Arvind *et al.*, 2005). A more detailed discussion of this work appears in chapter 3.

Kornfeld *et al*. found a second interaction motif through a genetic screen in *C. elegans* (Jacobs *et al.*, 1999). Originally found as FxFP, interactions also occur if the sequence lacks the terminal proline. Binding to FxF motifs appears to be greater in ERK1/2 than for other MAPKs (Jacobs *et al.*, 1999). Lee *et al*. concluded the FxF motif binds to a hydrophobic pocket formed between the P+1 site, an α-helix, and the MAPK insert of ERK2 (Lee *et al.*, 2004). Further crystallographic studies suggested how proteins discriminate between binding to ERK1/2 and p38 (Chang *et al.*, 2002; Zhou *et al.*, 2006). Work from our laboratory on this problem is discussed in chapter 3.

*Regulation of EKR1/2 by phosphatases*

ERK1/2 are regulated by a wide variety of protein phosphatases. Interactions with phosphatases may serve multiple regulatory roles. Clearly interactions with

phosphatases can control the temporal activation of ERK1/2. However, these interactions may also play a role in the spatial control of ERK. The protein-tyrosine phosphatase PTP-SL contains a D motif and is known to sequester ERK in the cytoplasm, thereby fulfilling the dual roles of spatial and temporal control (Karlsson *et al.*, 2006). Dual specificity MKPs provide a major source of regulatory control of MAP kinase cascades in mammalian cells. These phosphatases can also provide negative feedback to the MAPK cascade. MKP3 a major regulator of cytoplasmic ERK is activated by ERK but not by the gain-of-function mutant sevenmaker (Camps *et al.*, 1998).

*Scaffolds*

Scaffolding proteins control and are controlled by the ERK1/2 cascade. These proteins can act to sequester signaling components in specific cellular compartments, increase the local concentration of signaling components, prevent interaction with other molecules that would interfere with signaling, and regulate the activation and efficiency of signaling proteins. The yeast protein Sterile-5p (Ste5p) was the first MAPK scaffold identified (Raman *et al.*, 2007). KSR, connector enhancer of KSR (CNK), MEK partner 1 (MP1), and paxillin are some of the scaffolding proteins known to regulate the ERK1/2 pathway (Cacace *et al.*, 1999; Claperon and Therrien, 2007; Raman *et al.*, 2007; Therrien *et al.*, 1996).

The KSR proteins were identified in genetic screens using *Drosophila* and *C. elegans*. Two mammalian genes encode KSR1 and KSR2, although the majority of work has been done on KSR1. The more widely expressed form KSR1 is known to associate with elements of the MAPK cascade and other regulatory proteins such as 14-3-3, cell

division cycle (cdc) 37, and heat shock protein 90 (Cacace *et al.*, 1999; Claperon and Therrien, 2007).

CNK was isolated during a genetic modifier screen for proteins that enhanced an eye phenotype caused by expression of the kinase domain of KSR in *Drosophila*. There are three genes that encode CNK proteins in mammals known as CNK1, CNK2/MAGUIN and CNK3/PIP-3E. CNKs 1 and 2 have been implicated in ERK1/2 signaling. CNK associates with the kinase domain of Raf through a binding motif known as the Raf-interacting motif (RIM). Adjacent to the RIM, CNK contains a regulatory sequence that inhibits Raf activation in the absence of RTK signaling (Douziech *et al.*, 2003; Laberge *et al.*, 2005).

Paxillin is a 68 kDa protein highly expressed in smooth muscle tissues and first purified from chicken gizzard (Turner *et al.*, 1990). Paxillin localizes to either focal adhesions or the analogous dense plaques of muscle tissue (Turner, 1994). In addition to its association with the ERK1/2 signaling cascade, paxillin interacts with other kinases such as focal adhesion kinase and proteins associated with the cytoskeleton. Like many scaffolds paxillin is itself a phosphoprotein with its association to focal adhesions regulated by its phosphorylation (Ishibe *et al.*, 2004; Salasznyk *et al.*, 2007; Turner, 1998). Paxillin is required for activation of ERK by Mos in *Xenopus* oocytes (Rasar *et al.*, 2006).

*Localization / Nuclear translocation*

It is widely accepted that cellular localization and compartmentalization is a key element in the regulation of the ERK1/2 signaling cascade. Using immunofluorescence

microscopic techniques, nuclear localization is perhaps the most dramatic and easily seen change in cellular morphology. Unfortunately, while it is easy to score a nucleus for the relative amount of ERK it contains, determining the mechanisms of transport and nuclear sequestration have not been trivial pursuits. Many factors including the phosphorylation state, activity, dimerization, and the direct interaction of ERK1/2 with nucleoporins have been cited as variables in the nuclear translocation problem. However, it appears that much like ERK1/2, with its dynamic and variable input and output regime, nuclear translocation of ERK1/2 is not the function of a linear transport cascade, but a dynamic amalgamation of transport modalities.

The canonical view of nuclear transport for proteins larger than 30 kDa is that they interact with a karyopherin-mediated transport complex driven by the gradient of RanGDP/GTP at the nuclear pore. Karyopherin βs are flexible molecules that consist of HEAT repeats, a motif named for the proteins in which it was initially found: **h**untingtin, **e**longation factor3, '**A**' subunit of protein phosphatase 2A, and **t**arget of rapamycin (TOR). When college textbooks describe nuclear transport, the Karyopherin $\alpha/\beta_1$ model is cited. This model is based on the substrate to be transported interacting via either a mono or bipartite nuclear localization sequence (NLS), made of positively charged residues, with karyopherin α. The association with its substrate causes a conformational change of the auto-inhibitory domain of karyopherin α to the karyopherin β interaction site / importin β binding domain. A complex of substrate, karyopherin β1, and karyopherin α is formed. This complex then interacts with nucleoporins through their numerous FxF motifs to transit the nuclear pore complex. Upon arrival in the nucleoplasm RanGDP interacts with a Ran guanine exchanger factor to exchange GDP

for GTP. In the presence of RanGTP, the transport complex falls apart with recycling of the karyopherins through the nuclear pore and delivery of their cargo. However, this is a simplified view of a system that we are just beginning to understand. In fact, the NLS for most nuclear proteins is not known. It has been shown in karyopherin β1 that some NLSs are structural and not sequence motifs. Karyopherin β1 binds to a dimer of sterol response element binding protein (SREBP) by a structural NLS formed upon homodimerization of SREBP (Lee *et al.*, 2003). Since the nuclear transport mechanism for most nuclear proteins is not known, there are sure to be more sequence and structurally based NLSs and NESs mechanisms discovered in the future.

*Drosophila* utilize the karyopherin D-Importin-7 (DIM7 / RanBP7) to transport activate ERK into cell nuclei (Lorenzen *et al.*, 2001). Mutations in moleskin, the gene that encodes DIM7, enhanced hypomorphic D-ERK phenotypes while suppressing hypermorphic phenotypes. The same study implicated the importin β homolog Ketel as being involved in transport of active D-ERK. However, these proteins had not been shown to be responsible for the transport of any ERKs in metazoans until a recent paper by Chuderland *et al*. The Seger group has found a new putative NLS for signaling proteins based on the phosphorylation of an SPS motif found within the kinase domain of ERK1/2, SMAD3, and MEK1. Phosphorylation of either of the serine residues in the SPS motif causes importin 7 mediated nuclear translocation of these proteins. They also showed that fusion of a 19 amino acid stretch containing the SPS motif was sufficient to cause nuclear translocation of non-diffusible proteins (Chuderland *et al.*, 2008).

The nuclear transport of ERK1/2 appears to rely on both active and passive means of nuclear entry. In 1998 Khokhlatchev *et al*. described the nuclear accumulation

of ERK2 showing that it depends on its phosphorylation state and not its activity. Additionally they found that ERK2 mutants deficient in their ability to form either hetero- or homodimers reduced the ability of ERK to be retained in the nucleus (Khokhlatchev *et al.*, 1998). In 1999 Adachi *et al*. described transport of monomeric ERK as driven by passive diffusion with dimeric forms of ERK transported via an active mechanism (Adachi *et al.*, 1999). Evidence of nuclear import of ERK1/2 without the requirement for karyopherin chaperones comes from work in our laboratory as well as the Nishida group. We showed that import of a green fluorescent protein (GFP) fusion ERK2 protein was blocked by the addition of wheat germ agglutinin (WGA), which has a high binding affinity for N-acetyl glucosamine moieties attached to the nucleoporins (Whitehurst *et al.*, 2002). Nishida's group obtained similar results and showed direct binding of *Xenopus* ERK2 to Nucleoporin 214 (Nup 214) while our lab demonstrated direct binding of rat ERK2 to Nup 153c (Whitehurst *et al.*, 2002). These data taken together with data showing competition between ERK2 and karyopherin β1 in import reconstitution assays provided evidence of a nuclear import mechanism that operates for both the active and inactive forms of ERK2 without the need for energy.

**PEA 15**

*History*

PEA15 is highly expressed in astrocytes and hippocampal neurons (Sharif *et al.*, 2004). It was originally identified by Araujo *et al.* in mouse brain astrocytes (Araujo *et al.*, 1993). Five years later Condorelli *et al*. used differential display to isolate PEA-15 in a screen for genes involved in type II diabetes. As a result of their studies they suggested

the name be changed to PED-15 for phosphoprotein expressed in diabetes (Condorelli *et al.*, 1998). Later that same year PEA-15 was also cloned using expression cloning to identify proteins that block the H-Ras mediated suppression of integrin activation (Ramos *et al.*, 1998). Although it is named for its expression in astrocytes, PEA15 is widely expressed in tissues as diverse as brain, breast, pancreatic β cells, lung and prostate (Araujo *et al.*, 1993; Danziger *et al.*, 1995; Estelles *et al.*, 1996).

*Structure and Function*

PEA15 is a 130 residue acidic protein consisting of an N-terminal death effector domain (DED) and a relatively unstructured C-terminal tail. It is a highly conserved protein with the amino acid sequence in mouse, rat, hamster, and human 100% identical (Krueger *et al.*, 2005). Within the DED PEA15 contains a strong NES. Figure 2-3 shows both a schematic diagram and a ribbon model of PEA15 based on the nuclear magnetic resonance data of Hill *et al* (Hill *et al.*, 2002). Two serine residues in the C-terminal tail can be phosphorylated as a function of intracellular calcium concentrations. Because differential phosphorylation of PEA15 regulates its function, PEA15s are classified by their phosphorylation state, with the designations N, Pa, and Pb representing unphosphorylated, monophosphate and diphosphate forms (Renault *et al.*, 2003). Changes in intracellular $Ca^{2+}$ concentration affect the phosphorylation states of S104 and S116 through the actions of PKC and calmodulin-dependent protein kinase II (CamKII). Evidence also points to phosphorylation of S116 by the growth factor regulated Akt kinase (Araujo *et al.*, 1993; Kubes *et al.*, 1998). Callaway *et al*. determined that residues in both the DED and the C terminus of PEA15 are important for binding to ERK1/2 as

show in figure 2-3 panel B. My work regarding differential phosphorylation of PEA15 and its effect on ERK1/2 function is presented in chapter 5.

PEA15 is a multifunctional protein controlling both response to apoptotic stimuli and regulating cell proliferation. During reactive gliosis, astrocytes are exposed to high levels of tumor necrosis factor α (TNFα), a condition that leads to apoptosis in most cells expressing TNF receptor 1 (TNFR1) or Fas. However, due to their expression of PEA15, astrocytes remain resistant to apoptosis under these extreme conditions (Renault *et al.*, 2003). It is hypothesized that the PEA15 DED associates with DEDs of pro-apoptotic molecules such as Fas-associated protein with death domain (FADD) and caspase 8, thereby interfering with the assembly of the death-induced signaling complex (Boldin *et al.*, 1996; Muzio *et al.*, 1996). In astrocytes cultured from PEA15 null mice, cells undergo apoptosis when exposed to TNFα, but are resistant to apoptotic stimuli upon the reintroduction of PEA15 through transfection (Kitsberg *et al.*, 1999).

In addition to its function as an anti-apoptotic regulatory protein, PEA15 directly binds to and regulates ERK1/2. It has been reported that binding to PEA15 does not interfere with either the activation or activity of ERK1/2, although PEA15 serves to sequester ERK1/2 in the cytoplasm (Formstecher *et al.*, 2001; Whitehurst *et al.*, 2004). However *in vitro* assays showed PEA15 inhibited activation of ERK2 by MEK1 (Whitehurst *et al.*, 2004). Kruger *et al.* presented a model of PEA15 phosphorylation in which phosphorylation of serine 104 or serine 116 acts as a switch committing PEA15 to anti-apoptotic activities or binding and sequestration of ERK in the cytoplasm in regulating cellular proliferation and inhibiting integrin activation (Krueger *et al.*, 2005).

There is evidence that PEA15 is involved in the regulation of glucose uptake. Valentino *et al*. found PEA15 expression to be two-fold higher in euglycemic first degree relatives of type II diabetics when compared to a euglycemic control group with no relation to individuals with insulin resistance (Valentino *et al.*, 2006). A genetic study of type II diabetes among Pima Indians found a link between the 1q21-22 locus and diabetes (Wolford *et al.*, 2000). This locus includes the gene for PEA15. Overexpression of PEA15 in transgenic mice inhibits transport of glucose (Condorelli *et al.*, 1998). Studies by Vigliotta *et al*. and Condorelli *et al*. suggest that overexpression of PEA15 may contribute to insulin resistance in type II diabetes (Condorelli *et al.*, 2001; Vigliotta *et al.*, 2004). Transgenic mice overexpressing PEA15 in pancreatic β-cells had reduced mRNA levels of Sur1 / Kir6.2 potassium channel components in islets cells as well impaired insulin secretion response to hyperglycemia (Miele *et al.*, 2007). Expression of PEA15 in pancreatic β-cells may affect cell survival by inhibiting apoptotic pathways, and influence the expression of genes involved in the glucose response due to regulation of ERK1/2 localization. These studies are intriguing and point to the need to examine the mechanisms of PEA15 and ERK1/2 interaction in pancreatic β cells. My work on this problem is discussed in chapter 5.

| MAPK | Aliases | References |
|---|---|---|
| ERK1 | p44, MAPK | (Boulton *et al.*, 1990) |
| ERK2 | p42, MAPK | (Boulton *et al.*, 1991b) |
| ERK3α | p63, rat ERK3 | (Boulton *et al.*, 1991b) |
| ERK3β | Human ERK3 | (Gonzalez *et al.*, 1992) |
| ERK1b | ERK4 | (Yung *et al.*, 2000) |
| JNK1 | SAPKγ | (Derijard *et al.*, 1994; Gupta *et al.*, 1996; Kyriakis *et al.*, 1994) |
| JNK2 | SAPKα | (Derijard *et al.*, 1994; Gupta *et al.*, 1996; Kyriakis *et al.*, 1994) |
| JNK3 | SAPKβ | (Derijard *et al.*, 1994; Gupta *et al.*, 1996; Kyriakis *et al.*, 1994) |
| p38α | p38, CSBP, SAPK2 | (Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994) |
| p38β | p38-2 | (Jiang *et al.*, 1996; Jiang *et al.*, 1997; Stein *et al.*, 1997) |
| p38β2 | | (Kumar *et al.*, 1997) |
| p38γ | ERK6, SAPK3 | (Lechner *et al.*, 1996; Li *et al.*, 1996) |
| p38δ | SAPK4 | (Goedert *et al.*, 1997; Jiang *et al.*, 1997; Kumar *et al.*, 1997) |
| Mxi | | (Zervos *et al.*, 1995) |
| ERK5 | | (Lee *et al.*, 1995; Zhou *et al.*, 1995) |
| ERK7 | | (Abe *et al.*, 1999) |
| NLK | Nemo-like kinase | (Brott *et al.*, 1998) |
| MAK | Male germ cell associated kinase | (Matsushime *et al.*, 1990) |
| MRK | MAK-related kinase | (Abe *et al.*, 1995) |
| MOK | | (Miyata *et al.*, 1999) |
| KKIALRE | | (Meyerson *et al.*, 1992) |
| KKIAMRE | | (Taglienti *et al.*, 1996) |

**Table 2-1**

The mammalian MAPKs, their aliases and references

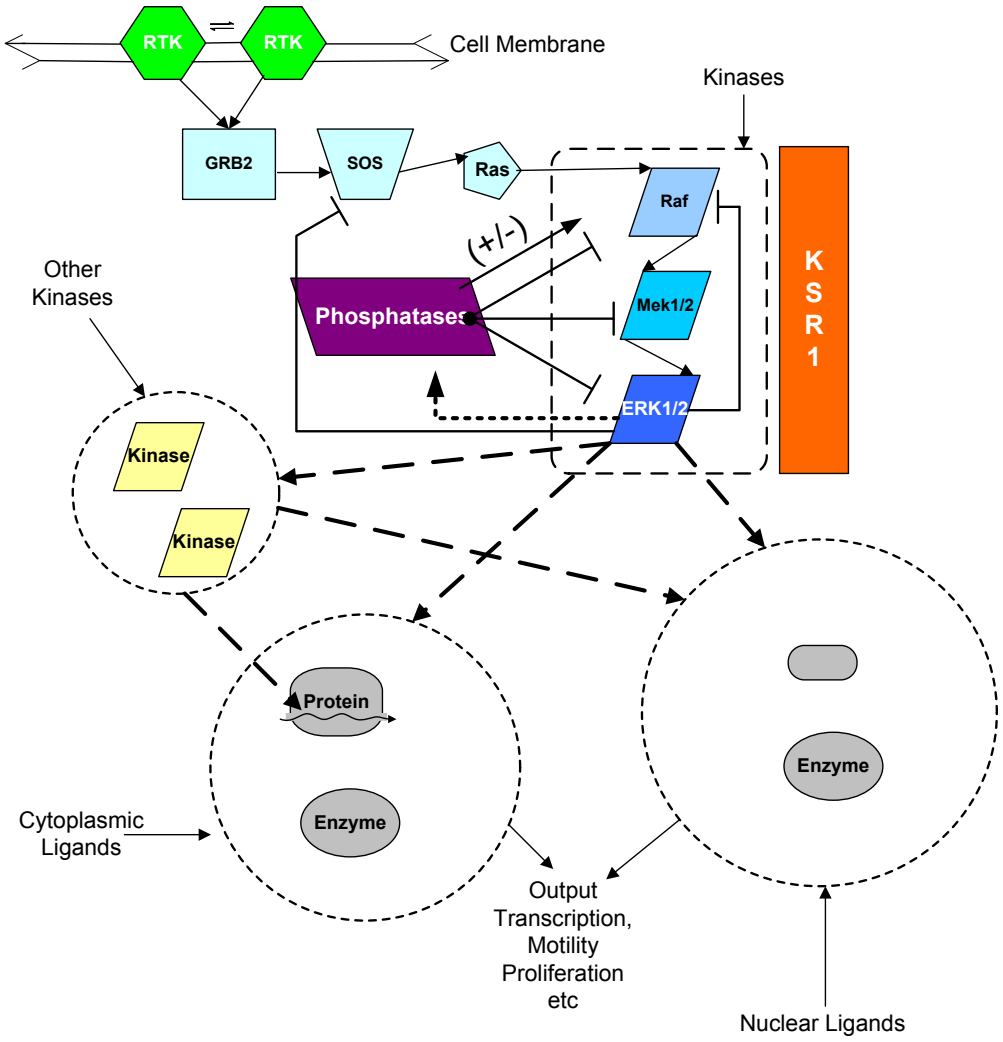



**Figure 2–1 Canonical MAPK Pathway**

This panel illustrates a reductionist view of activation of RTKs and the transduction of the signal from the RTK to the final cellular output acting through the ERK1/2 MAPK module. (Goad unpublished)

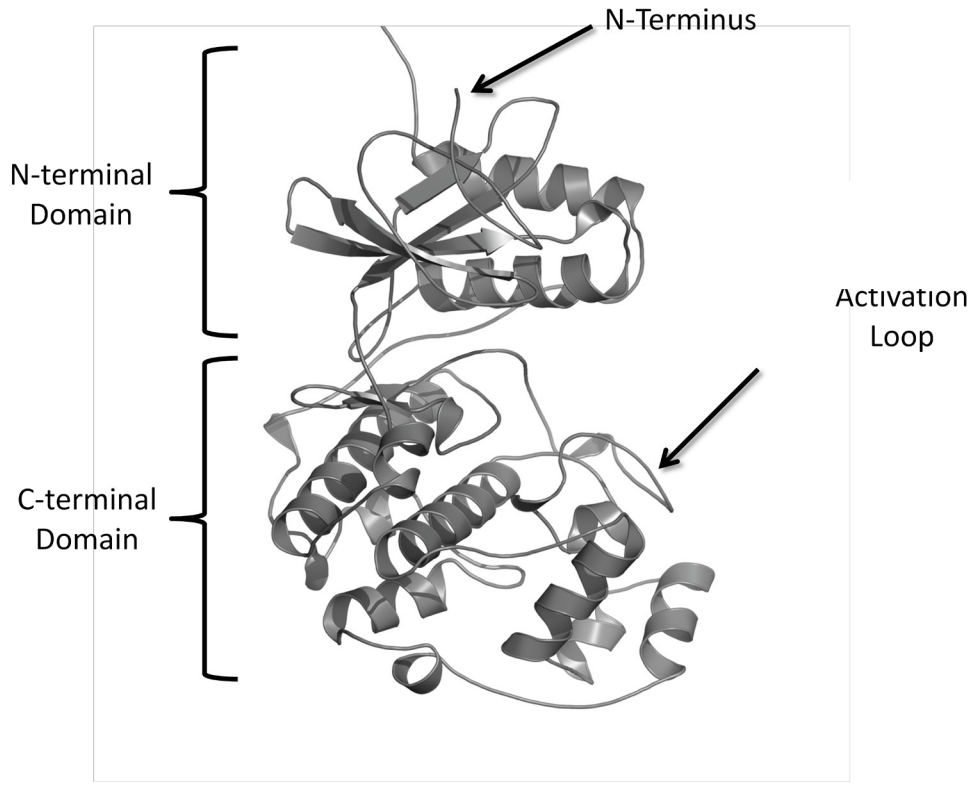
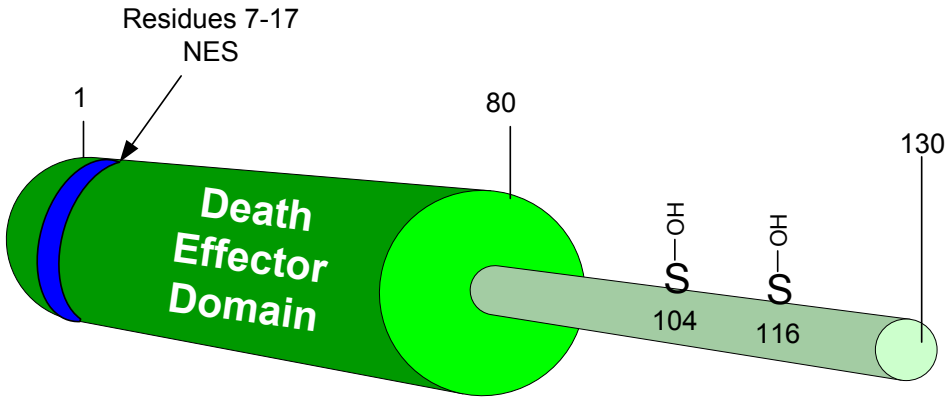



**Figure 2-2**

Major structural elements of inactive ERK2 based on the RCSB 1ERK file. (Goad unpublished)

A


Residues 7-17
NES
1
80
130
Death
Effector
Domain
HO
S
104
HO
S
116

B

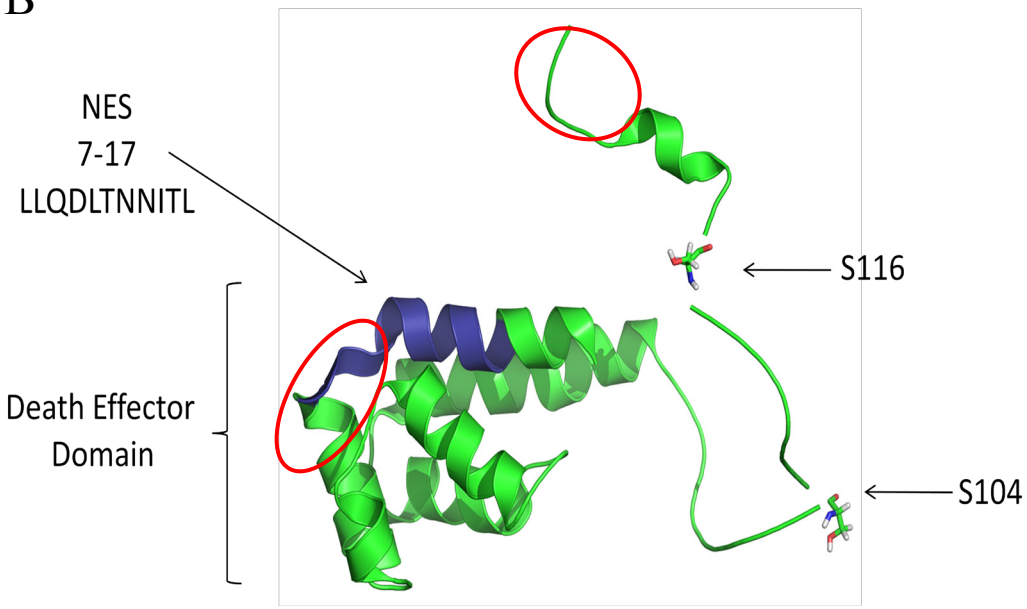

NES
7-17
LLQDLTNNITL
Death Effector
Domain
S116
S104

**Figure 2-3 Structural Elements of PEA-15**

Panel A shows a schematic diagram of PEA15. The NES is colored blue. Panel B shows a ribbon diagram of PEA 15based on the NMR data for PEA-15 (RCSB 1N3K). The NES is colored blue, and red ovals indicate regions important for ERK1/2 binding. Callaway *et al*. described residues N14, T16, E18, C27, and D74 within the DED as residues involved in ERK interaction (Callaway *et al.*, 2005). They also determined that residues L121, L123, K128, and K129 form a 'reverse' D motif at the C-terminus. (Goad unpublished)

## CHAPTER THREE

### Mutational analysis of ERK2 import


**Abstract**

A central question in our laboratory is how the MAPK cascade integrates multiple inputs into distinct outputs. Many proteins interact with MAPKs to regulate them temporally and spatially. Nuclear translocation is a major aspect of the spatial regulation of ERK1/2. Previous work has suggested multiple import mechanisms are utilized for the nuclear translocation of ERK1/2.

We made the assumption that protein-protein interactions between ERK1/2 and other proteins would be essential for its nuclear translocation. Mutation of residues in ERK1/2 known to be important for protein docking should cause changes in ERK1/2 nuclear translocation. Our strategy was to mutate residues identified during structural analysis of ERK1/2 as possible docking interactors. We also examined mutations that affected the activity of ERK1/2 as well as its ability to dimerize.

During our survey of ERK2 mutants we identified three modes of transport: 1. ERK1/2 interact directly with nucleoporins without the need for energy. 2. Nuclear import of some mutants is dependent on the phosphorylation state of ERK2. 3. Nuclear import of phosphorylated ERK2 is affected by the presence of energy. These observations led us to suggest karyopherins and motor proteins are involved in the transport of active ERK1/2.


Arvind *et al*. described a novel mutation in the common docking region of human ERK2 (Arvind *et al.*, 2005). I recapitulated the mutation in rat ERK2 and further

characterized it in transport reconstitution assays and biochemically. I found the mutation affected the basal activity of ERK2 but had no effect on its nuclear translocation.

In this chapter I will discuss our mutational screen and our findings, discuss ERK2-E320K a mutation that leads to an increase in basal ERK2 activity, and suggest three models of nuclear transport of ERK1/2.

**Introduction**

The localization and transport of both the active and inactive forms of ERK1/2 is a primary means of cellular control. Aspects of the cellular response to external stimuli are often governed by the translocation of ERK1/2 through the nuclear pore. The mechanisms governing the transport of ERK1/2 across the nuclear pore are poorly understood. ERK2 can transit the nuclear pore in an energy-independent process involving direct interactions with nucleoporins. From published studies it appears that the import of ERK2 is complex and involves multiple import mechanisms.

We hypothesized that residues within docking regions of ERK1/2 are involved in protein-protein interactions that affect the ability of ERK1/2 to transit the nuclear pore complex. We mutated residues within known docking regions and screened recombinant proteins in import reconstitution assays. The import reconstitution assays allowed control of the concentration and presence of transport factors and energy while evaluating nuclear translocation. We expected to identify residues and regions required for nuclear import of ERK1/2.

Two regions of ERK1/2 have been implicated in docking of ligands to ERK1/2. The CD region consisting of residues L311-A325 in rat ERK1/2 interacts with basic hydrophobic motifs known as D motifs. Mutation of an aspartic acid residue in the CD region of ERK is responsible for the gain-of-function sevenmaker phenotype in *Drosophila*. The second docking region in ERK1/2 consists of residues in the αG -helix and the MAPK insert. The MAPK insert consists of the α1L14 and α2L14 helices of ERK2 which encompass residues 241-272 (refer to figure 3-7). Based on the structures of phosphorylated and unphosphorylated ERK2, we selected residues for mutation that

appeared to be involved in docking. We included the previously described ERK2-K52R kinase-deficient mutant and $L_4$A/H176E dimerization-deficient mutant in our survey as controls.

Our laboratory has probed these mechanisms using import reconstitution assays, and immunofluorescence microscopy, using recombinant ERK2 proteins mutated in key residues involved in protein-protein interactions. We found that ERK2 molecules carrying mutations in or near the activation loop or regions that interact with the D motif had no significant impairment in their ability to transit the nuclear pore. However, several mutants that were defective in FxF motif binding were significantly impaired in nuclear import in our import reconstitution assays. Our immunofluorescence microscopy was not sensitive enough to detect these subtle changes in ERK2 import in any but the most severely affected mutant.

Mutant ERK2s were also characterized biochemically by kinase assay and binding competition assays with nucleoporin 153. During the biochemical examination of mutants we found that mutation of a residue in the CD region of ERK2 significantly changes the basal kinase activity of ERK2 without any impact on nuclear localization. Our results indicate that there are multiple nuclear import systems for ERK2.

**Experimental Methods**

*Plasmid constructs and expression of recombinant proteins*

The parental plasmids were: NPT7-5-$His_6$, NPT7-5-$His_6$-GFP, and pCMV5-myc containing wild type ERK2. Polymerase chain reaction (PCR) mutagenesis was used to create the majority of ERK2 mutants. The ERK2-E320K was made using two complementary DNA oligomers. The forward oligomer is gacccaagtgataagcccattgctgaag, and the reverse complement is cttcagcaatgggcttatcacttgggtc. I used NPT7-5-$His_6$-ERK2 vector as the template and performed PCR using the DNA polymerase pfu from *Pyrococcus furiosus*. After 19 cycles of PCR the product was digested with DpnI for three hours. *E. coli* strain DH10-α was transformed with the PCR product. The transformed *E. coli* were plated on LB agar plates containing 100 μg/ml of carbenicillin. After 24 hours at 37°C, viable colonies were selected and small scale preparations of the DNA were made. DNA was digested with NcoI and XbaI and analyzed by agarose electrophoresis using a 1% gel. DNA that produced the appropriate bands was selected for sequencing. The sequence files were analyzed to ensure that the DNA carried the desired mutation and that it did not carry any other mutations that affected coding. After verification, larger stocks of DNA were produced.

The BL-21 strain of *E. coli* was transformed using mutant DNA and grown on LB-carbenicillin plates for 24 hours. A colony was selected and grown in LB containing 100 μg/ml of ampicillin and allowed to grow overnight at 37°C. The next day 10 ml of the *E. coli* culture was added to 1 L of LB-ampicillin medium. The bacteria were grown to an $OD_{600}$ of 0.4-0.8 before the allolactose mimetic isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 400 μM then the

culture was incubated at 30°C overnight. The pellet was harvested by centrifugation, and resuspended in 50 mM $NaPO_4$ / 0.3 M NaCl, pH 8.0, buffer containing 20 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine (lysis buffer). Bacteria were incubated with lysozyme before being mechanically sheared using a Polytron homogenizer. Membranes were further disrupted by 4 rounds of sonication. The lysed cells were centrifuged at 70,000g for 45 minutes to separate the soluble proteins from the insoluble material. The supernatant was incubated $Ni^{2+}$- nitrilotriacetic acid (NTA)-agarose resin beads at 4°C overnight. The beads were washed in 90 volumes of lysis buffer and loaded in a column. The beads were washed with 100 volumes of 20 mM imidazole. The protein was eluted in 40 drop fractions using a gradient of 20 mM to 250 mM imidazole. A sample of every $3^{rd}$ fraction was analyzed on a 12% polyacrylamide gel in sodium dodecyl-sulfate (SDS). The gel was stained with Coomassie blue. Fractions containing ERK2 were pooled. The protein was dialyzed overnight in 20 mM Tris, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 10 mM benzamidine, and 10% glycerol pH 7.5. After dialysis the protein was snap frozen in liquid nitrogen and stored at -80°C.

*Cell Culture*

HeLa, human embryonic kidney 293, and human foreskin fibroblasts immortalized with h-tert (BJ cells) (Ramirez *et al.*, 2004) were grown on glass coverslips in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. The cells were incubated at 37°C in a 10% $CO_2$ atmosphere. In some experiments the medium also contained 100 U/ml penicillin and 100 μg/ ml

streptomycin. To minimize the amount of active endogenous ERK2 in import reconstitution assays, cells were placed in serum-free medium for 2 – 4 hours.

*Import reconstitution assays and fluorescence microscopy.*

Cells were grown on glass coverslips in serum-free medium for 2 – 4 hours before the start of the assay. Cells were placed on ice, rinsed twice with ice cold 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT (TB). The cells were permeabilized in a solution of TB and 70 μg/ml digitonin for 5 minutes on ice. The cells were then washed two times with TB and transferred to a humidified chamber. ERK2 mutants, the import substrates, were added at a concentration of ~ 0.04 mg/ml to initiate the import assay. After 15 minutes or the indicated time at room temperature, assays were terminated by fixing in 3% para-formaldehyde (PFA) for 15-20 minutes on ice. In experiments requiring an energy regeneration system 1 mM ATP, 1 mM GTP, 5 mM phosphocreatine and 20 units/ml of creatine phospho-kinase were added to the solution containing the import substrate. If the protein of interest was tagged with a fluorophore such as GFP, the coverslips were washed, and then mounted on glass slides using Aqua-polymount. Otherwise, coverslips were washed in TB and incubated with 0.5% Triton-X 100 in 20 mM Tris, 100 mM NaCl (TBS) for 10 minutes at room temperature. After further washing in TBS, the cells were treated with 1% BSA in TBS plus 0.1% Tween 20 (TBST) for 30 minutes at room temperature. The BSA / TBST solution was the carrier solution used for the primary antibodies to the protein of interest, either anti-pERK (Sigma) or anti-serum Y691. Antibody dilutions ranged from 1:200 to 1:10,000. Coverslips were transferred to

humidified chambers and incubated with primary antibody at 4°C overnight. After washing the coverslips three times in TBST, the coverslips were incubated with secondary antibody diluted 1:3000 and 4', 6-diamidino-2-phenylindole (DAPI) diluted 1:7500 for 1 hour at room temperature. The coverslips were washed three times in TBST and rinsed in deionized water. The coverslips were mounted on glass slides using Aqua-polymount.

Import reconstitution assays were visualized using epi-fluoresence microscopy. Images were captured using a Zeiss Axioskop2 plus microscope and a Hamamatsu digital charge-coupled device (CCD) camera (C4742 – 95). To compare the relative fluorescence intensities, exposures for all conditions in an experiment were identical. Relative fluorescence intensities were quantified either using Slidebook® 4.1 software (Intelligent Imaging Innovations Inc.) or using ImageJ software obtained from the National Institutes of Health at http://rsb.info.nih.gov/ij/ (Rasband, 1997-2004).

*Protein kinase assays*

Activity of recombinant GFP-ERK2 proteins against MBP was measured using *in vitro* kinase assays. The recombinant proteins were diluted to 70 μg/ml in 20 mM Tris, pH 8.0. ERK2 proteins were assayed using a final concentration of 0.6 mg/ml MBP, 5 mM $MgCl_2$, and 200 μM ATP 104 μCi/mol. The reaction was incubated at 30°C for 1 hour. Reactions were terminated by the addition of 5x electrophoresis sample buffer containing 0.3 M Tris, 10% SDS, 50% glycerol, 0.3 M β-mercaptoethanol and 2.5 mg/ml bromophenol blue and incubation at 95°C for 5 minutes. The samples were analyzed by electrophoresis in a 12% polyacrylamide gels containing SDS. The gels were stained

overnight in Coomassie blue, rinsed in water then destained in a solution of 20% methanol and 10% acetic acid for 3 – 8 hours at room temperature. Dried gels were subjected to autoradiography before the bands were excised for scintillation counting. The constitutively active MEK1-R4F mutant was used to activate ERK2.

**Results**

*Import of ERK2 carrying mutations in the CD region*

We performed mutational analysis of the two major regions of ERK1/2 involved in protein docking including D316, D319, and E320 three residues in the CD region of ERK2. Mutations in this region were used to test the hypothesis that proteins containing a D motif regulate nuclear translocation of ERK1/2. D-motif containing proteins may sequester ERK2 or may escort ERK2 through the nuclear pore. Initially we selected two acidic residues from the CD region as candidates in our screen based on data from a yeast two hybrid screen and a *Drosophila* gain-of-function phenotype. A yeast two hybrid screen performed by Robinson *et al*. confirmed that mutation of D316 and D319 (sevenmaker) disrupted the binding of MKP3, a regulatory phosphatase, but did not affect the ability of MEK1 to bind to and activate ERK2 (Robinson *et al.*, 2002a). Based on these observations we included the ERK2-D316A / 319A mutant in our survey. Figure 3-4 shows no significant affect on the nuclear translocation of the kinase-deficient K52R mutant, the dimerization deficient $L_4$A/H176E mutant or of the D316 /319 mutation of ERK2 in our import reconstitution assays.

Arvind *et al*. discovered a naturally occurring mutation of E320 (rat ERK2 numbering) to lysine in a human cancer cell line (Arvind *et al.*, 2005). This residue is adjacent to the two key aspartic acid residues that define the CD region. Figure 3-1 shows both a schematic diagram and a ribbon diagram illustrating the position of the sevenmaker mutant, the CD region, and the E320K mutation. Figure 3-2 shows a Coomassie blue stain of fractions containing GFP-ERK2-E320K purified using $Ni^{2+}$-NTA-agarose column chromatography.

Although mutations in the CD region of ERK2 were shown to have a small effect on the activity of ERK2, I quantitated the activity of the E320K ERK2 mutant kinase toward the substrate MBP. As can be seen in figure 3-3, the activity of the ERK2-E320K mutant toward MBP is about 20-fold greater than the basal activity of wild type ERK2 (Yazicioglu *et al.*, 2007). I did not detect any significant difference in activity toward MBP between t phosphorylated wild type ERK2 or phosphorylated E320K mutant.

I evaluated the nuclear transport of GFP-ERK2-E320K mutant using import reconstitution assays. We showed previously that fusion of GFP moiety to ERK2 has no discernable effect on import reconstitution assays (Robinson *et al.*, 2002a). Figure 3-5 shows that there is no significant difference in the nuclear import of either the active or inactive forms of the GFP-ERK2-E320K mutant, as was the case for ERK2-D316A / D319A (figure 3-4). These results indicate that the acidic residues of the CD region do not affect nuclear transport of ERK1/2

*Import of ERK2 mutants impaired in the putative nucleoporin binding site*

The MAPK insert along with residues in α-helix G have also been identified as a region involved in ERK2 protein-protein interactions. This region binds to FxF motifs and PEA15 (Jacobs *et al.*, 1999; Lee *et al.*, 2004; Whitehurst *et al.*, 2004). Our laboratory has shown that ERK2 can bind directly to nucleoporins and that binding to PEA15 blocks ERK2 interaction with nucleoporins (Whitehurst *et al.*, 2004). Since binding to PEA15 prevents the translocation of ERK2 from the cytosol to the nucleus, we hypothesized that mutations in ERK2 that proved deleterious to the interaction of PEA15

and ERK2 would also affect nuclear transport of ERK2. While identifying mutants of ERK2 that had reduced binding to interactors, like PEA15, our laboratory showed ERK2 mutants with a deletion of the MAPK insert residues were defective in binding to PEA15 and in nuclear import (Whitehurst *et al.*, 2004). Additionally mutation of Y261 to N in ERK2 caused reduced binding of ERK2 to PEA15 (Robinson *et al.*, 2002b; Whitehurst *et al.*, 2004). Lee *et al*. used hydrogen-deuterium exchange to identify residues involved in the binding of an FxF containing peptide from Elk-1 to phosphorylated ERK2 (Lee *et al.*, 2004). Their data suggested several residues that might be important for interaction with other FxF motif containing proteins such as the nucleoporins.

To determine residues required for nuclear translocation of ERK2 we made mutants of residues based on our earlier work and suggested by our collaborator Betsy Goldsmith. All of the mutant proteins were screened using import reconstitution assays in both their unphosphorylated and phosphorylated forms. Many of the import assays included an ATP / GTP energy regeneration system to provide an energy source. To confirm that the mutant proteins were phosphorylated by MEK1-R4F, blots were probed with an antibody against phosphorylated ERK1/2. All of the mutant proteins with the exception of the MAPK insert deletion were phosphorylated as shown in figure 3-6. Table 3-1 provides a summary of our import reconstitution assay results. We found that in cases such as mutants W190A and E320K the nuclear transport of ERK2 was independent of its phosphorylation state and unaffected by energy. In other cases, such as the mutants N199A / S200A, the phosphorylation state of ERK2 affected mutant transport. Perhaps the most interesting finding was that transport of some phospho-ERK2 mutants such as Y261N was affected by the presence of energy. Figure 3-7 shows

the residue positions and their effect on nuclear transport in the structures of both the phosphorylated and unphosphorylated forms of ERK2.

**Discussion**

We used import reconstitution assays to analyze nuclear translocation of ERK2 mutants with deficiencies in sites of protein-protein interactions to examine import under controlled conditions. At least three modalities are utilized by cells for the nuclear translocation of ERK2. Previous work showed that both the active and inactive forms of ERK2 interact directly with nucleoporins to facilitate their nuclear transport regardless of the phosphorylation state of ERK2 (Matsubayashi *et al.*, 2001; Robinson *et al.*, 2002b). Our laboratory also reported that the nuclear transport of phospho-ERK2 increased in the presence of energy (Ranganathan *et al.*, 2006). The data in table 3-1 show that in some mutants the phosphorylation state of ERK2 alone determines the extent of nuclear import regardless of the presence of energy and that mutations impairing binding of FxF motifs have the greatest affect on nuclear transport of ERK2.

Biochemical characterization of our mutant proteins revealed a point mutation E320K that causes a relatively large increase in basal activity of ERK2. The increase in activity is larger than was observed with mutation of D316 / 319. Currently another student is attempting to crystallize the ERK2-E320K mutant to conduct further structural analysis. One possibility offered by the current structure of unphosphorylated ERK2, as seen in figure 3-1, is that the positively charged lysine residue may form a hydrogen bond with the negatively charged carboxyl terminus of D316 since these residues are in close proximity. Such an interaction could act to occlude binding of proteins with the D motif and might also affect the structure of the ERK2 activation loop as has been suggested by Goldsmith and others (Chang *et al.*, 2002).

Three different mechanisms may account for the import phenomena we have observed. Figure 3-8 serves to illustrate our proposed models. As previously reported the first model is predicated on the interaction of either inactive or active ERK2 directly with nucleoporins and does not require energy. The second paradigm is predicated on the assumption that import of phospho-ERK2 occurs as part of an import complex and requires energy. In our third paradigm, cytoskeletal elements in conjunction with molecular motors such as dynein transport ERK2 from distant sites to the region surrounding the nuclear pore. The increased concentration of ERK2 at the nuclear pore, concurrent with sequestration and anchoring of nuclear ERK2, would drive the import process. Evidence for this model comes from the finding that energy-dependent pERK2 import in reconstitution assays occurs even without an exogenous source of transport factors; either residual factors in permeabilized cells account for import or other energy-dependent mechanisms are at work.

In chapter 4 of this dissertation I will discuss additional experiments regarding the regulation and transport of ERK2 across the nuclear pore.

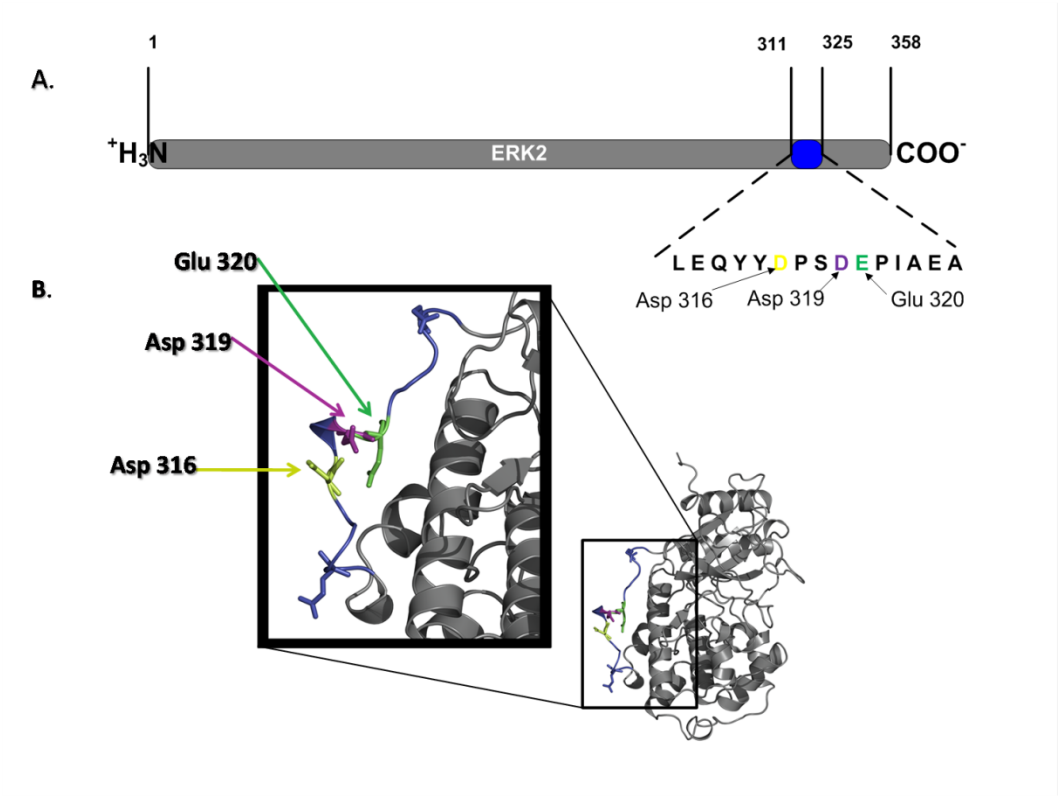



**Figure 3-1 Structure of ERK2**

Panel A: Schematic diagram of ERK2 showing the location of the CD region located between residues 311-325. The CD region is colored blue, with an inset that shows the sequence of residues. Panel B: Ribbon diagram based on the crystal structure of inactive ERK2 (RBCS file 1ERK). Note the ERK molecule has been rotated to show the CD region. The inset shows all the acidic residues as molecular stick figures with D316 colored yellow, D319 colored magenta, and E320 colored green.

(Goad unpublished)

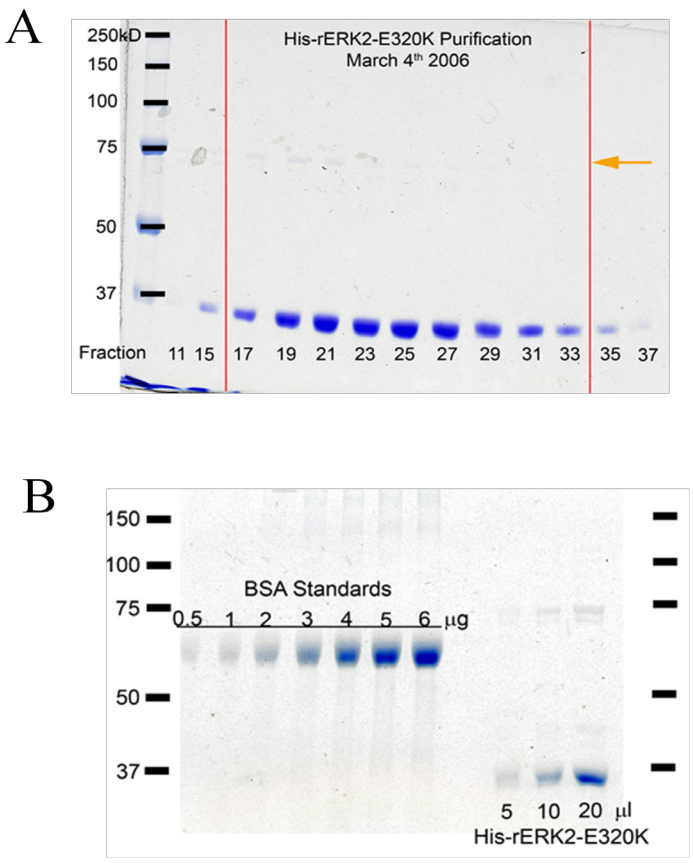



**Figure 3-2 Purification of mutant proteins**

Panel A: Coomassie blue stain of an SDS polyacrylamide gel with fractions of His-ERK2-E320K eluted from a $Ni^{2+}$-NTA-agarose column using an imidazole gradient (20-250 mM). Fractions 17 – 33 (between the red lines) were pooled and dialyzed in purification buffer. The yellow arrow points to very faint bands of contaminating protein(s). Panel B: Coomassie stain of 5, 10, and 20 µl aliquots of the pooled and dialyzed fractions from the top panel. The recombinant protein concentration was determined by densitometry after calibration of a standard curve using BSA standards.

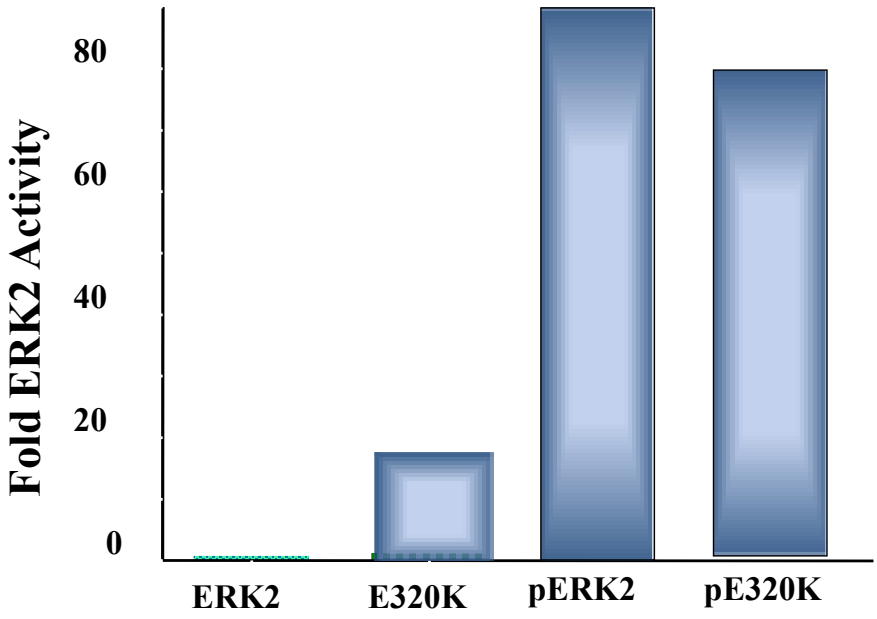



**Figure 3-3 ERK2 wild type and ERK2-E320K activity toward MBP**

Comparison of protein kinase activity of ERK2 and ERK2 E320K with and without phosphorylation by MEK1 *in vitro*. Activity was measured using myelin basic protein as substrate and is shown relative to unphosphorylated ERK2 (Yazicioglu *et al.*, 2007).

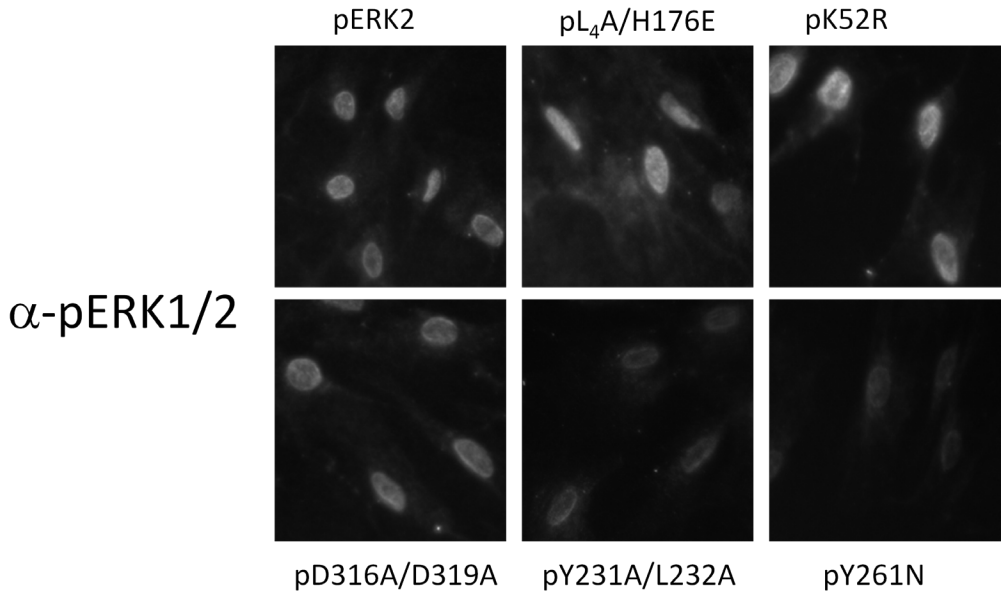



**Figure 3-4 Import reconstitution assays of ERK2 mutants in BJ cells**

Comparison of nuclear import of other ERK2 mutants probed with anti-phospho ERK1/2 antibody. Upper left panel: Import of wild type phospho-ERK2. Lower left panel: Import of the phosphorylated CD region mutant ERK2-D316A / D319A. Upper middle panel: Import of the phosphorylated monomeric mutant ERK2-$L_4$A / H176E. Lower middle panel: Import of the phosphorylated mutant ERK2-Y231A / L232A which is impaired in binding FxF motifs. Upper right panel: Import of the phosphorylated, kinase dead, mutant ERK2-K52R. Lower right panel: Import of the phosphorylated mutant ERK2-Y261N which is impaired in binding FxF motifs. Figure courtesy of Mustafa Yazicioglu (Yazicioglu *et al.*, 2007).

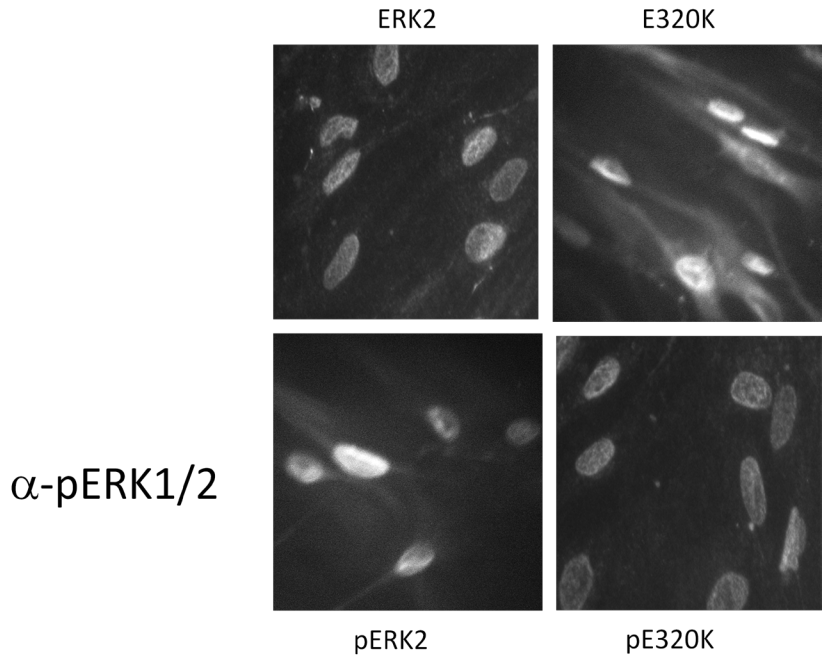



**Figure 3-5 Import reconstitution assays of ERK2-E320K in BJ cells**

Import reconstitution assay of wild type ERK2 and ERK-E320K. Upper left panel: Import of inactive wild type ERK2. Lower left panel: Import of wild type phospho-ERK2 probed with anti-phospho-ERK1/2 antibody. Upper right panel: Import of unphosphorylated ERK2-E320K. Lower right panel: Import of phospho-ERK2-E320K probed with anti-phospho-ERK1/2 antibody. Analysis of this import data shows that import of ERK2-E320K is not import impaired regardless of its phosphorylation state. (Yazicioglu *et al.*, 2007)

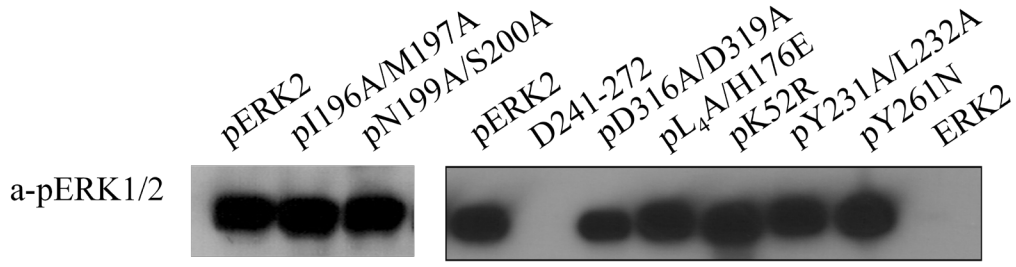



**Figure 3-6 pERK blots of *in vitro* phosphorylated ERK2 and mutants.** The last lane contains unphosphorylated ERK2. Figure courtesy of Mustafa Yazicioglu (Yazicioglu *et al.*, 2007)

| Mutation | ERK2 – Energy | pERK2 – Energy | pERK2 + Energy | Mutant type |
|---|---|---|---|---|
| ERK2 | 100 | 100 | 100 | None – Wild Type |
| Y261N or A | 35 | 35 | 75 | FXF binding |
| I196A / M197A | 45 | 75 | 80 | FXF binding |
| N199A / S200A | 55 | 100 | 100 | FXF binding |
| Y231A / Y232A | 100 | 45 | 75 | FXF binding |
| Δ241-272 | 25 | -- | -- | MAPK insert |
| D316A / D319A | 90 | 95 | 100 | D motif binding |
| E320K | 100 | 100 | | D motif binding |
| W190A | 100 | 95 | 90 | Substrate site |
| P224G | 95 | 80 | | Substrate site |
| P227G | 95 | 90 | | Substrate site |
| K52R | 100 | 100 | | Inactive |
| $L_4$A / H176E | 100 | 100 | | Monomeric |

**Table 3-1 Import of ERK2 and mutants**

This table summarizes the data from multiple *in vitro* import reconstitution assays. 100 cells were analyzed for each condition. All assays were normalized to the import of wild type ERK2. Conditions included 1) No addition of energy and no phosphorylation, 2) No addition of energy, but phosphorylation of ERK2 and its mutants, and 3) Addition of an energy regeneration system, and phosphorylation of ERK2 and its mutants in import reconstitution assays. This table summarizes work from Angelique Whitehurst, Aarati Ranganathan, Mustafa Yazicioglu, and Daryl Goad (Yazicioglu *et al.*, 2007).

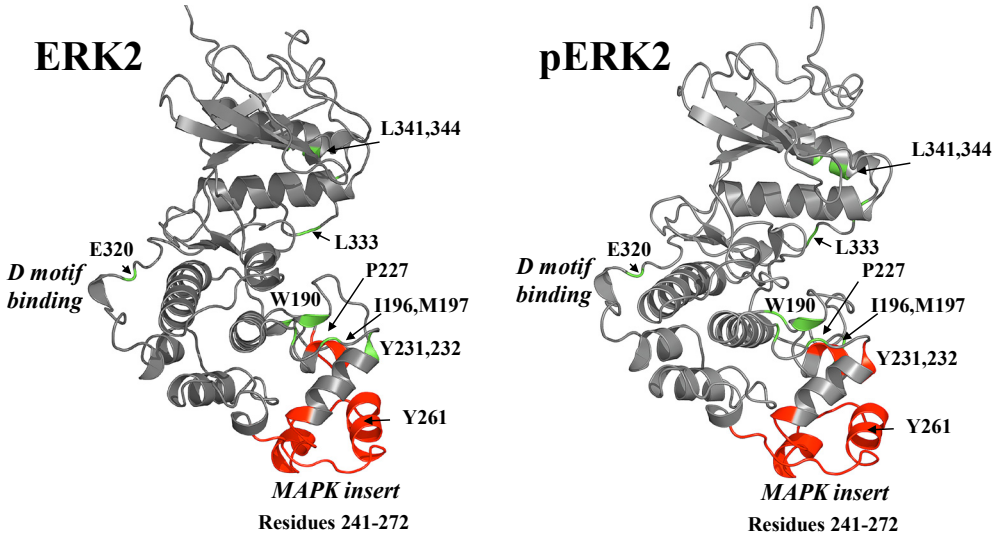



**Figure 3-7 Structure of ERK2 with mutated residues indicated.**

Comparison of the structure of unphosphorylated ERK2 and phosphorylated ERK2 based on the RBCS structure files 1ERK and 2ERK respectively. Similar views of ERK2 and pERK2 are shown with residues mutated in import assays color coded. Mutation of red-colored residues caused reduced nuclear import while mutation of residues colored green caused no discernible effect on translocation. The residue numbers are indicated in the above figure; however, note that residues N199, S200, P224, and L336 are not visible in this view (Yazicioglu *et al.*, 2007).

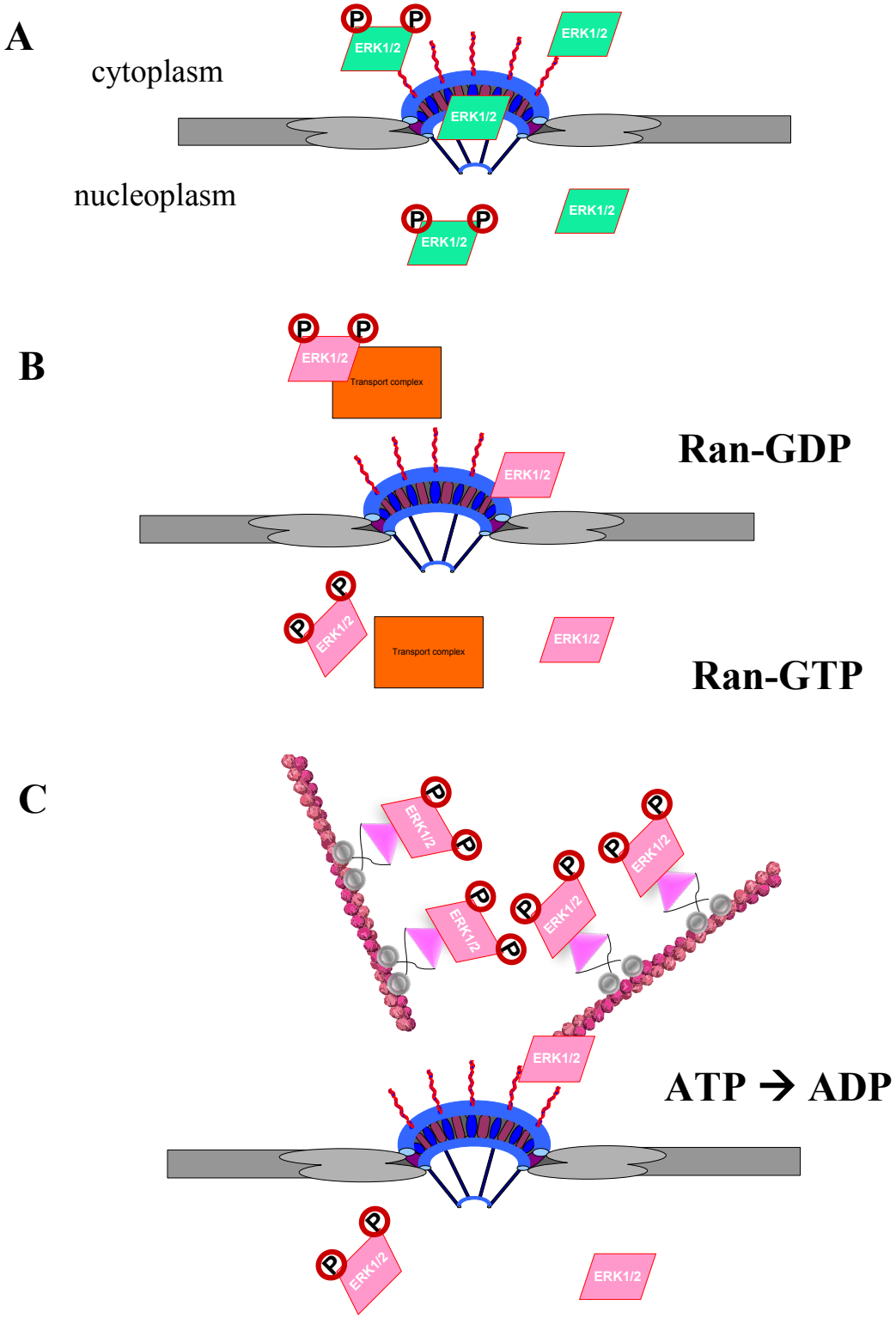

A
cytoplasm
nucleoplasm
ERK1/2
B
Transport complex
Ran-GDP
Ran-GTP
C
ATP → ADP

**Figure 3-8 Mechanisms of ERK2 nuclear import**

Panel A: Direct interaction of ERK2 and phospho-ERK2 with nucleoporins. ERK interacts with the FxF motifs of nucleoporins without a requirement for energy or phosphorylation of ERK. Panel B: Interaction of a pool of phospho-ERK2 with a transport complex. ERK2 interacts either directly or indirectly with proteins actively transiting the nuclear pore as part of a larger complex facilitating the active transport of ERK2. This scenario requires the expenditure of energy in the form of a Ran-GDP / Ran-GTP gradient across the nuclear membrane in order to drive the active translocation of the larger complex. Panel C: Phosphorylated ERK2 is selectively transported to the nuclear pore using elements of the cytoskeleton, such as microtubules, and molecular motors. The increased local concentration of phospho-ERK2 at the cytoplasmic face of the nuclear pore in conjunction with the sequestration and anchoring of phospho-ERK2 by proteins within the nucleus drives translocation of phospho-ERK2 through the nuclear pore.

## CHAPTER FOUR

## Active nuclear translocation, cytoplasmic association, and cellular distribution of active ERK2


### Abstract

I examined the roles of karyopherins, molecular motors, and cytoskeletal elements in the regulation of ERK1/2 nucleocytoplasmic transport. There is an energy-dependent nuclear transport mechanism with specificity for phosphorylated ERK1/2 in mammalian cells (Ranganathan *et al.*, 2006; Yazicioglu *et al.*, 2007). From this energy requirement I developed two hypotheses: first, phosphorylated ERK may preferentially bind to a nuclear transport complex containing a karyopherin; and second, molecular motors such as dynein selectively may bind to phosphorylated ERK1/2 and transport them to nuclear pore complexes. Either of these hypotheses could explain the need for energy. In the first case, ATP is hydrolyzed to ADP driving the molecular motors, while in the second scenario a gradient of RanGDP to RanGTP is maintained across the nuclear membrane to facilitate the binding and release of karyopherins from their cargoes. Additionally I also tested the possibility that, the activity of the karyopherins might be also regulated by phosphorylation by active ERK1/2.

Preliminary data from solution binding assays indicates that both the phosphorylated and unphosphorylated forms of ERK2 bind to immobilized karyopherins β1 and β2, and are released in the presence of Ran-GTP. Our laboratory will continue to test the nuclear transport of pERK1/2 and the need for a karyopherin and energy.

While examining molecular motors and cytoskeletal regulation of pERK1/2, I observed the that disruption of microtubules causes activation of ERK1/2 in the absence of a stimulating ligand as was observed by the Nishida group in 1989 (Shinohara-Gotoh *et al.*, 1991). Unexpectedly, however was the finding that phorbol 12-myristate 13 acetate (PMA) was necessary for the nuclear accumulation of active ERK1/2, even when microtubules were disrupted. I also demonstrated differential cytoplasmic staining of total ERK1/2 and pERK1/2 pools.

The work I have completed on molecular motors, the cytoskeleton, and regulated transport of pERK1/2 by karyopherins provides platforms for ongoing research in our laboratory.

**Introduction**

Our laboratory seeks to understand the mechanisms involved in producing diverse cell fates from signals transduced through the MAPK cascade. I have concentrated my research on systems that control the nuclear localization of ERK1/2. During our mutational analysis of ERK2 import, it became apparent that there is a system(s) for nuclear translocation that requires energy and that has specificity for the active forms of ERK1/2. Based on our previous observations we put forward three models of nuclear import of ERK1/2 (figure 3-8). In this section I will discuss the roles of the karyopherins, molecular motors, and cytoskeletal elements in our models and the experiments we are conducting to test our hypotheses.

As I reported in chapter three, there is an energy-dependent nuclear transport system that influences the localization of activated ERK1/2. We observed that mutation of tyrosines 261, 231, and 232, which impair ERK1/2 binding to FxF motifs and nuclear uptake in unphosphorylated ERK2, produced pERK2 phenotypes in which nuclear translocation was increased in the presence of an energy regeneration system (refer to table 3-1). From our data, we deduced three possible models of ERK1/2 nuclear transport (refer to figure 3-8). The first model of nuclear transport does not require energy and is based on direct interactions of both the inactive and active forms of ERK1/2 with nucleoporins. The second model proposes the selective transport of active ERK1/2 by transport complexes containing karyopherins. In this model energy is required for the maintenance of a RanGDP / RanGTP gradient that spans the nuclear membrane. The third model proposes the selective transport of active ERK1/2 by

retrograde molecular motors to the nuclear pore complex. This model also requires energy to maintain adequate reserves of ATP to drive the motor proteins.

The karyopherin-βs are an evolutionarily conserved protein family that functions as regulators of nuclear translocation. Table 4-1 shows the 20 known mammalian karyopherins, their yeast orthologs, and classifies them according to function. The karyopherin-β family is characterized by repetition of 18-20 helix-loop-helix domains known as HEAT repeats, a molecular weight range of 95 – 145 kDa, and an acidic isoelectric point (Pemberton and Paschal, 2005) . The HEAT repeat is composed of approximately 40 amino acid residues and is named for the proteins in which the motif was initially identified; huntingtin, elongation factor 3, 'A' subunit of protein phosphatase 2A, and target of rapamycin (Chook and Blobel, 1999). High resolution crystal structures of karyopherins β1 and β2 show the HEAT repeats forming an orthogonally opposed set of arches. However, low resolution X-ray scattering studies suggest these molecules are extremely flexible with large differences in conformations between ligand bound molecules and unbound molecules (Mosammaparast and Pemberton, 2004). The repeated HEAT motifs provide a large surface area for protein-protein interactions. Consequently, given the flexibility and large surface area, crystal structures of karyopherin β1 bound to three different cargoes show marked differences in binding conformations.

Karyopherin-βs act as nuclear transport chaperones forming transport complexes with cargoes, the GTPase Ran, and adaptor proteins such as karyopherin α and non-karyopherin family Ran binding proteins (RanBPs). Karyopherins recognize cargoes that display either a NLS or NES. In the canonical import pathway karyopherin α recognizes

its cognate NLS, a basic sequence of residues, on the cargo protein. On binding to the cargo protein karyopherin α undergoes a conformational change, in which its auto-inhibitory domain folds into the importin β binding domain. Karyopherin β1 then interacts with cargo bound karyopherin α. Cargo bound karyopherin β1 then interacts with FxF motifs in the nucleoporins that make up the nuclear pore complex thereby effecting nuclear transport of the cargo. After transiting the nuclear pore, Ran-GDP interacts with the Ran guanine exchange factor (GEF) regulator of chromosome condensation 1 (RCC1). RCC1 catalyzes the exchange of GDP for GTP. The presence of RanGTP causes dissociation of the proteins in transport complex and nuclear import is complete.

Given the rather linear model presented in the previous paragraph it is important to realize that most karyopherins interact directly with their cargoes without the need of an adapter protein and the NLS and transport mechanism for most nuclear proteins is still unknown. The dynamic rearrangement and changes in affinity of the nucleoporins in response to the cell cycle, before nuclear envelope breakdown, adds yet another layer of complexity that should be considered when discussing nuclear translocation.

Molecular motors function to transport organelles, vesicles and proteins along cytoskeletal elements throughout the cell. The motor protein families dynein and kinesin are uni-directional transporters that travel the microtubule network. The motor proteins obtain the energy required for transport through the hydrolysis of ATP. Kinesin proteins are plus-end directed motors, moving cargoes in an in anterograde (toward the plasma membrane) direction. Dyneins are minus-end directed motors that move cargoes in a retrograde (toward the nucleus) fashion. Because dyneins move cargo toward the nucleus

and require ATP for energy, I focused my efforts on the effect they have on ERK1/2 nuclear transport.

The microtubule-associated motor proteins were also interesting in the context of the work of Reszka *et al*. which showed half of all the detectable ERK1/2 activity in NIH 3T3 mouse fibroblasts was associated with microtubules (Reszka *et al.*, 1995). Dyneins are at the convergence of a pool of microtubule associated ERK1/2, which might be directed to the nucleus for translocation by retrograde transport, and require energy in the form of ATP for transport.

In addition to a cargo and ATP, dyneins also require dynactin, an adaptor complex to link the motor to the cargo. Dynactin is a multi-protein complex containing an F-actin like filament composed of actin related protein 1 (Arp1) and capped at the ends by a 62 kDa subunit and by actin capping protein (Burkhardt *et al.*, 1997). The dynactin complex also requires the presence of a 50 kDa subunit known as dynamitin for its assembly. Dynamitin is present in a ratio of 4-5 moles / mole of dynactin, but has not been localized within the complex. Overexpression of dynamitin has been found to disrupt dynactin complexes, and abrogate transport by dynein in mammalian cells (Burkhardt *et al.*, 1997).

Based on our previous work and the current literature I hypothesized that karyopherins are involved in the selective nuclear transport of active ERK1/2. I also hypothesized that the dynein family of molecular motors is involved in the transport of active ERK2 to the nuclear pore complex. Both of these models fit our previous data suggesting a system(s) that requires energy for the nuclear transport of active ERK1/2.

**Experimental Methods**

*Cell culture*

HeLa and human foreskin fibroblasts immortalized with h-tert (BJ cells) (Ramirez *et al.*, 2004), were grown on glass coverslips in Dulbecco's modified Eagles medium supplemented with 10% FBS and 1% L-glutamine at 37°C in a 10% $CO_2$ atmosphere.

*Sub-cloning of karyopherins and dynamitin*

Karyopherin constructs were acquired from Yuh-Min Chook or from the American Type Culture Collection (ATCC). Table 4-2 lists the karyopherins we obtained and their sources. The karyopherin clones were sequence verified before sub-cloning into the pGEX-4T-TEV vector. Karyopherins β1 and β2 were already in the appropriate vector and their sequences verified.

Human dynamitin cloned into pCMVβ was a gift from Richard Vallee. I digested both pCMVβ-dynamitin and $pHis_6$-parallel with NcoI and HindIII restriction enzymes for 1 hour at 37°C. The reactions were then subjected to electrophoresis using a 1% agarose gel. The resulting bands were excised and extracted using BioRad freeze and squeeze columns. The purified vector and insert were ligated using T-4 DNA ligase. After ligation the DNA was used to transform strain DH-10 of *E. coli*. The transformed bacteria were cultured on LB plates containing 100 μg/ml carbenicillin overnight at 37°C. Colonies were selected for small scale DNA preparations. The resulting DNA was digested with NcoI and HindIII restriction enzymes and analyzed by gel electrophoresis

on a 1% agarose gel. DNA producing the appropriate banding pattern was selected for large scale DNA preparation and stored at -20°C.

*Protein expression and purification*

*E.coli* strain BL-21 was transformed with the plasmid DNA and grown on LB plates containing 100 μg/ml carbenicillin for 18 hours. A colony was selected and used to inoculate a solution of LB containing 100 μg/ml of ampicillin. The culture was incubated overnight at 37°C. The next day 10 ml of the *E.coli* culture was added to 1 L of LB medium containing 100 μg/ml ampicillin. The bacteria were incubated at 37°C and grown to an $OD_{600}$ of 0.6 – 0.8 then cooled to 30°C. IPTG was added to a final concentration of 200 μM, and the culture was incubated at 30°C overnight. The pellet was harvested by centrifugation and resuspended in 20% glycerol, 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM EGTA, 2 mM magnesium acetate, 2 mM DTT, 1 mM PMSF, 10 μg/ml Leupeptin, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM benzamidine (TB buffer). The resuspended bacteria underwent 4 passes in a cell disrupter set to 100,000 kPa. The material was centrifuged at 50,000 g for 45 minutes to separate the soluble proteins from the insoluble material. Glutathione-Sepharose beads were added to the supernatant and incubated at 4°C overnight. The beads were then washed with 90 volumes of TB buffer resuspended in TB buffer, and stored at -20°C.

Recombinant $His_6$-Ran and $His_6$-dynamitin were purified from bacterial expression plasmids as described in chapter 3.

*Protein kinase assays*

Equal amounts of recombinant proteins were incubated in 30 µl reactions containing 10 mM HEPES pH 8.0, 10 mM magnesium chloride, 50 µM ATP (200 cpm / pmol [$\gamma^{32}$P] ATP), 1 mM DTT, and 1 mM benzamidine for 1 hour at 30°C. After termination with 10 µl 5x electrophoresis sample buffer, (0.3 M Tris, 10% SDS, 50% glycerol, 0.3 M β-mercaptoethanol, and 2.5 mg/ml bromophenol blue) samples were heated at 95°C for 5 minutes and resolved by SDS-PAGE. Dried gels were subjected to autoradiography before bands were excised for Cerenkov counting.

*RanGTP*

A reaction mixture of 7.2 mg/ml recombinant Ran, 34 mM DTT, 3.4 mM GTP, and 17 mM EDTA was incubated with 69 mM magnesium acetate for 15 minutes at 4°C. The reaction was aliquoted, snap frozen in liquid nitrogen, and stored at -80°C. In reactions with karyopherins the Ran-GTP solution was diluted to produce a final concentration of 5 mM magnesium.

*Solution binding experiments and western blotting*

Recombinant proteins were diluted to equal concentrations in 20 mM Tris pH 7.5, 1 mM DTT, 1 mM EGTA, 10 mM benzamidine, 10% glycerol (purification buffer). Reactions containing ERK2 and karyopherins immobilized on glutathione-Sepharose beads were incubated 30 minutes at 4°C with continuous inversion and washed three times in 2.5 volumes of purification buffer. The beads were resuspended in purification buffer plus or minus 0.116 µg of RanGTP. Reactions were incubated 40 minutes at 4°C.

Beads were washed in 90 volumes of purification buffer. Reactions were terminated by the addition of 5X SDS sample buffer and incubated at 95°C for 5 minutes. The samples were resolved by electrophoresis in 12% polyacrylamide gels in SDS and transferred to nitrocellulose. Anti-serum Y691 diluted 1:1000 in 0.5% milk, 20 mM Tris, and 500 mM NaCl was used to detect ERK2.

*Cytoskeletal perturbations*

BJ cells were grown to 85-95% confluence on glass coverslips in 6 well plates as above. The culture medium was replaced with serum-free medium 2 – 4 hours before treatment with nocodazole or PMA. Nocodazole was diluted to 100 nM or 1 μM in serum-free medium. Culture medium was replaced using nocodazole-containing medium for 15 minutes. Then the coverslips were treated with 100 nM PMA in dimethyl sulfoxide (DMSO) or 0.6% DMSO alone and incubated for an additional 15 minutes. The coverslips were then processed for immunofluorescence microscopy as described below.

To minimize the amount of active ERK1/2 in the cells, they were placed in serum-free medium for 4 hours. Cells were treated with 0.5 μM jasplakinolide in DMSO, 0.5 μM latrunculin b in DMSO, or 0.5% DMSO and incubated for 30 – 45 minutes. Selected wells were treated with 750 nM PMA. Cells were incubated an additional 15 minutes before fixation and immunostaining.

*Immunofluorescence microscopy*

Cells were placed on ice and the coverslips washed in cold TBS. Cells were fixed in 4% para-formaldehyde diluted in 10 mM Na phosphate pH 7.4, 137 mM NaCl, 2.7 mM KCl, (PBS) for 10 minutes at room temperature. The coverslips were washed 3 times in TBS before treatment with 0.5% TritonX-100 in TBS for 10 minutes at room temperature. The coverslips were washed twice in TBS, once in TBST, and incubated with 1% BSA in TBST for 30 minutes at room temperature. The coverslips were then transferred to humidified chambers and incubated with primary antibody, either anti-serum Y691 diluted 1:1000 or a monoclonal antibody against phospho-ERK1/2 (Sigma) diluted 1:500 in BSA / TBST. After overnight incubation at 4°C, coverslips were washed in TBST prior to incubation with Alexa-Fluor 488 goat anti-rabbit antibody 1:3000, Alexa-Fluor 546 goat anti-mouse antibody 1:3000, and DAPI diluted 1:7,500 in BSA/TBST for 1 hour at room temperature. The coverslips were washed in TBST, and then rinsed in deionized water before mounting onto glass slides with Aqua-polymount.

Micrographs were produced using epi-fluorescence microscopy. Images were captured using a Zeiss Axioskop2 plus microscope and a Hamamatsu digital CCD camera (C4742-95). Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.), Photoshop CS2 (Adobe Systems, Inc.) and Image J (Rasband, 1997-2004) were used for image capture, processing and annotation.

**Results**

*Karyopherins*

To search for the energy-dependent mechanism responsible for the selective import of phosphorylated ERK1/2, I first examined the role of karyopherins. In *Drosophila* S2 cells the mammalian importin 7 ortholog, DIM-7 is necessary for the active nuclear transport of phosphorylated D-ERK (Lorenzen *et al.*, 2001). Thus the hypothesis was that importin 7 functioned as a transporter of active ERK1/2 in mammalian cells. We tested our hypothesis by knocking down importin 7 in HeLa cells. After knockdown of importin 7 with three different dsRNAi oligonucleotides, the cells were stimulated with PMA or ultra-violet radiation. Antibodies raised against the phosphorylated forms of ERK1/2, p38, and JNK in conjunction with immunofluorescence microscopy were used to probe the effect of importin 7 on transport of these MAPKs. Immunoblots of cell lysates showed substantial knockdown of importin 7 with all three dsRNA oligomers. However, we never saw any effect on the import of ERK1/2 or any of the other MAPKs we tested under these conditions (Yazicioglu *et al.*, 2007). That led us to the hypothesis that one or more other carriers are responsible for the transport of active ERK and I began testing other karyopherins in the context of active ERK2 transport.

Initially, I focused my efforts on karyopherins β1 and β2. Karyopherin β1 transports SREBP2 though the nuclear pore complex (NPC) without the need for karyopherin α or any other adaptor proteins, although SREBP2 does not contain a canonical NLS (Lee *et al.*, 2003; Nagoshi *et al.*, 1999; Nagoshi and Yoneda, 2001). Karyopherin β1 recognizes a structural helix-loop-helix NLS formed by the homo-

dimerization of SREBP2 (Lee *et al.*, 2003; Nagoshi and Yoneda, 2001). ERK1/2 can form dimers *in vitro* and there is evidence that indicates active ERK1/2 may form homo- or hetero-dimers before nuclear translocation occurs. Lee *et al*. reported a C-terminal sequence based NLS consisting of R/K/H-$X_{(2-5)}$-P-Y (PY-NLS) recognized by karyopherin β2 (Lee *et al.*, 2006). An examination of ERK1/2 sequences revealed a C-terminal PY motif that bore some similarities to the PY-NLS. Thus I hypothesized that karyopherin β1 or karyopherin β2 was responsible for the energy dependent nuclear translocation of active ERK1/2.

I performed an *in silico* analysis of putative ERK binding motifs within the sequences of karyopherins β1 and β2 found several possible sites as shown in table 4-3. These ideas were tested using *in vitro* solution binding assays. If my hypothesis was correct active ERK1/2 should selectively bind to immobilized karyopherin β1 or β2, and the addition of Ran-GTP should cause release of ERK1/2 from the karyopherin.

Figure 4-1 illustrates the results from a representative purification of karyopherins β1 and β2. I expected the solution binding assay to show one of the karyopherins binding to, and being released from active ERK2 and not to show any significant binding to unphosphorylated ERK2 based on our observations that showed an energy-dependent import mechanism only for active ERK2. Figure 4-2 panel A shows the results of an experiment in which I found that both active and inactive ERK2 bound to karyopherin β1 and β2. Figure 4-2 panels B and C show that RanGTP caused release of the active and inactive forms of ERK2 from both karyopherins. Additional experiments under slightly different conditions showed a similar overall trend in that both forms of ERK2 bound and were released from immobilized karyopherins β1 or β2 upon

addition of RanGTP. No selectivity between phosphorylated and unphosphorylated ERK2 was detected. Another student in the lab has been able to replicate these results.

My *in silico* analysis predicted two putative ERK phosphorylation sites in karyopherin β2. Figure 4- 3 demonstrates that karyopherins β1 and β2 are not substrates of ERK2. Control reactions of ERK2 toward the substrate MBP showed robust ERK2 activity. Other karyopherins and nucleoporins may be targets of ERK1/2 as it transits the NPC; however, these experiments make it unlikely that karyopherins β1 and β2 are regulated by ERK2 via phosphorylation.

*Molecular motors and the cytoskeleton*

Molecular motors like dynein transport their cargos toward the minus end of microtubules. The dynein family of proteins also requires the adapter complex dynactin to facilitate binding of its cargo. Transport of activated ERK1/2 from the cell periphery or from distant compartments to the proximity of nuclear pores could drive nuclear transport via a concentration gradient. This hypothesis is predicated on the assumption that active ERK1/2 are sequestered in the nucleus by binding partners, thereby reducing the local, free concentration of ERK21/2 on the nucleoplasmic side of the nuclear pore.

The approach I used to study the effect of molecular motors on nuclear translocation of active ERK1/2 was to disrupt microtubules and the dynactin complex. I considered RNAi and expression of dynamitin. Dynamitin is part of the dynactin adapter complex and overexpression of dynamitin 'blows up' the dynactin complex (Burkhardt *et al.*, 1997).

My basic approach was to express and purify the dynamitin protein and use it in import reconstitution assays in experiments that showed energy-dependent nuclear transport for active ERK in the presence and absence of recombinant dynamitin. We theorized that molecular motors traversing the microtubules might not be washed out of the cell after permeabilization of the plasma membrane in import reconstitution assays. Remaining motor proteins could act to increase the local concentration of active ERK at the nuclear pore in the presence of energy. If dynein motors are involved, pre-treating permeabilized cells with dynamitin should disrupt the function of dynein motors and should abrogate the increased transport of active ERK1/2 in the presence of energy.

I decided to disrupt microtubules using a pharmacologic approach. I theorized the disruption of microtubules would also disrupt transport of cargo by molecular motors. *In vitro* experiments have shown the drug nocodazole disrupts microtubule dynamic instability by stabilizing microtubules and preventing polymerization of tubulin when used at concentrations equal or greater than the tubulin concentration (Vasquez *et al.*, 1997). Nocodazole is often used at nanomolar concentrations to block mitosis by stabilizing the microtubules. At low doses, such as 4 – 100 nM, nocodazole slows the dynamic turnover of tubulin in microtubules without causing the ensuing catastrophe seen at higher doses (Vasquez *et al.*, 1997).

Micromolar doses of nocodazole cause massive depolymerization of microtubules. After treatment with either 100 nM or 1 μM nocodazole, the cells were treated with PMA to activate ERK1/2. The localization of both active and inactive ERK1/2 was examined using immunofluorescence microscopy. I anticipated an abrogation of active ERK1/2 nuclear transport in cells treated with 1 μM nocodazole.

Instead, as can be seen in figure 4-4, I found that nocodazole treatment does not affect nuclear translocation of active ERK1/2, however it does cause activation of a cytoplasmic pool of ERK1/2 without the presence of a stimulating ligand as reported previously by the Nishida group (Gotoh *et al.*, 1990). I also observed active ERK in both the cytoplasm and nucleus of nocodazole treated cells stimulated with PMA.

To determine if pharmacologic disruption of actin filaments would affect the localization or activation of ERK1/2, I treated serum deprived cells with jasplakinolide, which acts to stabilize actin *in vitro* but causes disruption of actin filaments and polymerization of monomeric actin into amorphous masses *in vivo* (Bubb *et al.*, 2000), in the presence or absence of PMA. I expected actin destabilization to result in a defect in transport of ERK1/2, figure 4-5 shows the results of this treatment. Jasplakinolide treatment did not affect the localization of active ERK1/2; however I did observe a 1.5 fold increase in the amount of total ERK1/2 in the nucleus after treatment with jasplakinolide with no significant change in nuclear fluorescence of pERK1/2 (figure 4-5 panel D). Although, I did not analyze many micrographs, I quantified the changes in relative nuclear fluorescence to determine the magnitude of change.

*Discontinuity of ERK1/2 pools in the cytoplasm*

When I observed fixed cells immuno-stained for both total ERK1/2 and pERK1/2 I would often see discontinuous distribution of the pools of active and inactive ERK1/2 (figure 4-6). The cells in this image showed moderate levels of activated ERK1/2 in the nucleus, with most of the activated ERK1/2 localized to the cell periphery.

**Discussion**

I began this series of experiments to test the following hypotheses 1. One or more members of the karyopherin-β family are involved in the selective nuclear translocation of ERK1/2; 2. Karyopherins β1 and β2 are substrates of ERK1/2; 3. The dynein family of molecular motors is responsible for the transport of active ERK1/2 from distant locations to the cytoplasmic face of the nuclear pore complex. Testing these hypotheses showed that karyopherins β1 and β2 bind to both ERK1/2 and pERK1/2 *in vitro* and addition of RanGTP causes release of both forms of ERK1/2. Although karyopherins β1 and β2 bind to ERK2, they are not substrates of ERK2. Nuclear localization of active ERK1/2 requires stimulation by an activating ligand, such as PMA, even when microtubules are disrupted. Disruption of the actin cytoskeleton causes a 1.5 fold increase in nuclear accumulation of unphosphorylated ERK1/2. And the distribution of active and unphosphorylated ERK1/2 is discontinuous in cells.

My experience with karyopherins leads me to believe that multiple karyopherins are involved in the regulated nuclear transport of ERK1/2. Although my early *in vitro* binding experiments did not demonstrate specificity for active ERK2, they did show binding and release of ERK2 in the presence of RanGTP. I initially discounted the possible role of importin 7 in nuclear import of active ERK1/2 based on our previous work. However, a recent paper by Chuderland *et al*. supports a role for importin 7. They report that ERK1/2, SMAD3, and MEK1 contain an SPS motif that can be phosphorylated and act as an NLS for importin 7 (Chuderland *et al.*, 2008). They used siRNA constructs to knockdown importin 7 in HeLa cells that were stimulated with EGF and showed decreased nuclear localization of ERK using a general ERK antibody.

Knockdown of importin 7 only caused a partial inhibition of nuclear transport in non-stimulated cells. They concluded that the SPS motif, which lies in a 19-residue stretch within the kinase domain of ERK2, can be phosphorylated at either serine residue to act as an NLS for importin 7. They also report knockdown of importin 7 affects the nuclear localization of SMAD3 and MEK1 as well. They supported their findings using GFP fusion constructs of ERK2, mutational analysis of the SPS motif, and cellular fractionation studies in conjunction with immunofluorescence microscopy.

Several factors may explain the difference in findings in RNAi of importin 7 in HeLa cells, including insufficient reduction in protein to detect a loss of function, differences in antibody selectivity and sensitivity, and the use of different stimuli for activation of ERK1/2. In our study we detected phosphorylated ERK1/2, while Chuderland *et al*. probed ERK1/2 localization with a non-phospho ERK1/2 antibody. A recent paper by Yao *et al*. demonstrate importins 7 and 8 preferentially transport SMADs through the NPC with importin 8 having the largest effect on SMADs 1,3, and 4 (Yao *et al.*, 2008). And a paper by Vomastek *et al*. demonstrates the nuclear pore protein translocated promoter region (Tpr) is an FxF motif bearing ERK2 substrate that binds preferentially to pERK1/2 (Vomastek *et al.*, 2008). Taken together our previous data, and the data of the Seger ,Xu, and Nandicoori groups support our current hypothesis that multiple mechanisms are responsible for the regulation and active nuclear translocation of active ERK1/2. It has been suggested that proteins that are critical to the function of nuclear processes have evolved multiple regulated pathways into the nucleus (Pemberton and Paschal, 2005). It is probable that ERK1/2 multiple pathways have arisen for nuclear entry via the karyopherins. For future studies the hypothesis that several karyopherins

are involved in the nuclear translocation of ERK1/2 appears most likely. Continuing solution binding assays using other importins appears to be a productive direction. Addition of RanGDP during the initial binding phase may stabilize the binding of the karyopherins to their cargo. RNAi both in cellular assays and import reconstitution assays should also be employed. Inhibition of karyopherin β2 with the inhibitor M9M (Cansizoglu *et al.*, 2007) in cells and import reconstitution assays may also provide clues regarding facilitated transport of ERK1/2. Although kinase assays indicate that karyopherins β1 and β2 are not substrates of ERK1/2, other karyopherins may be regulated by ERK1/2 and could be tested as potential ERK1/2 substrates.

I expected that the disruption of the cytoskeleton would lead to a decrease in the nuclear translocation of active ERK1/2. However, pharmacological disruption of microtubules or polymerization of actin into amorphous masses caused either no change or an increase in nuclear ERK1/2. Active ERK1/2 accumulated in the nucleus in the presence of stimulating ligands in spite of disturbed cytoskeletal function. Nevertheless, two interesting observations came from treatment of cells with nocodazole. First, nocodazole caused activation of a cytoplasmic pool of ERK1/2 in the absence of a stimulating ligand as suggested earlier. The striking increase in pERK1/2 implies that microtubule binding suppresses a substantial activation of ERK1/2. The underlying mechanisms are unknown. Addition of a ligand then caused translocation of pERK1/2. Treatment of cells with jasplakinolide caused nuclear accumulation of inactive ERK1/2 in the nucleus in the absence of a stimulating ligand. The likely explanation for the increase in cytosolic levels of active ERK1/2 in nocodazole-treated cells, based on the work of Reszka *et al*., is that disruption of the microtubules released a large pool of

ERK1/2 that is normally part of a complex of microtubule-associated proteins. However, release of this cytoplasmic pool was not sufficient to cause a substantial increase in nuclear active ERK1/2. The implication is that ligands such as PMA trigger an import mechanism. The concerning the localization of inactive ERK1/2 in cells treated with jasplakinolide indicates the actin cytoskeleton may play a role in regulating the localization of ERK1/2. Further studies are needed to determine the roles of nuclear and cytoplasmic actin on ERK1/2 localization and transport. Another possibility is that disruption of the actin filaments activated protective mechanisms that caused the translocation of inactive ERK1/2 to the nucleus.

These findings do not exclude molecular motors in transport of active ERK to the nuclear pore complex. The use of dynamitin to probe a role for dynein remains a worthwhile strategy. Immunofluorescence experiments and import reconstitution assays using dynamitin could be performed to determine if dynein plays a role in ERK1/2 transport. Two alternate strategies for disrupting the function of dynein are RNAi oligonucleotides to suppress its expression, or antibodies to interfere with its function. The use of recombinant dynamitin in the context of import reconstitution assays may provide the most interpretable data. If dynamitin is transfected or if RNAi oligomers are used against dynein in cells, interpretation of the resulting phenotype will be hard to distinguish from global effects caused by disrupting retrograde transport. Strategies for examining the kinesin and myosin families of molecular motors in the context of ERK1/2 transport may also be of value.

Finally, the discontinuity of cytoplasmic pools of active ERK1/2 and inactive ERK1/2 in cells grown to a moderate confluence suggests that much of the ERK1/2 in the

cytoplasm is constrained and not freely diffusible. Given that cells have evolved many scaffolding proteins and other proteins that act to sequester both the active and inactive forms of ERK1/2, a reasonable supposition is that ERK1/2 exists in many heterogeneous pools within the cell. Much remains to be done to elucidate mechanisms regulating ERK1/2 localization.

| Mammalian Karyopherin Name | Alias | Function | Yeast Ortholog |
|---|---|---|---|
| Karyopherin β1 | Importin β | Import | Karyopherin 95 |
| Karyopherin β2 | Transportin | Import | Karyopherin 104 |
| Transportin SR1 | | Import | Karyopherin 111 |
| Transportin SR2 | | Import | |
| Importin 4 | | Import | Karyopherin 123 |
| Importin 5 | | Import | Karyopherin 121 |
| Importin 7 | RanBP 7 | Import | Karyopherin 119* |
| Importin 8 | RanBP 8 | Import | Karyopherin 119* |
| Importin 9 | | Import | Karyopherin 114 |
| Importin 11 | | Import | |
| Importin 13 | | Import / Export | |
| Crm1 | | Export | Crm1 |
| Exportin-t | | Export | Los1 |
| CAS | | Export | Cse1 |
| Exportin 4 | | Export | |
| Exportin 5 | | Export | |
| Exportin 6 | | Export | |
| Exportin 7 | | Export | |
| RanBP 6 | | Unknown | |
| RanBP 17 | | Unknown | |

**Table 4-1 Mammalian karyopherins**

Karyopherins highlighted orange import into the nucleus. The karyopherins highlighted green export. No function has been defined for karyopherins highlighted blue. *The yeast ortholog karyopherin 119 is similar in sequence to both mammalian importin 7 and 8.

| Karyopherin | Source | Parental Vector |
|---|---|---|
| Importin 4B | Yuh-Min Chook | pGEX-4T-TEV-ZZ |
| Importin 4B | ATCC clone 6164334 | pCMV-Sport6 |
| Importin 11 | Yuh-Min Chook | pGEX-4T-TEV |
| Importin 13 | Yuh-Min Chook | pGEX-4T-TEV-ZZ |
| Importin 13 | ATCC clone 298572 | pCMV-Sport6 |
| Transportin SR | Yuh-Min Chook | pGEX-4T-TEV-ZZ |
| Transportin SR | ATCC clone 6313452 | pCMV-Sport6 |
| Karyopherin β1 | Yuh-Min Chook | PGEX-4T-TEV |
| Karyopherin β2 | Yuh-Min Chook | PGEX-4T-TEV |
| Importin 7 | Yuh Min Chook | pCR-Blunt |

**Table 4-2 List of karyopherins subcloned**

The coding sequence of the karyopherins was verified before they were sub-cloned into the GST epitope tagged bacterial expression vector pGEX-4T-TEV.

| Putative D binding motifs | | | |
|---|---|---|---|
| Karyopherin β1 | | **Karyopherin β2** | |
| Site | **Sequence** | **Site** | **Sequence** |
| L352 | CKAAGVCLMLLATCC | I821 | FRGICTMISVNPSGV |
| V95 | RREVNKYVLQTLGTE | L211 | SRTQALMLHIDSFIE |
| V734* | EFKKYLEVVLNTLQQ | L645 | KDFMIVALDLLSGLA |
| L714 | RSVKPQILSVGGDIA | L878* | RFSDQFPLPLKER |
| V387 | WRYRDAAVMAFGCIL | L72 | RSLSGLILKNNVKAH |
| I407 | SQLKPLVIQAMPTLI | L239 | RKNVCRALVMLLEVR |
| V275 | MKSDIDEVALQGIEF | V303 | HLPKLIPVLVNGMKY |
| L736* | KKYLEVVLNTLQQAS | I301 | VRHLPKLIPVLVNGM |
| V716 | VKPQILSVFGDIALA | V305 | PKLIPVLVNGMKYSD |
| I61 | RVAAGLQIKNSLTSK | I176 | DRPLNIMIPKFLQFF |
| L16 | KTVSPDRLELEAAQK | I315 | MKYSDIDIILLKGDV |
| V837 | GKDVLKLVEARPMIH | L383 | RKCSAAALDVLANVY |
| **Putative FP Binding Motifs** | | | |
| | | P585 | DEDKDLFPLLECLSS |
| | | P84 | KAHFQNFPNGVTDFI |
| **Putative Phosphorylation recognition sties P-S/T** | | | |
| | | S103 | LNNIGDS**S**PLIRATV |
| | | T763 | EIINRPN**T**PKTLLEN |

**Table 4-3 *In silico* analysis of karyopherins β1 and β2**

Putative ERK1/2 interacting sites on karyopherins β1 and β2 as determined by Scansite (http://scansite.mit.edu/). Entries denoted with an asterisk are most similar to the canonical D binding motif.

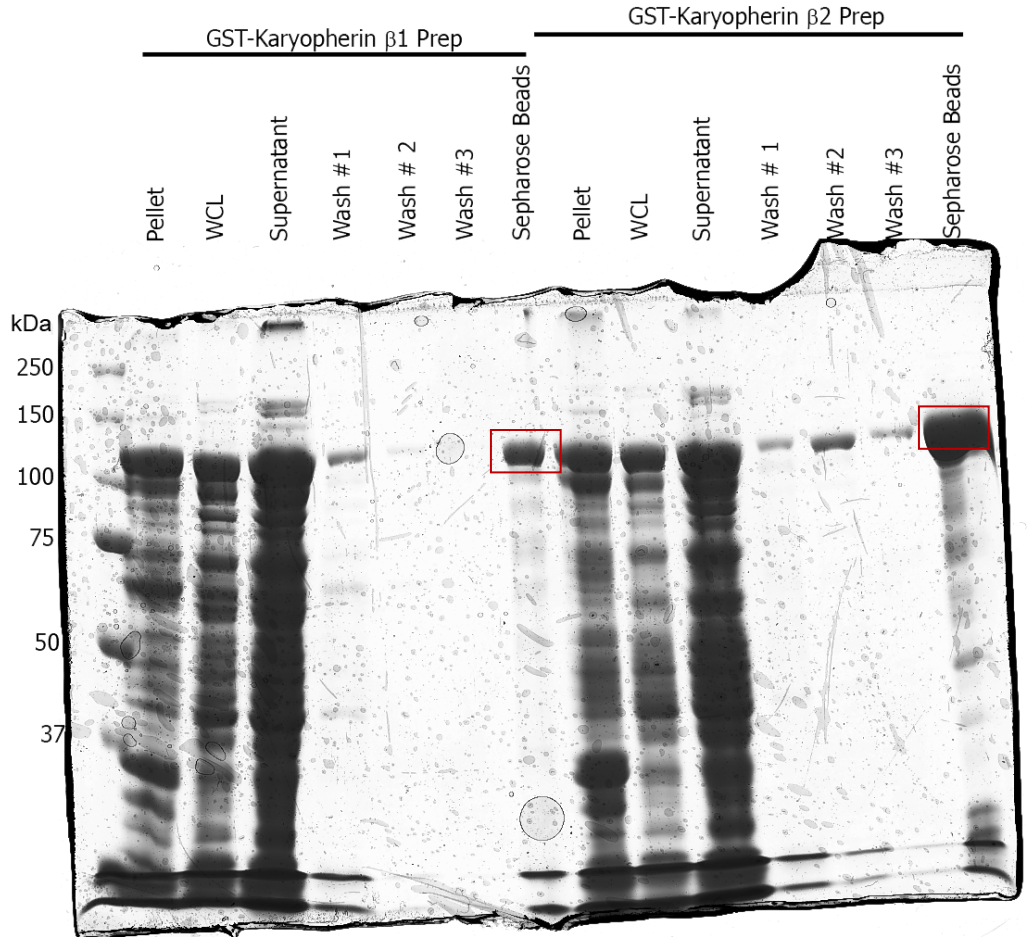
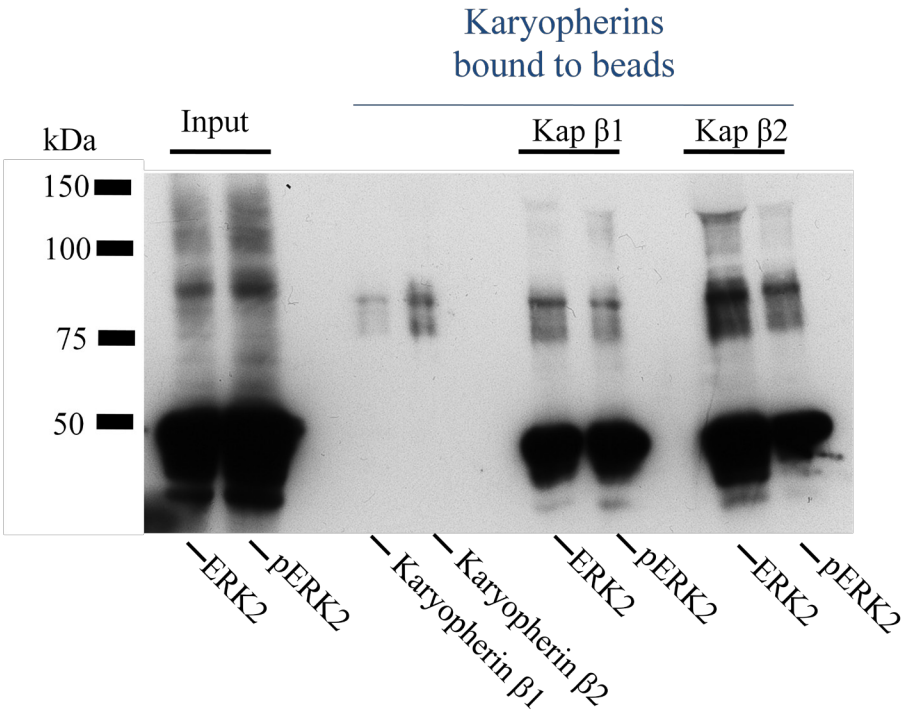


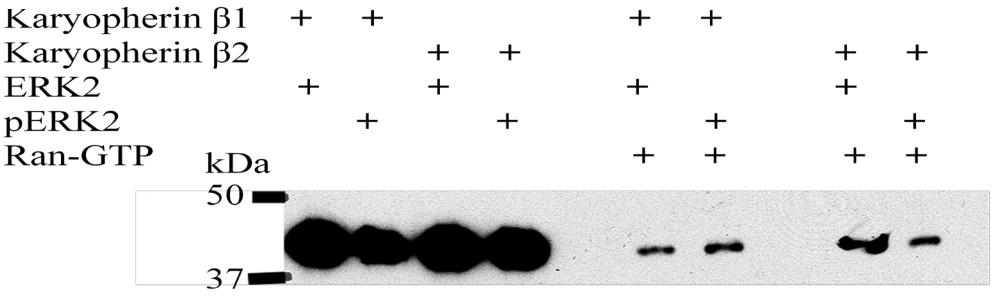

**Figure 4-1 Expression and purification of karyopherins β1 and β2**

A 12% polyacrylamide gel in SDS after staining in Coomassie blue. Bands outlined in red are karyopherin products bound to glutathione-Sepharose beads. WCL = whole cell lysate. Final lane of each product is of the karyopherins released from the glutathione-Sepharose-GST-karyopherin after denaturation.

A


Karyopherins
bound to beads
Input
Kap β1
Kap β2
kDa
150
100
75
50
ERK2
pERK2
Karyopherin β1
Karyopherin β2
ERK2
pERK2
ERK2
pERK2

B


Karyopherin β1 + + + +
Karyopherin β2 + + + +
ERK2 + + + +
pERK2 + + + +
Ran-GTP + + + +
kDa
50
37

C

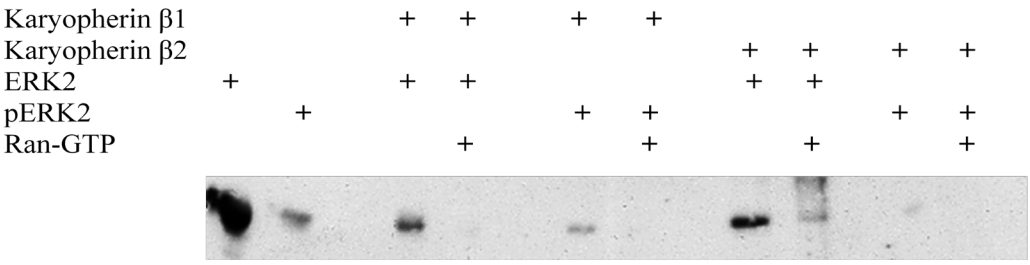

Karyopherin β1 + + + +
Karyopherin β2 + + + +
ERK2 + + + +
pERK2 + + + +
Ran-GTP + + + +

**Figure 4-2 Solution binding assays**

Panel A: Binding assay of ERK2, pERK2, karyopherin β1, and karyopherin β2. Immunoblot, 12% polyacrylamide gel, using the anti-serum Y691 to detect ERK2. Panels B and C: Two binding assays of ERK2, pERK2, karyopherin β1, karyopherin β2, and Ran-GTP. Immunobloted as in A.

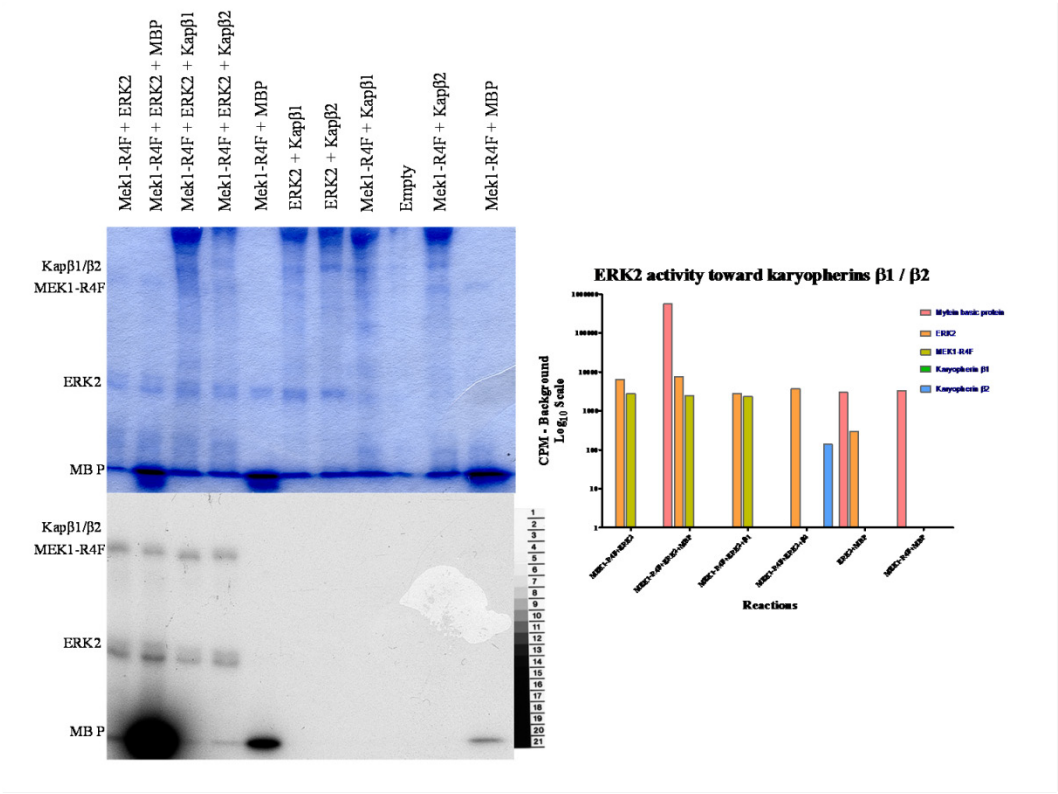



**Figure 4-3 Kinase assay of the karyopherins β1 and β2 as substrates of ERK2**

Upper left panel: Coomassie blue stain of kinase reactions. Lower left panel: Autoradiogram of kinase reactions. Right panel: Quantification of the kinase reactions by scintillation counting

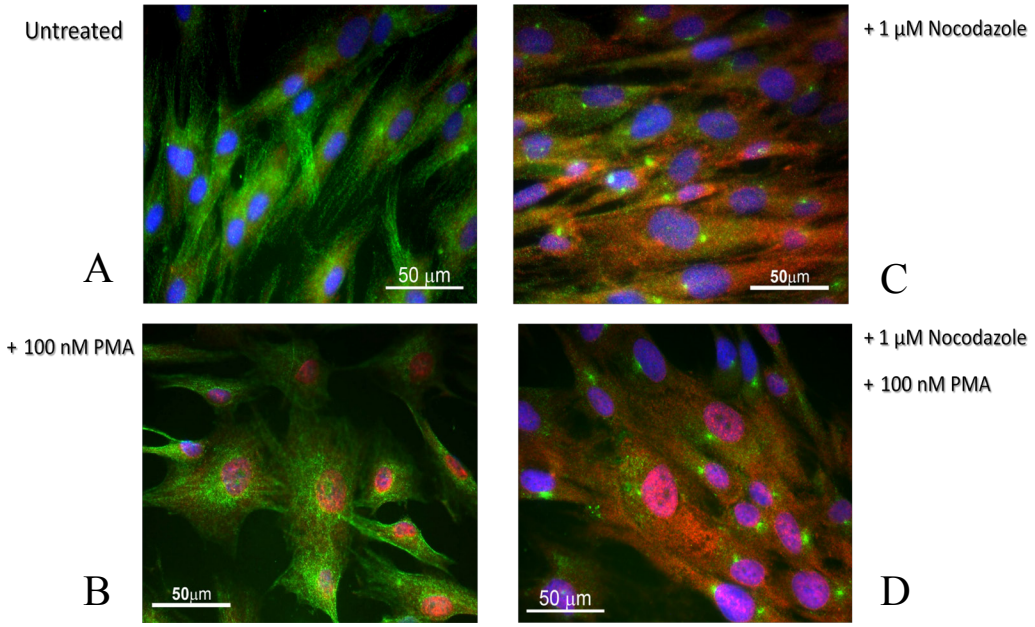



**Figure 4-4 Effect of nocodazole on ERK1/2 localization**

Panel A: Serum deprived BJ cells. Panel B: Serum deprived BJ cells treated with 100 nM PMA. Panel C: Serum deprived BJ cells treated with 1 µM nocodazole. Panel D: Serum deprived BJ cells treated with 100 nM PMA and 1 µM nocodazole. Colors in all panels: Green = anti-tubulin staining mono-clonal anti-αtubulin Red = anti-phsopho-ERK1/2 antibody (Sigma) staining. Blue = DNA staining by DAPI

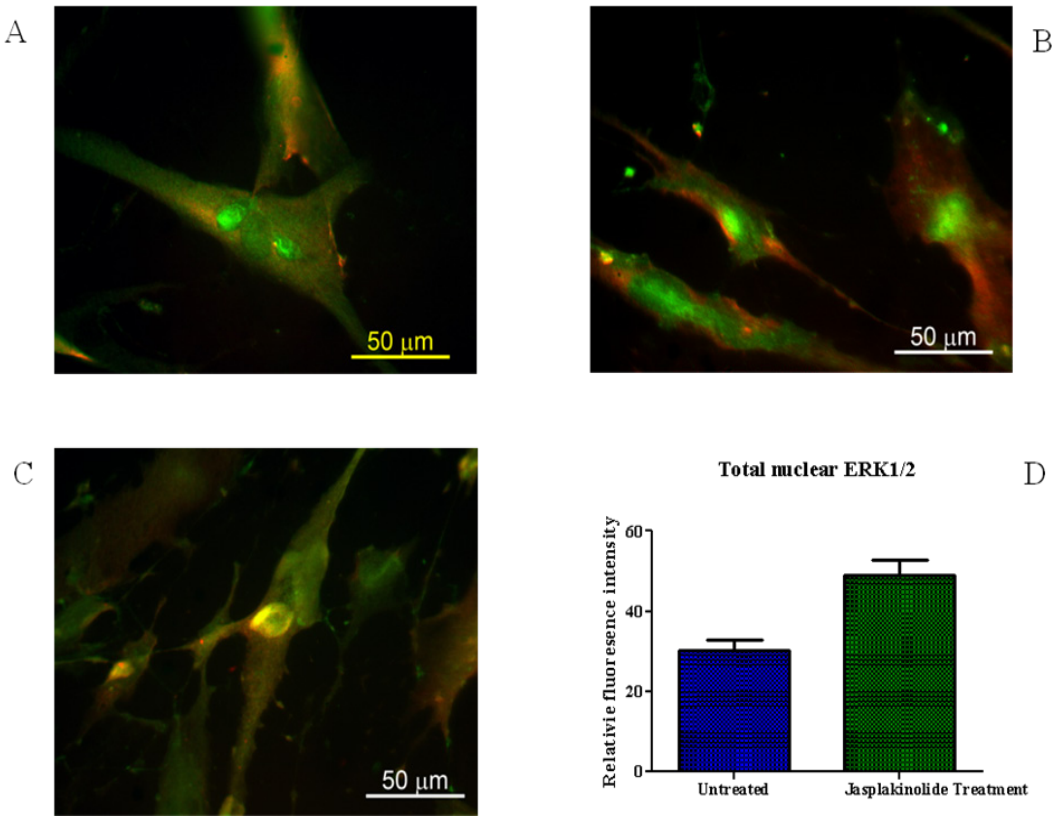



**Figure 4-5 Localization of ERK1/2 in cells treated with Jasplakinolide**

Panel A: Serum deprived BJ cells. Panel B: Serum deprived BJ cells treated with 0.5 µM jasplakinolide for 45minutes. Panel C: Serum deprived BJ cells treated with 0.5 µM jasplakinolide for 45 minutes and stimulated with 750 nM PMA for 15 minutes. Panel D: Quantification of relative fluorescence intensity in the nuclei. Untreated n = 6 coverslips, treated n = 4 coverslips, error bars = standard error of the mean approximately 6 nuclei counted per coverslip. Colors in all panels: Red = phospho-ERK1/2 (Sigma mAb), Green = Total ERK1/2 (anti-serum Y691), Blue = DNA (DAPI)

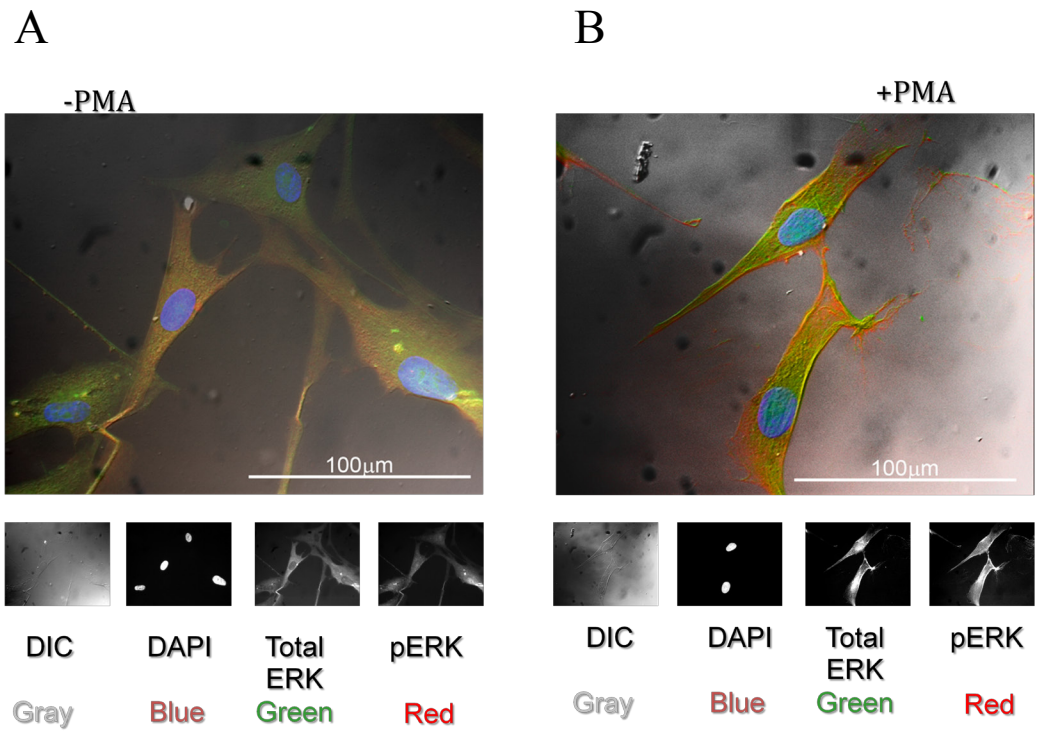



**Figure 4-6 Comparison of pERK and ERK showing relative discontinuity**

Upper panels composite images with DIC overlay. Lower panels individual channel images used to create the composite images. Panel A: serum deprived BJ cells. Panel B: PMA treated BJ cells. Total ERK detected with anti-serum Y691. pERK detected with monoclonal pERK antibody (Sigma)

## CHAPTER FIVE

### The regulatory effects of PEA15 expression and phosphorylation on ERK2

PEA15 is a 15 kDa protein that has regulatory effects on proliferation and programmed cell death. PEA15 associates with and regulates ERK1/2, RSK2, FADD (Ramos, 2005). Expression of PEA15 activates the Ras pathway, PKC and has been reported to increase the concentration of the glucose transporter Glut1 at the cell membrane of skeletal muscle cells (Condorelli *et al.*, 1998; Ramos *et al.*, 2000; Renault-Mihara *et al.*, 2006). PEA15 is overexpressed in type II diabetes and affects both glucose sensitivity generally and insulin secretion from β-cells. Additionally, expression of PEA15 is known to affect cell motility perhaps through regulation of integrin receptors at the plasma membrane. While it may seem PEA15 acts to regulate many different cellular pathways, it is more probable that PEA15 acts as a signal integrator, allowing crosstalk among pathways and affecting cellular outcomes by modulation of the cross-pathway traffic.

PEA15 contains an N-terminal death effector domain (DED) and a relatively unstructured C-terminus. PEA15 is regulated both translationally and by post-translational modifications. Two serine residues, S104 and S116, are targets of $Ca^{2+}$ activated protein kinases; additionally S116 is a target of Akt. Phosphorylation of these residues affects the stability and function of PEA15.

Our laboratory is pursuing two lines of inquiry regarding PEA15. First, does phosphorylation of the serine residues of PEA15 confer any specificity to the binding and regulation of ERK1/2 and phospho-ERK1/2? Second, how does PEA15 expression and

interaction with ERK1/2 regulate pancreatic β-cell function? To address these questions we are using mutational analysis of PEA15 and RNAi mediated knockdown of endogenous PEA15 in the context of *in vitro* assays including import, immunofluorescence microscopy, and cell motility.

Our laboratory strives to answer the question of how the MAPK cascade acts to integrate diverse signals into distinct cellular outputs by examining molecules that control the localization or activity of ERK1/2. One molecule that exerts spatial control over ERK1/2 is the DED-containing protein PEA15. PEA15 acts as cytoplasmic anchor and as a scaffolding protein for ERK1/2. In addition, PEA15 inhibits assembly of the death-inducing signaling complex by binding to FADD and caspase 8.

Messenger RNA encoding PEA15 has been found in all tissues examined; however, protein expression seems to be limited, suggesting that translation of the PEA15 message is under inhibitory control (Ramos, 2005). PEA15 is highly conserved with 100% amino acid identity among the proteins from hamsters, mice, and man. Like many proteins PEA15 is regulated by post-translational modifications. Phosphorylation of the C-terminal PEA15 residues S104 and S116 can occur through calcium concentration-dependent mechanisms. PKC phosphorylates S104 CamKII phosphorylates S116. The growth factor stimulated kinase Akt can also phosphorylate S116. PEA15 interacts directly with ERK1/2, RSK2, and FADD. Through these interactions PEA15 exerts direct regulatory influences over cell death and proliferation, (figure 5-1).

PEA15 is differentially phosphorylated, unphosphorylated, monophosphorylated and diphosphorylated forms are found (Kubes *et al.*, 1998). Kevin Dalby provided some

preliminary *in vitro* binding data that indicated differentially phosphorylated forms of PEA15 might have different affinities for phosphorylated and unphosphorylated ERK2. Work in our lab showed PEA15 acts as a cytoplasmic anchor for ERK1/2. I hypothesized that differential phosphorylation of PEA15 acts to change its specificity for active or inactive ERK1/2.

Aberrant expression of PEA15 has been identified in diseases such as squamous cell carcinoma, glioma, breast cancer, astrogliosis, type II diabetes, and most recently in polycystic ovary syndrome (PCOS) (Bera *et al.*, 1994; Condorelli *et al.*, 1998; Hwang *et al.*, 1997; Ramos, 2005; Savastano *et al.*, 2007; Sharif *et al.*, 2004; Tsukamoto *et al.*, 1999; Vigliotta *et al.*, 2004). A paradox of PEA15 expression profiles is that overexpression of PEA15 is known to inhibit cellular proliferation; however, PEA15 is highly expressed in tissues known for their proliferative capacity such as some tumors and astrocytes. The apparent dissociation between the high expression of PEA15 and proliferation in these tissues may in part be explained by the differential phosphorylation of PEA15 by its regulatory kinases.

PEA15 is a 15 kDa product of a gene at human locus 1q21-22. While the PEA15 message appears to be expressed in all tissues, the protein is most abundant in astrocytes and testis. As illustrated in figure 2-3, the protein consists of an N-terminal death effector domain, spanning residues 1 – 80, with residues 7 – 17 encoding a nuclear export signal. The C terminus of the molecule is relatively unstructured but contains the two serine residues that are kinase targets. It has also been reported that the very C terminus of the molecule encodes a 'reverse' D motif consisting of the sequence LxLxxxxKK (Callaway *et al.*, 2005). A peptide derived from the D motif in Elk1 competes with

PEA15 binding to ERK2. Suggesting the reverse motif of PEA15 is required for its docking interaction with ERK1/2.

PEA15 is a multifunctional molecule and the first DED-containing protein known to regulate cellular processes beside apoptosis (Ramos *et al.*, 2000) . In its anti-apoptotic role PEA15 binds to the death effector molecules FADD and caspase 8 blocking their association with the death initiator signaling complex. Phosphorylation is required for PEA15 to fulfill its role as an anti-apoptotic protein.

Ramos *et al*. cloned PEA15 in an expression screen for molecules that prevented Ras suppression of integrin activation in fibroblasts (Ramos *et al.*, 1998). Thus, expression of PEA15 is also known to affect integrin activity. At least part of the mechanism is due to binding of PEA15 to ERK1/2 and preventing their translocation to the nucleus. More recently PEA15 has been found to scaffold the activation of RSK2 by ERK1/2 (Vaidyanathan *et al.*, 2007). PEA15-regulated activation of RSK2 by ERK1/2 allows for activation and translocation of RSK2 with no concomitant nuclear localization of activated ERK1/2.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of $^{32}P$ labeled proteins by Araujo *et al*. showed that phosphorylation of PEA15 in astrocytes exposed to neurotransmitters is tightly regulated (Araujo *et al.*, 1993). Danziger *et al*. suggested the names of Pa, Pb, and N be given to the phospho-S104, phospho-S116, and the non-phosphorylated forms of PEA15 respectively based on the original names given to the spots on the 2D-PAGE gel run by Araujo *et al*. (Danziger *et al.*, 1995). In a review of PEA15 Renault *et al*. claim unpublished data from high resolution 2D-PAGE in conjunction with micro sequencing shows Pa actually consists of two sub-isoforms, Pa1

only phosphorylated on S116, and Pa2 only phosphorylated on S104 (Renault *et al.*, 2003). Phosphorylation of S104 on PEA15 has been implicated in the regulation of glucose metabolism, while phosphorylation of S116 has been shown to inhibit apoptosis and be required for the recruitment of PEA15 to the death-inducing signaling complex (Renganathan *et al.*, 2005). Other studies show the effects of phosphorylation of the serine residues are not quite so straightforward. One study suggested that phosphorylation of PEA15 at S104 is sufficient to prevent its binding to ERK1/2, while another study suggested phosphorylation of both S104 and S116 is required to disrupt ERK1/2 binding (Krueger *et al.*, 2005; Renganathan *et al.*, 2005). The effect PEA15 has on insulin sensitivity and Glut 1 and 4 translocations is based on observations in differentiating L6 skeletal cells (Condorelli *et al.*, 1999; Condorelli *et al.*, 1998; Condorelli *et al.*, 2001). A recent study of untransfected C5N keratinocytes and transfected HEK293 cells showed that phorbol esters can induce phosphorylation of S116, thereby increasing the stability of PEA15 by preventing its ubiquitinylation and subsequent proteasomal degradation (Perfetti *et al.*, 2007). The authors of that study were able to block the effect of phorbol ester by blocking activation of PKC-ζ or expression of a dominant negative form of PKC-ζ. In light of these conflicting studies, it is clear that phosphorylation of serine residues in PEA15 control both its function and stability; however, more studies are required to discern the function(s) of phosphorylation of the individual and combined residues and to resolve the apparent contradictions in the current body of literature.

A study of glioblastoma cells found PEA15 phosphorylated on S116 expressed in perinecrotic areas of tumors. The authors concluded that expression of PEA15 protects glioblastoma cells from glucose deprivation-induced apoptosis (Ekerot *et al.*, 2008).

High expression of PEA15, and its effect on cell survival was recently demonstrated in a comparison of testes from wild type and testis from PEA15 knock-out mice. Both the PEA15 protein and its mRNA were localized to the cytoplasm of Sertoli cells, all types of spermatogonia, and spermatocytes until the zygotene phase of meiosis in wild type testis. ERK2 was also localized to the cytoplasm of these cells. In contrast, in PEA15 knock-out mice ERK2 was primarily located in the nucleus of these germ cells. Testis from PEA15 knock-out mice also produced a significantly high number of apoptotic spermatocytes (Mizrak *et al.*, 2007). These studies lead to the idea that the regulation of ERK localization by PEA15 in conjunction with its anti-apoptotic activities is part of a cooperative system that ensures cell survival.

Overexpression of PEA15 has been linked to several diseases. PEA15 is expressed in some glioma and breast cancers. It has been suggested that these cancers rely on the anti-apoptotic function of PEA15 to extend cell survival. PEA15 is overexpressed in diabetes and PCOS. Savastano *et al*. found the increased expression of PEA15 in white blood cell lysates of PCOS patients that was correlated with increased insulin resistance regardless of body mass index (Savastano *et al.*, 2007). Several studies have linked overexpression of PEA15 to type II diabetes. Transgenic mice ubiquitously expressing PEA15 had mildly elevated blood glucose levels and decreased glucose tolerance. These mice developed diabetes on a fatty diet. Insulin reduced the blood glucose 35% after 45 minutes in the diabetic transgenic mice while, control mice had a 70% drop in blood glucose 45 minutes after insulin treatment. The effects of PEA15 overexpression were also examined in cultured Min6 β-cells. Excess PEA15 caused activation of PKC and a decline in glucose-stimulated insulin secretion. Blockage of the

endogenous PEA15 increased glucose sensitivity by 2.5-fold in the Min6 cells. It appears that PEA15 can act to impair the secretion of insulin in β-cells (Vigliotta *et al.*, 2004). Islets from transgenic mice that only overexpressed PEA15 in β-cells had reduced mRNA expression of the potassium channel subunits Sur1 and Kir6.2, and their upstream regulator Foxa2, as well as limited glucose-stimulated induction of PKCζ (Miele *et al.*, 2007).

We hypothesize that PEA15 has a direct role in regulating the glucose-stimulated activity of ERK1/2 in β-cells by controlling ERK2 localization. A key question I set out to answer was if phosphorylation of PEA15 had any effect on the binding affinity that might change its affinity and specificity for active or inactive ERK (Callaway *et al.*, 2005). It seems reasonable to conclude that the phosphorylation states of both the anchor and substrate molecules can affect the affinity. My plan was to create phospho-mimetic aspartic acid mutations at both PEA15 serine sites in conjunction with alanine mutations, which cannot be phosphorylated, at the same sites in order to test the change in affinity of ERK2 for PEA15 in the context of different phosphorylation states of both proteins. I also planned to examine the effect of these mutants on import and localization of both forms of ERK2 using import reconstitution assays and immunofluorescence microscopy of fixed cells. Unfortunately, Glading *et al*. published results showing mutation of S104 to aspartic acid did not inhibit invasion of breast cancer leading them to conclude phosphorylation of S104 abrogates binding to PEA15 (Glading *et al.*, 2007) . Although they demonstrated S104 phospho-mimetic mutations were adequate to prevent the spatial regulation of ERK2, it seems there may still be merit in studying the effects of the individual and combined phosphorylation of PEA15. By creating permutations of PEA15

that are differentially phosphorylated and expressing them in β-cells, the effect of pools of monophosphorylated and unphosphorylated PEA15 can be measured in the context of normal and diseased states. These data may be helpful in understanding the effect of PEA15 on localization of ERK1/2, as well as, provide tools for separating the anti-apoptotic role PEA15 from its role as an ERK regulator and scaffold.

**Experimental Methods**

*Plasmid constructs and sub-cloning*

We received a gift of hamster PEA15 from Joe Ramos. I designed DNA oligomers containing the mutations at each site and used pfu DNA polymerase in a PCR reaction to make the changes. Table 5-1 lists the mutations and the sequence of the oligomers used to make the mutants. After 19 cycles of PCR the reaction was digested for three hours with DpnI. After the digestion the reaction mixture was used to transform *E. coli* strain DH-10α. Bacterial colonies were grown on LB agar plates containing 100 μg/ml carbenicillin. After 18 hours viable colonies were selected and grown in small cultures for small scale DNA preparation. The DNA was digested with BamH1 and EcoR1. DNA that produced the appropriate bands on a 1% agarose gel was selected for sequencing. The sequence file was analyzed to ensure the presence of the mutation, and to ensure no other mutations occurred that affected the protein coding sequence. After sequence verification, a large scale DNA preparation was performed and stored at -20°C.

A cassette of the mutant cDNAs was created using PCR to insert flanking EcoR1 and HindIII restriction sites in the coding sequence. The sense primer containing the EcoR1 restriction enzyme site was, ccggaattcatggccgagtacgggactctc, while the antisense primer containing the HindIII restriction enzyme site was, ccaagctttcaagccttctttggtgggggagccaatttg. 5 ng of template DNA was used in a reaction containing 4 μM of each oligomer. After 30 PCR cycles the DNA was extracted in a 50% mixture of Tris-saturated phenol and chloroform containing a 24:1 dilution of isoamyl alcohol. Following extraction the DNA was precipitated and resuspended in deionized water. The PCR product was then digested with the restriction enzymes EcoR1

and HindIII. The digested DNA was gel purified and used in a ligation reaction containing $pHis_6$-parallel vector previously digested with EcoR1and HindIII and treated with calf alkaline phosphatase prior to gel purification. The digested plasmid and the PCR product were ligated using T4 ligase. The DNA was used to transform *E. coli* DH-10α cells. After 18 hours of growth at 37°C on LB plates containing100 μg/ml carbenicillin, viable colonies were selected for small scale preparations of DNA. The DNA was digested with EcoR1 and HindIII and analyzed on a 1% agarose gel. Samples that showed the appropriate banding pattern were selected for sequencing. Sequences files were then analyzed to ensure the insert was properly cloned into the vector and that no deleterious mutations had occurred. All PEA15 mutants and wild type DNA were subcloned into the $pHis_6$-parallel vector using the above described oligomers and procedure.

*Cell culture*

HeLa and BJ cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% FBS and 2 mM L-glutamine. In some experiments the medium also contained 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified incubator at 37°C in a 5% $CO_2$ atmosphere.

Ins1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 11 mM glucose, 10 mM HEPES, 2 mM L-glutamine, 1 mM Na-pyruvate, and 50 nM β-mercaptoethanol. Cells were incubated in a humidified incubator at 37°C in a 5% $CO_2$ atmosphere

*Transfection*

Cells were grown in 6 well plates with 3 glass coverslips in each well using standard medium without penicillin / streptomycin. When cells had reached the desired confluence of 50 – 75%, they were transfected with the appropriate DNA and Fugene6 transfection reagent. The Fugene6 was diluted in Opti-mem serum-free medium and allowed to equilibrate for 5 minutes at room temperature as per the manufacturer's instructions. Then 2 μg DNA was added and allowed to incubate for a further 15 minutes at room temperature. After incubation the DNA mixture was added dropwise to the individual wells containing the cells. Cell cultures were then returned to the incubator and harvested 18-36 hours post-transfection.

*Immunofluorescence microscopy*

Cells were placed in serum-free medium 2-4 hours before being treated with inhibitors or inducers such as U0126, PMA, and glucose. The cells were then placed on ice and rinsed in ice cold TBS. Cells were fixed with 4% para-formaldehyde in PBS for 10 minutes at room temperature. Following fixation cells were washed three times with TBS at room temperature. The cells were incubated with a 0.5% Triton X-100 in TBS at room temperature for 10 minutes then washed in TBS at room temperature. The cells were washed one time in TBST for 5 minutes at room temperature. Following the washes the cells were incubated in freshly prepared 1% BSA in TBST for 30 minutes at room temperature. The coverslips were transferred to a humidified chamber and incubated at 4°C overnight with primary antibody diluted 1:250 – 1:1,000 in BSA / TBST. Coverslips were washed three times in TBST at room temperature. The

coverslips were incubated for 1 hour at room temperature in the dark with secondary antibody consisting of a 1:3000 dilution of Alexa-Fluor 488 goat anti-rabbit antibody, a 1:3000 dilution of Alexa-Fluor 546 goat anti-mouse antibody, and a 1:7500 dilution of DAPI in BSA / TBST. After incubation, coverslips were washed three times in TBST. The coverslips were rinsed in deionized water and mounted on glass slides using Aqua-polymount.

Microscopic examination of the samples was accomplished using a Zeiss Axioskop2 plus microscope for epi-fluorescent examination or a Leica TCS SP5 confocal microscope. The Zeiss Axioskop2 plus was equipped with a Hamamatsu digital CCD camera (C472-95) and images were acquired using Slidebook 4.1 software (Intelligent Imaging Inovations Inc.) and analyzed using Adobe photoshop CS2 to expand the histogram and make composite images. Leica TCS confocal images were captured using a photo-multiplier tube integral to the microscope system and Leica imaging software. Confocal image histograms were expanded, and analyzed, and the final output produced was using Image J software from the NIH (Rasband, 1997-2004).

*RNAi*

Cells were grown to a confluence of 30 – 50% in medium free of penicillin / streptomycin. dsRNA oligomers were diluted to 1 μM in Opti-mem serum-free medium and incubated for 5 minutes at room temperature. Oligofectamine reagent diluted in Opti-Mem medium was then added to the dsRNA solution and incubated for 20 minutes at room temperature. The normal cell culture medium was replaced by Opti-mem serum-free medium after rinsing the cells in serum-free medium. After the addition of the

dsRNA solution, the cells were incubated at 37°C in 10% $CO_2$ for 4 hours, and then the transfection medium was replaced with normal growth medium. Cells were then treated and harvested according to the indicated experimental conditions.

*Wound healing assays*

Cells were grown in 6 well culture dishes to near confluence. The monolayer of cells was disrupted in a crossed line pattern using a pipette tip. The cells were then rinsed with fresh medium to wash away the dislodged cells. Differential interference contrast (DIC) microscopic images were captured near a readily identifiable intersection of wounds. Cells were returned to the incubator for the indicated time, then the previously imaged area was located and a new set of DIC images captured using a Zeiss Axiophot2 microscope.

**Results and Discussion**

*Differential phosphorylation of PEA15*

PEA15 is phosphorylated by two different $Ca^{2+}$ sensitive protein kinases as well as the growth factor induced protein kinase Akt. PKC phosphorylates PEA15 at S104 while either CamKII or Akt phosphorylate PEA15 at the S116 site. In our quest to identify mechanisms responsible for the differential nuclear translocation of the non-phosphorylated and phosphorylated forms of ERK2, PEA15 stood out as a cytoplasmic anchoring protein that could regulate this process. The plan was to examine effects of the mutants on ERK1/2 localization before and after stimulation of the cells with epidermal growth factor (EGF) or PMA, ligands that cause differential ERK1/2 localization in BJ cells. Additionally, I planned on testing the recombinant proteins in nuclear import reconstitution assays using both phosphorylated and unphosphorylated ERK2 as the import substrates.

Table 4-1 illustrates the eight mutations I made and the DNA oligomers used to produce these mutations in PCR-mutagenesis reactions. I embarked on a strategy of sub-cloning the mutations into the $pHis_6$-parallel vector for protein purification. In retrospect, although my strategy produced mutants to test, concentrating on the molecular biology and trying to finish all of my mutations before starting the cell-based experiments was a critical mistake. Shortly after I completed the initial mutations, Glading *et al*. published a paper examining the affect of PEA15 and mutation of S104 on tumor invasiveness. Their work indicated phosphorylation of S104 was sufficient to disrupt the binding of ERK2 to PEA15 and increase the invasiveness of tumors expressing PEA15.

After publication of their paper, we decided to pursue other aspects of ERK1/2 localization.

The PEA15 mutants I made will be used to further examine the role of PEA15 interaction with ERK1/2 in pancreatic β-cells. PEA15 may act to modulate the glucose-induced changes in ERK1/2 localization in these cells. Stimulation of pancreatic β-cells by glucose causes insulin secretion through triggering and amplification of signaling pathways. β-cells metabolize glucose by oxidative glycolysis which causes a change in the ATP / ADP ratio, leading to the closure of ATP sensitive $K^+$ channels. Closure of $K^+$ channels causes membrane depolarization which causes the opening of voltage-gated $Ca^{2+}$ channels. Increase in the free cytoplasmic $Ca^{2+}$ concentration activates the exocytotic machinery. Amplification of the signal occurs after membrane depolarization, requires glucose metabolism and is independent of protein kinase A and PKC activation (Henquin, 2000). Concomitantly the increase in $Ca^{2+}$ concentration causes activation of ERK1/2, and their nuclear translocation (Lawrence *et al.*, 2008a). Ins 1 cells are cultured with 11 mM glucose in the medium to prevent de-differentiation of the β-cells, however physiologic levels of blood glucose range from 2-10 mM (Gibson *et al.*, 2006). A blood glucose level of 5.5 mM is considered to be the normal fasting glucose concentration (Tirosh *et al.*, 2005). The confocal micrographs in figure 5-2 show the distribution of phospho-ERK1/2 and total ERK1/2 after stimulation of β-cells with glucose. Ins 1 cells in medium containing 11 mM glucose had little activation of ERK1/2 with all of the phosphorylated species confined to the cytoplasm. While cells cultured in 11 mM glucose, placed in 2 mM glucose medium for 2 hours, then reintroduced to 11 mM glucose for 15min showed activation and nuclear localization of active ERK1/2 in some

cells. The most robust activation and nuclear localization of active ERK1/2 occurred in cells that were cultured in 5.5 mM glucose, placed in 2 mM glucose containing medium for 2 hours, then placed in 11 mM glucose containing medium for 15 minutes. As expected there is minimal phosphorylated ERK1/2 in cells cultured in 5.5 mM glucose, transferred to 2 mM glucose medium for 2 hours, treated with the MEK1 inhibitor U0126 for 30 minutes, then transferred to 11 mM glucose containing medium in the presence of U0126. These are promising results. Cell fixation and transfection will need to be optimized in order to ensure an adequate number of transfected cells in the microscope field for meaningful statistical analysis.

Clinical and genetic studies have implicated overexpression of PEA15 in type II diabetes. One possible cause for PEA15 overexpression in these patients may be as an anti-apoptotic response to chronic inflammation caused by the disease process. Although, the transgenic mouse model of diabetes in which overexpression of PEA15 caused diabetes, may argue against the chronic inflation argument, it is important to note the mice did not develop diabetes unless they were fed a 60% fat diet. However, humans are different than mice therefore study of cultured human cells in conjunction with genetic screens of single nuclear polymorphisms within the 1q21-22 locus of normal, diabetic, and pre-diabetic populations may augment our understanding of this disease process and the cellular processes co-opted by it.

*Migration*

We began a pilot study to identify and validate putative ERK1/2 interactors using RNAi. Our strategy was to knock down both *bona fide* ERK1/2 regulatory proteins such

as KSR1 and PEA15 along with proteins identified in yeast two-hybrid experiments, by Svetlana Ernest in our laboratory, as having a putative interaction with ERK1/2. Our plan was to evaluate cells transfected with the interfering RNA oligomers for physical phenotypes that could be easily scored using standard microscopic techniques. We scored cells based on the following: changes in localization and activation of ERK1/2, gross changes in cellular morphology, and cell migration.

We selected HeLa cells for the initial study because of their ERK1/2 profile and due to the ease of RNA transfection. Since we did not have antibodies for many of the proteins we wished to screen, we also designed DNA primers to be used in conjunction with reverse transcriptase PCR (RT-PCR) to determine if messenger RNA levels of the target proteins were being reduced. Three first year graduate students assisted with the pilot study during their laboratory rotations. Rachel Greer knocked down KSR1, MP1 and PEA15 in the presence and absence of lysophosphotidic acid (LPA); Paul Dutchak knocked down KSR1, MP1, Sur 8, Sprouty 2, and PEA15 in the presence or absence of EGF. After knocking down protein expression, they screened the cells using epi-fluoresence microscopy in conjunction with immunohistochemical staining of total ERK2 and phosphorylated ERK1/2. They also harvested cells for analysis by RT-PCR. No clear conclusions could be drawn from these experiments.

Another rotating first year student Bingke Yu, used wound healing assays to study changes in cell migration. Bingke used dsRNA oligomers to knockdown the proteins listed above. After the initial wounding and microscopy she incubated the cells for 8 hours and took micrographs of the same area she originally wounded. Bingke measured the width of each wound 3-5 times taking vertically displaced measurements

each time. I analyzed her data normalizing to compare different wounds. The most straightforward method was to average her measurements and convert them to a percentage wound recovery by dividing the final wound width by the initial wound width and multiplying by 100. Knock down of PEA15 gave the most robust results as can be seen in figure 5-3. When PEA15 expression was attenuated, the HeLa cells migrated farther into the wound. The large error bar in the migration of control cells makes it difficult to interpret unequivocally, therefore additional experiments are clearly warranted to determine if there is a difference migration in cells with reduced PEA15.

I expected that knock down of endogenous PEA15 would lead to an increase in cell motility and a decrease in cell proliferation. My expectations were based on the assumption that active ERK1/2 constrained to the cytoplasm would regulate elements of the cytoskeleton and focal adhesions, while active ERK that was translocated to the nucleus would activate transcription factors and genes involved in proliferative processes would be up regulated. Our knock down of endogenous PEA15 caused an increase the rate of wound healing over the course of the study; however it is difficult to determine the role of proliferation versus migration in the wound healing assays. I would assume that the greatest change in cells in the wound would be due to an increase in cell migration given the short 8 hour time course. Additionally analysis cell density from the edge of the wound to the edge of the microscopic field before and after wounding decreases after 8 hours, indicating a change in cell mobility and not increase proliferation. Consistent with these results, in 1998 Ramos *et al*. used a screen for suppression of integrin activation to clone PEA15, while in 2006 Renault-Mihara *et al*. showed expression of

PEA15 in astrocytes inhibited migration via a PKC dependent mechanism (Ramos *et al.*, 1998; Renault-Mihara *et al.*, 2006).

The functions of PEA15 as a regulator of apoptosis and as a regulator of ERK1/2 localization and activity are certainly coupled. However, in the context of the paper by Vaidyanathan *et al*. that shows PEA15 directs activity of ERK towards RSK2 by acting as a molecular scaffold, we have expanded our paradigm and are now considering the effects of PEA15 on ERK1/2 activity as well as the control it exerts on ERK1/2 cellular location.

**Future directions**

In retrospect, the Chuderland *et al*. paper did not address all of the possibilities regarding regulation of ERK1/2 by PEA15. It has been suggested that phosphorylation of serine 116 is required for both the stability of PEA15 and for its role as an anti-apoptotic protein. Glading *et al*. focused on phosphorylation of serine 104 in the disruption of the ERK1/2 PEA15 complex. It is entirely possible that a phosphorylation 'code' exists for PEA15 governing its stability, selectivity, and localization. Furthermore, given the effects of PEA15 expression in pancreatic β-cells, the differential phosphorylation of PEA15 may have cell effects dependent on cell type and context. The mutant PEA15 construct may yet be useful in probing the regulatory mechanisms of pancreatic β-cells and their effects on glucose sensitivity and insulin secretion. An examination of RSK activity and localization within β-cells expressing PEA15 is also warranted in light of the recent work by the Ramos group.

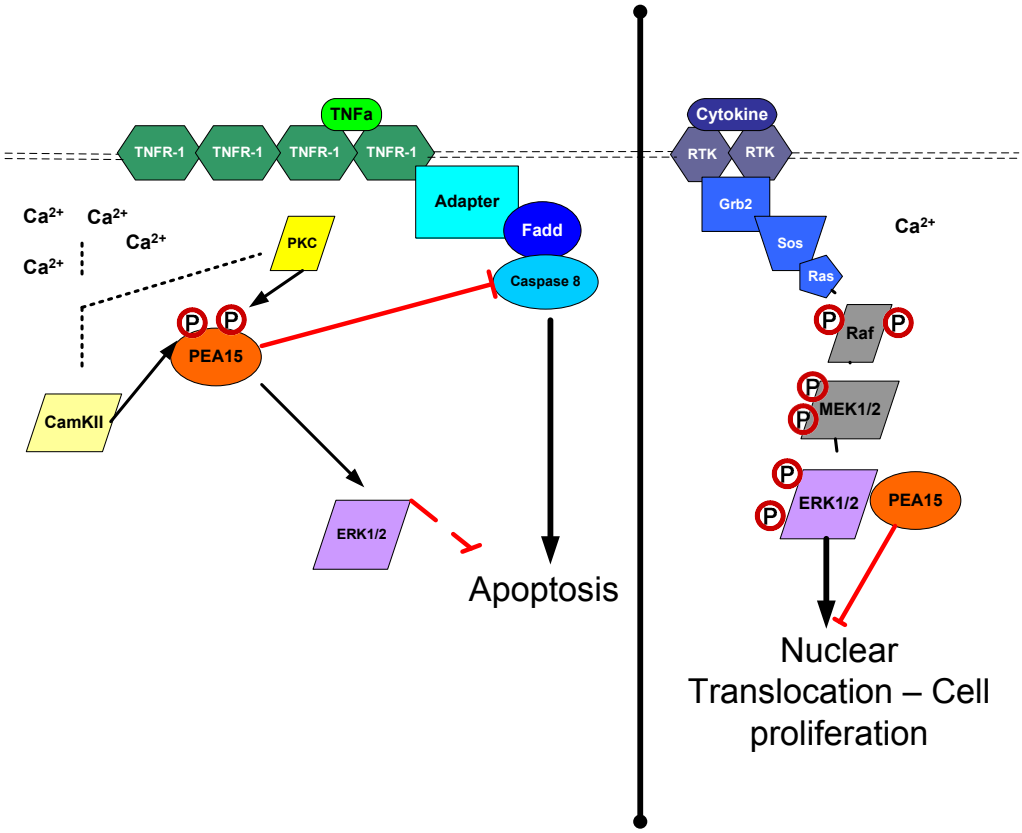

TNFa
TNFR-1
TNFR-1
TNFR-1
TNFR-1
Adapter
Fadd
Caspase 8
Ca2+
Ca2+
Ca2+
Ca2+
PKC
P
P
PEA15
CamKII
ERK1/2
Apoptosis
Cytokine
RTK
RTK
Grb2
Sos
Ras
Ca2+
P
Raf
P
P
MEK1/2
P
P
ERK1/2
PEA15
Nuclear
Translocation – Cell
proliferation

**Figure 5-1 Model of PEA15 function**

Left side of figure: TNFα binds to and activates TNFα-receptors. Adapter proteins bind to TNFα-receptors and the death effector FADD. FADD causes cleavage and activation of pro-caspase 8 and FADD and caspase 8 form components of the death-initiating signaling complex – which leads to the induction of apoptosis. However, if intracellular $Ca^{2+}$ concentrations are high enough they activate PKC and CamKII which phosphorylate PEA15 on serine 104 and 116. The pPEA15 binds to FADD and caspase 8 thereby disrupting the assembly of the death-inducing signaling complex and causing continued cell proliferation in an otherwise apoptotic environment

Right side of figure: In the absence of activation of CamKII or PKC, PEA15 binds to ERK1/2 acting as a cytoplasmic anchor and prevents activation of nuclear proteins regulated by ERK1/2.

| MUTANT | SENSE OLIGOMER | ANTI-SENSE OLIGOMER |
|---|---|---|
| PEA15 WILD TYPE | ---- | ---- |
| S104A | ctaacccgtatccccgctgccaagaagtacaaagac | gtctttgtacttcttggcagcggggatacgggttag |
| S104D | ctaacccgtatccccgatgccaagaagtacaaagac | gtctttgtacttcttggcatcggggatacgggttag |
| S116A | ccggcagcccgctgaggaagaaatcatc | gatgatttcttcctcagcgggctgccgg |
| S116D | ccggcagcccgatgaggaagaaatcatc | gatgatttcttcctcatcgggctgccgg |
| S104A / S116A | I used a previously made single mutation such as S104A as my template DNA and the appropriate sense and antisense oligomers to make the second mutation. | |
| S 104A / S116D | | |
| S104D / S116A | | |
| S104D / S116D | | |

**Table 5-1 PEA15 Mutants and primers**

Mutations made to the PEA15 serine 104 and serine 116 sites and the sequence of the DNA oligonucleotides used.

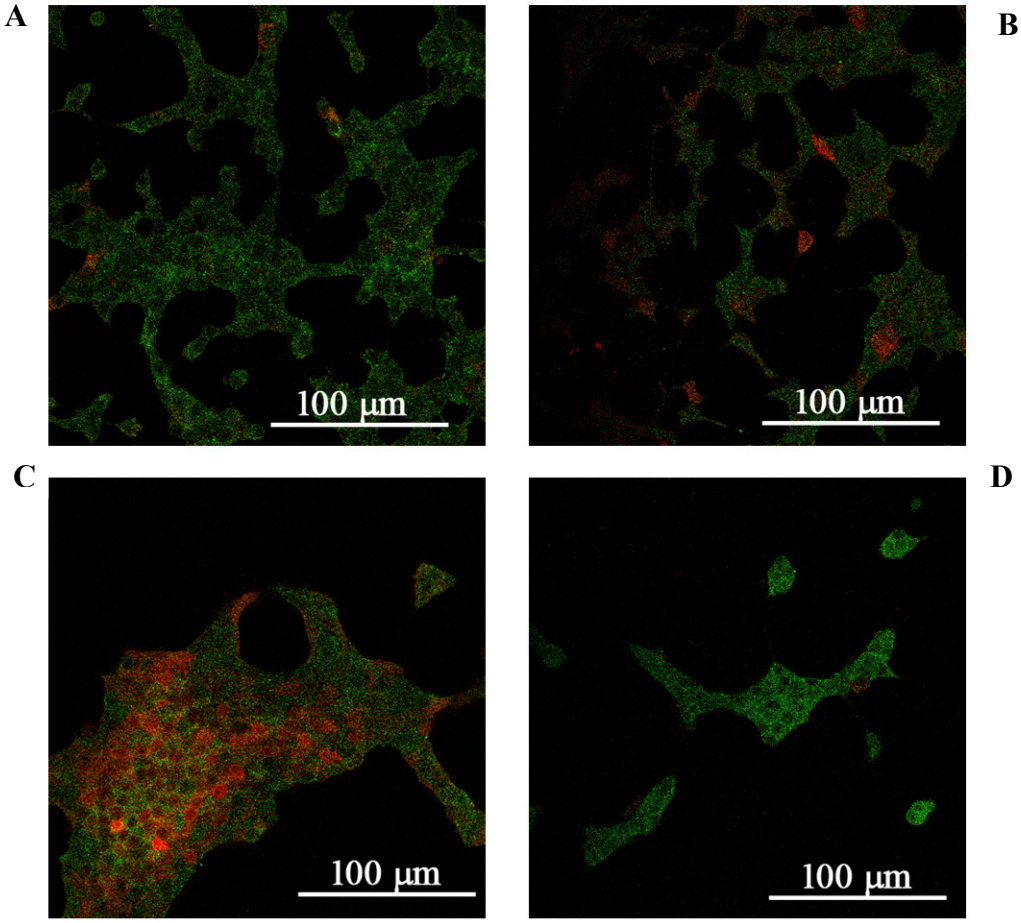
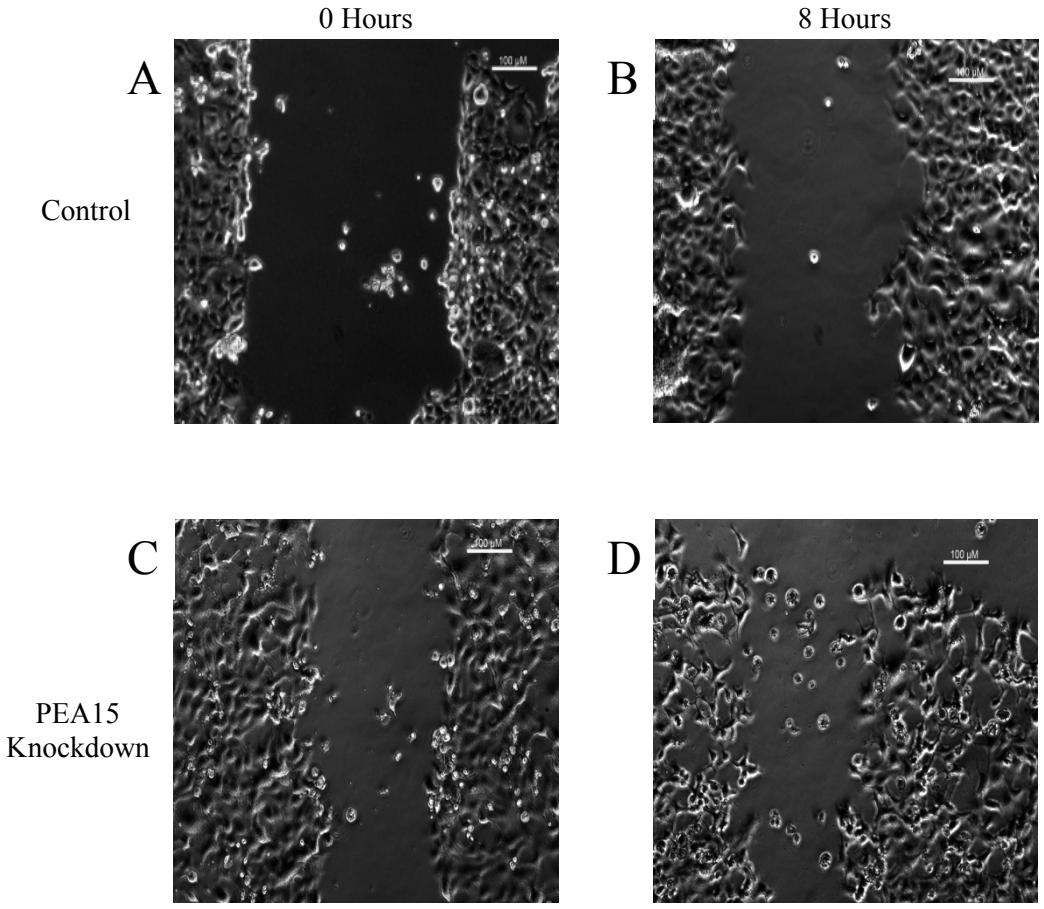

A
B
C
D
100 µm
100 µm
100 µm
100 µm

**Figure 5-2 Immunofluorescence Confocal microscopy of INS 1 cells stimulated by glucose**

Panel A: upper left. Ins 1 cells cultured in 11 mM glucose. Red = phospho-ERK1/2, Green = total ERK1/2. Panel B: upper right. Ins 1 cells cultured in 11 mM glucose, moved to 2 mM glucose 2 hours, then stimulated with 11 mM glucose 15 minutes. Panel C: lower left. Ins 1 cells cultured in 5.5 mM glucose, moved to 2 mM glucose for 2 hours, then stimulated with 11 mM glucose 15 minutes. Panel D: lower right. Ins 1 cells cultured in 5.5 mM glucose, moved to 2 mM glucose for 2 hours, treated with U0126 for 30 minutes, and stimulated with 11 mM glucose for 15 minutes in the presence of U0126.


0 Hours
8 Hours
A
B
Control
C
D
PEA15
Knockdown
100 µM

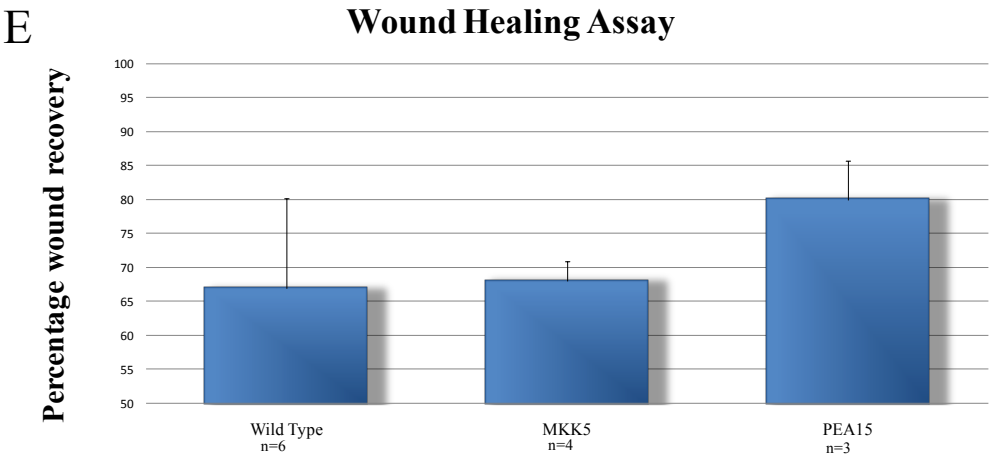

E
Wound Healing Assay
Percentage wound recovery
100
95
90
85
80
75
70
65
60
55
50
Wild Type
n=6
MKK5
n=4
PEA15
n=3

**Figure 5-3 Effect of PEA15 knockdown on migration**

Panel A: Initial wound in HeLa cell monolayer – control cells. Panel B: Wound after 8 hours in control cells. Panel C: Initial wound in HeLa cell monolayer, PEA15 knockdown cells. Panel D: Wound after 8 hours in PEA15 knockdown cells. Panel E: Quantification of migration as a percentage of the change in wound width, error bars are standard error of means$\left(SE = \frac{SD}{\sqrt{n}}\right)$. n = number of wounds counted.

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