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SCRIBBLE: A POTENTIAL DUAL KINASE INHIBITOR

Steven Christofakis
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SCRIBBLE: A POTENTIAL DUAL MAP KINASE INHIBITOR

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University.

by

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Richmond, Virginia
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Cheers.
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Abstract

SCRIBBLE: A POTENTIAL DUAL MAP KINASE INHIBITOR

By Steven C. Christofakis, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University.

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Extracellular signal-regulated kinases (ERKs) modulate cellular activities in response to extracellular stimuli and play important biological roles. Thus, perturbed kinase pathways induce pathological conditions, such as tumor development. Rit, a novel member of the Ras family GTPases, activase ERK6, and its over-expression confers tumorigenicity. We hypothesized the presence of scaffolding molecules specific to ERK6, similar to other known MAP kinases. We performed yeast two-hybrid assays using ERK6 as bait, and Scribble was identified as a binding partner. Scribble contains 16 LRR domains and four PDZ domains. We performed immunoprecipitation (IP) assays and discovered ERK2 as another binding partner. Surprisingly, no interaction was observed
with the highly homologous MAP kinase, ERK1. No other representative kinases showed binding capabilities with Scribble. IP data confirmed that both ERK2 and ERK6 bind to Scribble through its LRR and PDZ domains. Deletion of ten amino acids from the C-terminus of ERK2 and ERK6 abolished these interactions. *In vitro* kinase assays indicated the kinase suppressing ability of Scribble. Focus formation assays were performed with RitQ79L and H-RasV12 as constitutive activators of ERK6 and ERK2, respectively, in the presence of Scribble. Results confirmed the role of Scribble as a tumor suppressor.
Introduction

1.1 Cancer

At a fundamental level, cancer can be defined as a continuous uncontrolled proliferation of cells. These types of cells are unique in the sense that they do not respond appropriately to cellular signaling events that normally control cell behavior. Instead, cancer cells continue to grow and divide in an unregulated manner. This unregulated proliferation may lead to cancer cell invasion of normal tissues, eventually spreading to distant organs, a process known as metastasis (Fidler 2003; Lodish, 2004).

Development of tumors is believed to evolve from a single cell that acquires an abnormal proliferative advantage. This single progenitor cell does not acquire all characteristics of a cancer cell or the ability to survive on its own, but rather, progressively attains malignant properties through an accumulation of preferential aberrant transformations (Klein, 1998; Lodish, 2004).

In general, several independent and cooperative characteristics can be attributed to cancer development. First, these cells have reduced growth requirements. Whereas many cells normally produce growth factors, cancer cells frequently produce an uncontrolled increase in amounts of growth factors and/or receptors, and therefore are often able to support their own growth independent of signaling pathways, a process known as autocrine signaling. These factors are typically employed for orchestrated cell growth and
maintenance of systemic organs. Second, cancer cells have a greater insensitivity to growth arrest mechanisms. When growth factors are present in abnormally high concentrations, normal cells typically undergo growth arrest. Cancer cells, on the other hand, are much less sensitive to these types of effects. Third, cancer cells lose anchorage dependency, typically required for growth in normal cells. This is often due to a reduced expression of cell adhesion molecules, ultimately contributing to the ability of malignant cells to metastasize and invade other tissues. Fourth, neoplastic cells frequently display morphological transformations, such as high nucleus to cytoplasm ratios, aberrant growth habits, a high mitotic index compared to normal cells, and a loss of anchorage dependency for growth. Lastly, cancer cells exhibit loss of contact inhibition. When two or more cells, derived from normal tissues, come in contact with each other, cell growth is commonly arrested. Cancer cells lose this characteristic and will continue to grow without any form of spatiotemporal regulation (Hanahan, 2000; Lodish, 2004; Ramaswamy, 2003).

In 1971, Knudson proposed an epoch-making hypothesis, which, derived from statistical analysis of 48 retinoblastoma cases, suggested that multiple “hits” to a cell’s DNA were required to causes cancer (Knudson, 1971). Tumorigenesis was later found to depend on two forms of “hits”, namely, activation or upregulated expression of proto-oncogenes, genes that code for proteins aiding in cell growth and differentiation regulation, as well as deactivation and/or downregulation of tumor suppressor genes, which protect cells from acquiring tumorigenic potential by acting as a sensor of regulated and controlled cell proliferation (Lodish, 2004).
Extensive studies from a wide variety of cancers aided researchers with the identification of numerous causalities of cancer, including radiation, diverse carcinogenic reagents, and certain types of viruses as well. Radiation and chemical carcinogens elicit damage to DNA, inducing genetic mutations in the process, which are commonly referred to as initiating agents, as these mutations of target genes are believed to cause initial events eventually leading to tumorigenesis. However, not all carcinogens contribute to cancer development through the induction of genetic mutations but rather by stimulating cell proliferation, which are referred to as tumor promoters, as they stimulate cell growth by mimicking a wide variety of growth-upregulating molecules required by cell populations in early stages of neoplastic growth (Lodish, 2004). Such carcinogens can potentially cause both genetic and epigenetic modifications in cells, promoting clonal expansion, induction of genomic instability, and finally, transformation into neoplastic cells. In such cases, carcinogens may induce genetic damage to cells that provoke an altered responsiveness to the surrounding microenvironment and a proliferative advantage to normal surrounding cells (Lodish, 2004; Loeb, 2008).

The effects of radiation on tumorigenesis were first recognized shortly after the discovery of X-rays. Since then, many studies have revealed certain fundamental effects of radiation on cancer development. Radiation has potential to induce several genetic alterations, including nucleotide base damage, DNA cross-linking, single and double strand breaks. In general, whenever such alterations occur, DNA repair systems are activated in an attempt to restore damaged DNA induced by radiation, which may
potentially cause specific loss of function or gain of function genetic mutations resulting in genetic instability and ultimately, carcinogenesis (Little, 2000).

Several viruses have been found to play a large role in the formation of tumors, as it is estimated that 15% of all human tumors are a result of viruses. Some retroviruses, using reverse transcriptase, an enzyme transcribing single stranded RNA into double stranded DNA, can physically integrate DNA into the chromosomal DNA of a host and transduce oncogene expression. Other retroviruses are able to modify gene expression by inserting a provirus near strong promoter and enhancer sequences of initially silenced proto-oncogenes to induce gene expression. Furthermore, certain DNA tumor viruses promote the expression of viral oncoproteins that bind and inhibit cellular tumor suppressing proteins, such as p53 and pRb, abolishing their normal suppressive functions and disrupting innate mechanisms involved in cell growth control (Butel, 2000).

1.2 Cell Surface Receptors: Receptor Tyrosine Kinases (RTKs) and GPCRs

Cell signaling is a form of cellular communication that is responsible for orchestrating complex and tightly regulated cell activities, including development, growth, damage repair, homeostasis, immunity, and death. Cells receive information from the extracellular environment via receptors activated by specific molecules, such as hormones, neurotransmitters, cytokines, and growth factors. These ligands bind to specific receptors distributed on the surface of lipid bilayers of target cells, consequently activating the receptors and ultimately a series of intracellular, downstream effector proteins or channels.
that control such processes as ion flux across the plasma membrane and DNA translation (Lehninger, 2005; Simon, 2000).

Some extracellular cell surface receptors have a direct physical contact with intracellular enzymes and substrate proteins. One such representative family includes members of the receptor tyrosine kinase (RTK) family. This family of receptors is able to phosphorylate substrate proteins on tyrosine residues. When extracellular ligand and receptor binding occurs, cytosolic kinase domains are activated by the autophosphorylation of specific intracellular receptor domains, leading to intracellular target protein phosphorylation, and allowing for the sequential activation signaling cascades (Gschwind, 2001; Lehninger, 2005).

Figure 1.1. RTK Dimerization and Autophosphorylation. This diagram shows the interaction that occurs between activated RTKs upon activation by, in this example, an arbitrary growth factor. Receptors dimerize and then are able to cross-phosphorylate one another.
In many receptor tyrosine kinases, upon activation via ligand binding, receptor dimerization occurs, where either two or more identical (homodimerization) or different (heterodimerization) receptors bind to each other to form functional receptors. This receptor conformation leads to the autophosphorylation of cytosolic domains of the receptor, allowing for upregulated receptor kinase activity and creating docking sites for additional proteins that can induce diverse signaling events further downstream. Binding between downstream signaling proteins and the receptor occurs via domains that bind to specific phospho-tyrosine containing peptides, such as the Src Homology 2 (SH2) domain, a conserved modular domain responsible for the moderation of specific protein-protein interactions, originally identified in studies of Src family of proto-oncogenic tyrosine kinases (Ozaki, 2002). Furthermore, this association tethers proteins to the plasma membrane, which can allow binding of additional proteins, promote phosphorylation of these proteins, and subsequently, induce enzymatic activities (Huse, 2002; Lehninger, 2005).

Another family of cell receptors, referred to as G Protein-Coupled Receptors (GPCRs) transmit extracellular signals to cytosolic targets through the binding of guanine nucleotide binding proteins, or G proteins. GPCRs are functionally and structurally characterized by seven membrane-spanning α-helices. Heterotrimeric G proteins consist of three distinct subunits: α, β, and γ. The α subunit, G_{α}, is the regulatory subunit that binds guanine nucleotides in one of two forms, guanosine diphosphate (GDP) or guanosine triphosphate (GTP), and has the potential to directly regulate ion channel activities. Furthermore, β and γ subunits form a tightly binding, propeller-like complex, referred to as
Gβγ, that acts as an independent signaling molecule, also activating other second messengers or ion channels. G proteins associated with these receptors can be either stimulatory or inhibitory, regulating distinct intracellular targets and playing specific roles in signal transduction events. These heterotrimeric G protein complexes are composed of unique combinations of Gα and Gβγ subunits, and together with GPCRs and their ligand, determine the extent to which downstream signaling occurs, qualitatively and quantitatively.

![Figure 1.2. GPCRs and Subsequent Signaling Events.](image)

**Figure 1.2. GPCRs and Subsequent Signaling Events.** After GPCRs are activated with ligand binding, Gα is activated, consequently activating adenylyl cyclase activities, and propagating downstream signaling with cAMP.

In resting states, GDP bound Gα and Gβγ subunits are tethered together, localized at intracellular side of the plasma membrane. Upon ligand binding, induction of a conformational change of the receptor allows intracellular domains to directly interact with G proteins, stimulating GDP release in exchange for GTP. This prompts the dissociation of the GTP bound Gα subunit from the Gβγ complex, both inducing specific intracellular
responses. Cyclic adenosine monophosphate (cAMP) production, an important second messenger involved in intracellular signal transduction, is dependent on adenylyl cyclase (AC), an enzyme activated by GTP bound $G_\alpha$. Triggered $G_\alpha$ activity ceases upon GTP hydrolysis, allowing GDP bound $G_\alpha$ to once again form a complex with the $G_{\beta\gamma}$ subunits (Lodish, 2004; Vetter, 2001).

### 1.3 MAP Kinases

Mitogen activated protein kinases, or MAP kinases, are a family of highly conserved eukaryotic serine/threonine kinases that play important roles in signal transduction in response to diverse extracellular stimuli, such as growth factors, signaling molecules, and other extracellular stimuli, including mitogens, pro-inflammatory cytokines, heat shock, osmotic stress, and mechanical stress. The downstream effects of activated MAP kinases include a variety of cellular responses such as mitosis, proliferation, cell growth, cell survival, response to stress stimuli, differentiation, and apoptosis (Garrington, 1999). Extracellular signal regulated kinases (ERKs), p38 isoforms, and Jun kinases are the best-known MAP kinase sub-families participating in such cellular activities described above. ERKs play central roles in cell proliferation via both receptor tyrosine kinases and GPCRs. In addition, ERK signaling generally has the ability to “cross-talk” with other independent signaling pathways, for example, cAMP pathways (Lehninger, 2005; Widmann, 1999).

Activation of the ERK signaling cascade occurs through phosphorylation by diverse upstream signaling molecules and events. From the time that a stimulus is
presented at the extracellular domain of a receptor, propagation of the stimulus continues through a series of phosphorylation events, activating subsequent molecules (Chang, 2001). These events begin with the activation of small G proteins with the exchange of GDP for GTP. Among these small G proteins, Ras is one of the best studied. As a consequence of Ras activation, the Raf protein, a serine/threonine kinase, is activated. In turn, MEK is activated, which is the dual-specific kinase responsible for the phosphorylation of threonine and tyrosine residues on ERK proteins, promoting further phosphorylation of downstream targets, such as other protein kinases and transcription factors (Avruch, 2001; Lehninger, 2005).

Figure 1.3. MAP Kinase Signaling Events. This simplified model highlights the major components of ERK signaling. From binding of an extracellular signal to the receptor, Ras activates Raf, which further activates MEK, and finally, ERK activation results in induction of downstream effects, such as cell growth and proliferation.
1.4 Ras and Rit

Historical studies of rat sarcomas led to the identification of the Ras protein (both Harvey, H-Ras, and Kirsten Ras, K-Ras, oncogenes), eventually recognizing the ERK signaling pathway as a mediator of Ras activated signaling events (Dunn, 2005). Ras studies further identified several Ras homologues, which comprise the Ras family members, and revealed the principal role of mutations of ras genes in tumorigenesis. The basic roles of Ras in oncogenesis was clarified in a set of experiments where over-expression of Ras, or expression of dominant active Ras, upregulated cell proliferation in normal mammalian cells, and, at the same time, inhibition of Ras expression, or expression of dominant negative Ras, downregulated cell proliferation in the presence of growth factors. This relationship further suggested that Ras is required for normal cell growth as well as abnormal cell growth in, for example, tumor development (Kerkhoff, 2001; Lodish, 2004).

Ras exhibits GTPase activity in which the protein alternates binding between GDP (inactive state) and GTP (active state). Ras, in an inactive state, is modified by proteins known as guanine nucleotide exchange factors (GEFs) which promote the active form of Ras through the exchange of GDP for GTP. Opposite to this effect, GTPase activating proteins (GAPs) counter the effects of GEFs and stimulate GTP hydrolysis, converting Ras back into an inactive, GDP bound state. When specific mutations in ras genes occur, such as RasG12V (known as H-RasV12) or RasQ61K (known as RasK61), GTP hydrolysis is inhibited and Ras becomes constitutively active, activating downstream targets in the MAP kinase signaling pathway, compelling an unregulated proliferation of cells (Ackermann,
2005; Moret, 2008). Other mutations decrease Ras functions, such as RasN17, a dominant negative Ras mutant that favorably binds GDP, allowing inhibition of endogenous Ras activation by sequestering Ras-GEFs, subsequently inhibiting cell proliferation (Stewart, 2000).

Figure 1.4. The Ras Cycle. Ras GTPase activities are reliant upon activation status. GEFs promote the exchange of GDP for GTP, activating Ras. GAPs promote the hydrolysis of GTP to GDP, inactivating Ras.

Ras activation begins upstream, with, for example, activation of a receptor tyrosine kinase. As previously mentioned, these receptors become autophosphorylated upon extracellular ligand binding. This results in the formation of an association between GEFs,
for example, the Son of Sevenless, or Sos, protein, and the intracellular domain of the receptor. When receptor phosphorylation occurs, Growth Factor Receptor-Bound Protein 2, or Grb2, an adaptor protein, binds Sos at the plasma membrane where it is then able to activate Ras, which is anchored to the inner leaflet of the plasma membrane (Egan, 1993; Lodish, 2004).

**Figure 1.5. Sos and Grb2 Dependent Ras Activation.** The Sos protein binds Ras at the plasma membrane, which is connected to a receptor via Grb2. Receptor activation induces downstream signaling events, from Grb2 to Sos, and ultimately leading to Ras and Raf activation.

The Ras superfamily is traditionally comprised of five sub-family members known as Ras, Rho, Rab, ARF, and Ran. Recently, Ras-like protein in tissues (Rit) was identified as a novel branch of the Ras superfamily. Rit has been found to be highly expressed in most embryonic and adult tissues but very little is known about the protein and its biological functions in eukaryotic cells. Recently, it has been shown that Rit, in
cooperation with Raf, has the ability to transform NIH3T3 cells independently of MAP kinase pathway signaling (Sakabe, 2002). Furthermore, over-expression of Rit alone can transform NIH3T3 cells and effectively promote p38γ (also known as ERK6) activation, a member of the stress activated p38 MAP kinases, which has been suggested to regulate the transforming ability of Rit via discrete pathways independent of other Ras signaling pathways (Sakabe, 2002).

1.5 PI 3-K/Akt Pathway Signaling

Eukaryotic cells exhibit specific evolutionarily conserved cell signaling pathways responsible for promoting apoptosis, as well as pathways for the inhibition of apoptosis and promotion of cell survival. Of these signaling pathways, the phosphoinositide 3-kinase (PI 3-K) induced pathway is one of the primary cell survival pathways. PI 3-K activation begins with receptor protein tyrosine kinase or GPCR activation via ligand binding, similar to the MAP kinase pathway. Upon activation, PI 3-K phosphorylates PIP2, an inner membrane phospholipid, to form PIP3, a second messenger (Cantley, 2002). A downstream effector of this newly phosphorylated phospholipid is Akt, a serine/threonine kinase that subsequently phosphorylates several downstream targets controlling apoptosis that inhibit the activities of proteins that promote cell death, such as the Bad protein (Datta, 1997). Furthermore, Akt, activated by an independent protein kinase known as phosphoinositide-dependent kinase (PDK), can activate several transcription factors promoting cell survival and inhibiting cell death (Belham, 1999; Lehninger, 2005).
Akt signaling begins with receptor activation via ligand binding. PI 3-K activation, through phosphorylation by the receptor, converts PIP$_2$ to PIP$_3$. PIP$_3$ and an independent kinase, PDK, can activate Akt kinase functions. Akt is commonly activated in many cancers, leading to amplified effects of cell survival, growth, and metabolism in parallel with an increased resistance to apoptosis. As described above, Akt activation is dependent on the activities of PI 3-K and its phosphorylation of phospholipids, and therefore, inhibition of PI 3-K activity restrains the kinase activity of Akt. It is now known that the PI 3-K/Akt and MAP kinase pathways have interdependence of some common proteins, such as Ras, which can promote the kinase activity of Akt when constitutively active, promoting cell proliferation and survival (Cantley, 2002). Akt has numerous possible downstream phosphorylation targets and can activate diverse signaling cascades. Many studies have revealed that one such representative target is be the mammalian target of rapamycin protein (mTOR), a kinase
involved in cell proliferation and carcinogenesis (Hay, 2005). The complex Akt and mTOR relationship is one that has not yet been fully elucidated. However, intensive studies have revealed several important aspects of this interaction. With the presence of PIP3 and PDK phosphorylation of Akt at a specific threonine residue, Akt becomes activated. mTORC2, an mTOR protein complex formed in cells, can also phosphorylate Akt, but on a serine residue. With phosphorylation at both sites, Akt becomes fully activated, leading to cell growth and division. However, mTORC2 is considered to be a downstream target of PI 3-K/Akt signaling as phosphorylation of the serine residue on Akt by mTORC2 can be prevented with PI 3-K inhibitors and stimulated with growth factors. Therefore, Akt is able to regulate the mTOR pathway, ensuring that proper signaling occurs for cell growth and division in response to a variety of growth factors (Feng, 2010).

1.6 **Scaffolding Complexes**

Biochemical signaling cascades are an essential feature of cells, allowing for a response to a specific extracellular stimulus. Proteins in these signaling cascades function in a spatiotemporal and concentration dependent manner, an effect generally proportional to the intensity of the input stimulus. One way that proteins are able to achieve specific downstream effects is through the formation of signaling complexes. Signaling complexes are an aggregate of proteins that are co-localized to specific intracellular regions and usually dissociate upon activation. The actual physical interaction between proteins may occur through either direct protein-protein interactions or are mediated through scaffolding, or anchoring, proteins. Several MAP kinases have been found to assemble
into these scaffolding complexes along with other signaling molecules, such as those shown in Table 1, examples which include β-arrestins, connector enhancer of KSR (CNK), kinase suppressor of Ras (KSR), MEK kinase 1 (MEKK1), MEK-binding partner 1 (MP1), suppressor of Ras-8 (SUR-8), and JNK-interacting proteins (JIP) -1, -2, and -4. These scaffolding complexes have three primary functions: first, they are able to tether and co-localize signaling components, second, they are responsible for regulating pathways and aiding in the phosphorylation of other proteins in the cascade, and third, to insulate involved proteins from competing proteins, preventing “cross-talk” with other possible unrelated, interacting proteins of independent signaling pathways (Kolch, 2005; Whitmarsh, 1998).

<table>
<thead>
<tr>
<th>Scaffolding Proteins</th>
<th>β-arrestins</th>
<th>CNK</th>
<th>KSR</th>
<th>MEKK1</th>
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<td>JNK/p38</td>
<td>JNK/p38</td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>JNK3</td>
<td>JNK3</td>
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</tbody>
</table>

Table 1.1. **Known Scaffolding Proteins.** Several examples of scaffolding proteins that Regulate MAP kinase signaling can be seen here, listed as scaffolding proteins followed by representative signaling proteins. The blank spaces are those proteins that have not yet been conclusively identified.
As previously described, MAP kinase signaling is essential for cellular outcomes including cell mitosis, proliferation, differentiation, and apoptosis. Several pioneer studies revealed that when these cascades are activated through a single stimulus, there are several downstream pathways that may be activated, suggesting that there are “branch points” mediating these different functions. It is now better understood that the signaling cascades are organized in a manner to communicate and integrate information from an extracellular stimulus to achieve specific functions. One good example of such a complex is the MP1 complex, which coordinates activity between Ras, Raf, MEK and ERK signaling. This small scaffold protein is able to enhance ERK activation by binding MEK1 to ERK, and MP1 can additionally form a tight heterodimer with p14, an adaptor protein, which directs the MP1 complex to late endosomes. Furthermore, these complexes are subjected to different levels of regulation via modulation of signal strength and duration, spatial constraints, and “cross-talk” with other pathways. By assembling proteins, and even complexes, together, these versatile scaffolds are able to provide a means for assimilating and distributing signals with varying levels of specificity and regulation (Kolch, 2005).

1.7  p38γ (ERK6)

The MAP kinase family is composed of several sub-families, one of which is the p38 MAP kinase family. Currently, four independent isoforms of p38 have been identified, namely, p38α, p38β, p38γ (ERK6), and p38δ. p38 MAP kinases are activated by upstream MEK kinase family members, and were originally known as a stress activated family that respond to such extracellular signals, such as cytokines and inflammatory
stress, and play important roles in immunoresponse, the inflammatory response, as well as proliferation, differentiation, and apoptosis. However, it is important to note that primarily analysis of p38α and p38β, referred to as p38, have revealed biological functions of the remaining isoforms, where very little information has been available regarding p38γ (ERK6), including its interactions, roles in signaling, and function, due in large part to the unavailability of a specific p38γ kinase inhibitor (Court, 2002; Tang, 2005).

p38γ is a unique member of the p38 MAP kinase family, sharing about 60% amino acid sequence homology with both p38α and p38β. Known upstream activators include MEK3 and MEK6. p38γ mRNA is found to be redundantly expressed in systemic skeletal muscle, compared to p38α which is expressed ubiquitously. It has also been observed that p38γ is strongly expressed in several cancerous cell lines. In addition, unlike any other MAP kinases, p38γ mRNA and/or protein is augmented via a differentiation-associated process. Also, exclusive transfection of p38γ can induce cell differentiation in C2C12 cells (Tang, 2005). In K-Ras dependent cell transformation, p38γ has been recognized as a Ras effector in a phosphorylation independent manner, while activities involved with stress response regulation are suggested to occur through transient phosphorylation processes (Tang, 2005). Furthermore, it has been suggested that K-Ras activates p38γ by increasing its expression volume without phosphorylation, which, in turn, reciprocally promotes Ras transformation. This transforming ability is also dependent on the formation of a complex between p38γ and the canonical MAP kinase, ERK2. However, this observation must still be confirmed in other representative cell systems, as confirmed results are still ambiguous (Tang, 2005).
It was previously described that over-expression of Rit alone can transform NIH3T3 cells and can effectively activate p38γ. Moreover, p38γ is exclusively activated by Rit, which induces activation of the c-jun promotor, a gene and protein required for the formation of a transcription factor regulating gene expression in response to stimuli such as stress, cytokines, and growth factors (Sakabe, 2002).

Heterotrimeric G proteins, such as those in GPCR complexes, are also involved in signal transduction and regulation of biochemical signaling in MAP kinase pathways. Further studies of these regulating activities have revealed that Rho, a family member of Ras proteins, is able to activate p38γ, and in conjunction, p38γ activation is primarily mediated by the Gα subunit (Goldsmith, 2007).

1.8 Cell Polarity and Cancer

During cell proliferation and cell growth processes, cells typically modify their shapes and forms utilizing highly complex signaling pathways in response to certain intracellular or extracellular stimuli. Throughout cell growth, cells take on particular forms to fit specialized functions. The forms that these cells take establish specific cytosolic biases, or gradients, required for cell communication and signaling, cytoskeletal organization, and for such processes as endocytosis and exocytosis. Modification of cytoskeletal shape occurs through dynamic interactions of microfilaments, intermediate filaments, and microtubules, ultimately aiding in cell morphogenesis and in the establishment of permanent cellular poles that play an important role in cellular processes.
such as mitosis and transportation of cellular contents throughout a cell (Lodish, 2004; Perez, 2010).

When epithelial cells grow and form tissues, they develop specialized junctions, such as adherens junctions and tight junctions, that function in tethering cells together, preventing any molecules or ions from improperly passing through, and maintaining apicobasal cell polarity by inhibiting integral membrane proteins from dispersing laterally, respectively. As the majority of cancers are categorized as carcinomas, those that arise from epithelial cells, it has been found that many cancerous tissues exhibit a loss of both epithelial character and apicobasal polarity, as found in a process known as epithelial mesenchymal transition (EMT), accompanied with several biological characteristics, such as loss of cell adhesion, increased vimentin expression, decreased E-cadherin expression, and increased epithelial motility. Furthermore, with loss of apicobasal polarity and increased cell growth, carcinogenesis may be promoted (Radisky, 2005; Wodarz, 2007).

1.9 Polarity Complex Proteins

To date, three protein complexes, acting as tumor suppressors, have been identified involved in establishing and maintaining apicobasal polarity in epithelial cells: the Scribble, Par, and Crumbs polarity modules (Bulgakova, 2009; Gopalakrishnan, 2007; Mdina, 2002). Of interest, the Scribble polarity module, which localizes to the basolateral region of cells, consists of three proteins, specifically, Scribble, Discs Large (Dlg) and Lethal giant larvae (Lgl), all of which were initially discovered through *Drosophila* studies. These three proteins act similarly, signaling via similar pathways, as mutants in
any of these three proteins exhibit a similar phenotypic outcome: loss of apicobasal polarity and abnormal cell proliferation (Humbert, 2008). The Par polarity complex consists of three proteins, Par-3, Par-6, and atypical protein kinase C (aPKC). This complex is the first of the three complexes to become localized in an asymmetrical manner, which then further controls downstream localization of other polarity complexes (Krahn, 2010). The Crumbs complex is also composed of three primary proteins, namely, Crumbs, Pals (or Stardust in *Drosophila*) and Patj. These three proteins center around the Pals protein which acts as a scaffolding protein for the other co-localizing proteins, serving to tether the complex to the plasma membrane (Bulgakova, 2009).

As previously established, tumorigenesis requires several mutations to cellular DNA, one of which can be loss of polarity, specifically, loss of function mutations of adherens junctions. In circumstances where these losses of function mutations occur, cells can undergo EMT, facilitating cellular invasive and metastatic properties. Furthermore, through studies of Human papillomavirus (HPV) proteins E6 and E7, Scribble, Dlg, and Lgl have all been identified as targets of these viral products, linking them to mammalian tumor development. In addition, screening efforts for proteins targeted for ubiquitination by HPV E6 and E7 have identified human Scribble as a degradation target, and moreover, Scribble over-expression in rodent epithelial cells has been shown to inhibit transformation by over-expressed E6 and E7 proteins (Humbert, 2008; Zeitler, 2004).

1.10 Scribble Polarity Module Localization and Structure
Scribble is categorized as a member of the LAP family of proteins, named for the presence of LRR and PDZ domains, of which Scribble has sixteen and four, respectively. LRR domains are Leucine Rich Repeats containing short repeating motifs of 23 amino acids that form several structures, typically α/β horseshoe folds, which are rich in the hydrophobic leucine, and are involved in protein-protein interactions (Kallay, 2006). PDZ domains, named for three proteins first discovered containing this domain (Post synaptic density-95, Dlg, and Zonula occludens-1), are responsible for organizing and localizing protein complexes and are composed of approximately 80 to 100 amino acid residues (Fan, 2002). Dlg is a member of the MAGUK, or Membrane-Associated Guanylate Kinases, family of anchoring proteins, containing three PDZ domains, where the second of the three is necessary for proper intracellular localization. Lgl contains several WD40 motifs, also known as β propellers, which are domains also involved in protein-protein interactions (Humbert, 2008).

Several characteristics of the three proteins in the Scribble polarity module allow for interactions with other molecules and for proper function. Scribble, Dlg and Lgl expression must be properly regulated in a specific spatiotemporal manner in order to function appropriately, which is dependent on specialized domains specific for protein-protein interactions. Scribble has been found to locate at adherens junctions and extend basally in mammalian epithelial cells, where both Scribble and Dlg are individually and constitutively localized at the plasma membrane, while Lgl localization depends on its phosphorylation status, in which Lgl is inactive and cannot localize to the cell cortex in a phosphorylated state (Humbert, 2008). In mitotic Drosophila neuroblasts, these three
tumor suppressor proteins are involved in cell asymmetry, all exhibiting cortical localization. Evidence exists that in order for Scribble localization to properly occur, Dlg is necessary, although, the interaction between these two proteins is most likely via a linker protein, GUKholder, or GUKh, which mediates this interaction by binding with PDZ domains of Scribble and GUK domains of Dlg (Kallay, 2006).

1.11 Epithelial Mesenchymal Transition (EMT)

Mammalian epithelial cells are those that are commonly associated with linings of cavities and surfaces and are categorized according to their morphology and distribution. Epithelial cells exhibit several important characteristics that distinguish them from many other cell types. In their most rudimentary form, epithelial cells develop a simple avascular monolayer atop a basement membrane fastened to a layer of connective tissue. The epithelial tissue structure is entirely dependent on close cell-cell interactions. More specifically, structures are reliant on tight junctions, adherens junctions with cadherin proteins, especially E-cadherin, gap junctions for allowing cell-cell communication, desmosomes for cell adhesion, and integrins for cell and extracellular matrix (ECM) interactions, all of which play a role in establishing apicobasal polarity (Lee, 2006; Radisky, 2005).

Mesenchymal tissue has several distinct features quite different from those of epithelial tissues. Two important characteristics most opposite from those of epithelial cells are that cell-cell contacts are rather uncommon and there is no distinct cell polarity present in mesenchymal cells. On the other hand, these two tissue types are similar in their
interactions with the ECM as well as with the presence of a distinct cytoskeleton. In addition, mesenchymal cells possess certain attributes allowing them to restructure the surrounding ECM via matrix metalloproteinases (MMPs), enzymes allowing for the degradation of extracellular matrix proteins. This cell type also forms morphologically irregular and inconsistently profiled structures, typically with an elongated configuration. Furthermore, mesenchymal cells secrete many signaling molecules, such as growth factors, that may influence epithelial cells and several pathological processes, including metastasis, the invasion of cancerous cells into other distant tissues (Lee, 2006; Radisky, 2005).

Epithelial mesenchymal transition (EMT) is the process whereby normal epithelial cells transform all morphological characteristics into those of mesenchymal cells, a process generally common in the formation of solid tumors. On a cellular level, EMT may be detected with increased production of several transcription factors capable of inhibiting E-cadherin, as well as increased expression of vimentin, an intermediate filament, N-cadherin, a protein commonly expressed in connective tissue, and confinement of β-catenin to the nucleus, a protein involved in adherens junction structure that enhances cell-cell contacts when forming associations with cadherin complexes (Lee, 2006; Radisky, 2005; Wodarz, 2007).

All signaling pathways and molecular relationships entailed in EMT have not been fully elucidated and is an important area of research in tumorigenesis. In accordance with this, several important associations have been revealed. The Met receptor tyrosine kinase has recently been identified as an activator of EMT, where upon ligand binding and receptor activation, migration of cells in vitro occurs. This receptor has also been
suggested to regulating the confinement of β-catenin to the nucleus (Lee, 2006).

Epidermal growth factor (EGF), a growth factor that activates the EGF receptor, which is responsible for cellular functions such as cell differentiation, growth, and proliferation, has also been found to play a role in EMT, where EGF was found to induce EMT with its ability to increase vimentin and decrease E-cadherin expression (Lee, 2006). Another example is transforming growth factor-β (TGF-β), an antiproliferative protein expressed in normal epithelial cells, playing a role in such cellular functions as cell proliferation and differentiation. This protein has also been suggested to serve as an EMT activator with the possible modification of histone structure, proteins responsible for packaging DNA into nucleosomes (Lee, 2006). Furthermore, TGF-β has been found to promote ECM protein expression, such as that of collagen I, which has been reported to reduce the ability of tethering E-cadherin at cell-cell junctions in pancreatic cancer cell lines, giving cells mesenchymal characteristics, potentially initiating EMT activity. Throughout the evolution of many different types of cancers, EMT is believed to mediate metastasis, in which non-invasive tumor cells permeate into surrounding tissues, changing benign growths into malignant, invasive tumors (Lee, 2006; Radisky, 2005). An example of this type of effect occurs with transcription factors, such as Snail1, an inhibitor of E-cadherin production, which is enhanced by several EMT regulators (Lee, 2006).

1.12 Cell Adhesion

Cell adhesion is a process whereby a cell binds to a particular surface, such as the extracellular matrix, utilizing specific molecules, including integrins, catenins, and
cadherins. Cell adhesion is critical for the organization of diverse three-dimensional morphologies that determine the overall architecture of a tissue. The specific morphologies that cells and tissues take is dependent upon a cooperation between events involving a physical adhesion of cells and the biochemical signaling that regulates a transduction of information between neighboring cells (Gumbiner, 1996; Lodish, 2004).

Transmembrane cadherins are important in cell recognition and sorting that occurs throughout the development of an organism. E-cadherin is highly expressed in epithelial tissues and allows for the formation of tight epithelial associations, where loss of E-cadherin expression and/or function results in increased cell invasiveness. In order to achieve desired functions, cadherins need to form associations with catenins, such as α-catenin, which can bind to actin cytoskeletons, and β-catenin, an intermediate protein linking α-catenin to transmembrane cadherins. Cadherins further function as receptors of adherens junctions, as they localize with actin filaments to form these junctional structures, an activity highly dependent on interdigitation amongst adhesion molecules from both cell surfaces. Moreover, occludin is a protein highly expressed in tight junctions, and its functional role is specialized to cell-cell contacts, regulating permeability between adjacent cells and dividing cellular interfaces into two distinct cell surfaces. In addition to cell-cell contacts, cell attachments to the ECM, known as focal adhesions, are necessary for maintenance of tissue integrity. The basal lamina and reticular lamina are the two primary layers of the ECM, both abundant in adhesive molecules, including collagens, laminin, fibronectin, and proteoglycans. Specific receptors, such as integrins, are required by cells
to properly attach to the ECM and the adhesive molecules present (Gumbiner, 1996; Lehninger, 2005; Lodish, 2004).

Cell adhesion molecules are subjected to a large variety of signal transduction events responsible for regulating a variety of cellular responses to specific stimuli, such as cell adhesion, cell motility, growth, and gene expression regulation. It is known that cell proliferation is dependent on anchorage to a cellular matrix, a characteristic that is no longer present when malignant cell transformation occurs. During cell cycle progression, checkpoint control mechanisms ensure that particular processes have been successfully completed before the cycle can continue into the next phase of growth. One such checkpoint is mediated by the proper attachment of cellular integrins to an appropriate substrate, a process involving the focal adhesion kinase (FAK). As previously described, Ras, which functions as an activator of MAP kinase signaling, forms a complex with Grb2 via the Sos protein. It is known that Grb2 forms a physical association with FAK, providing a direct connection between cell adhesion and MAP kinase signal transduction events. Furthermore, regulation of tissue morphogenesis, cell growth, and differentiation is accomplished through several complex levels which require interactions between adhesion receptors, the cytoskeleton, and networks of signaling pathways, factors that may encourage the onset of carcinogenesis (Gumbiner, 1996; Lehninger, 2005; Lodish, 2004).
Methods and Materials

2.1 Cell Cultures

293FT cell lines were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) (4.5 g/L glucose, L-glutamine, and sodium pyruvate) (Cellgro, Manassas, VA) supplemented with 10% Serum Supreme (SS) (Lonza, Walkersville, MD) and 1% Penicillin – Streptomycin Solution (10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin) (Cellgro, Manassas, VA). Cells were preserved in an incubator containing 90% air and 10% CO₂ at 37°C in humid conditions.

2.2 Cell Lysis and Protein Extraction

Cell cultures were prepared for lysis according to their storage conditions prior to lysis. Cell cultures stored at -80°C were removed from the freezer, immediately placed on ice, and allowed to thaw. Cell cultures incubated at 37°C had their media removed, were washed with phosphate buffered saline (PBS) (8 grams NaCl, 0.2 grams KCl, 1.44 grams Na₂HPO₄, and 0.24 grams KH₂PO₄ per 800 mL distilled water, with pH 7.4 adjusted by HCl) and cell plates were incubated on ice.

To obtain protein samples, lysis buffer solution (1 µL 1M DTT (Fisher Scientific, Fair Lawn, NJ), 10 µL 0.1M PMSF (Sigma, St. Louis, MI), 1 µL 10 mg/mL aprotinin, 1 µL 10 mg/mL leupeptin, and 2 µL 1M orthovanadate per mL of kinase assay buffer containing
10 mL 1M HEPES (J.T. Baker, Phillipsburg, NJ) with pH 7.5, 10 mL 0.5 M EGTA with pH 8, 4.32 grams 1M β-glycerophosphate, 5 mL NP-40 lysis buffer, and 625 µL MgCl₂ per 500 mL of solution) was added to cell cultures drop-wise according to cell plate size. 200 µL of lysis buffer was added to 6 cm cell plates and 400 µL of lysis buffer was added to 10 cm cell plates. Plates were incubated on ice for 20 minutes, gently swirling every five minutes to ensure that contents were continually covered with lysis buffer. Cell plate contents were recovered with a cell scraper and transferred to pre-chilled micro-centrifuge tubes. Samples were then centrifuged at 16,100 rpm for 20 minutes at 4°C. Cleared cell lysates were transferred new to pre-chilled tubes. Protein concentrations of recovered supernatants were then measured with a NanoDrop ND-1000 Spectrophotometer.

2.3 Total RNA Extraction

To extract total RNA from samples, 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA) per 10 cm² was added to cell cultures drop-wise and samples were incubated for five minutes at room temperature. Cells were recovered and transferred to micro-centrifuge tubes. 200 µL of RNA grade chloroform per mL of TRIzol was added to each sample, tubes were shaken vigorously for 15 seconds, and samples were incubated at room temperature for two minutes. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous layer (top layer) of samples were transferred to new tubes, 500 µL of 2-propanol per mL of TRIzol was added to samples which were incubated at room temperature for ten minutes and then centrifuged at 12,000 rpm for ten minutes at 4°C. Supernatants were removed and RNA pellets were washed with 1 mL of 70% ethanol per
30 mL of TRIzol by vortexing. Samples were centrifuged at 7,500 rpm for five minutes at 4˚C. RNA pellets were air dried and dissolved with 100µL of RNAase-free distilled water.

2.4 Western Blot Analysis

Protein samples with already measured concentrations were resolved on 8%, 10%, or 12% SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) gels depending on the molecular weight of target proteins, where relatively larger proteins (> 100 kDa) of interest were resolved on 8% gels and smaller proteins (~60 kDa) of interest on 12% gels. Sufficient quantities of protein solutions were added to dilute 5x protein loading buffer to a 1x concentration and to make a total sample size of 18 µL for gel loading. Adjusted protein samples were boiled at 100˚C for five minutes and then immediately incubated on ice for five minutes. After briefly centrifuging, samples were applied into individual sample lanes submersed in 1x SDS running buffer (3.02 grams Tris, 18.8 grams glycine, and 10 mL 10% SDS per liter of distilled water) and resolved at 120 V for 1.25 hours. The transfer apparatus was then assembled and equilibrated to ensure that equal pressure was applied from gel to membrane, allowing for even transfer of proteins across the surface of the membrane. With the transfer apparatus set on ice, proteins were transferred to a PVDF (Polyvinylidene fluoride) membrane in 1x transfer buffer (5.85 grams Tris, 2.95 grams glycine, and 20% methanol per liter of distilled water) for two hours at 100 V. Membranes containing protein samples were then blocked for one hour with a 5% milk/1x T-TBS solution (2.5 grams Nestle Carnation Instant Nonfat Dry Milk per 50 mL T-TBS containing 1.21 grams Tris, 8.77 grams NaCl, and 500 µL Tween-20 per
liter of distilled water) at room temperature. Membranes were then exposed to 3 mL primary antibody solution overnight at 4°C with a 1:1000 starting concentration of antibody to T-TBS, a dilution dependent on concentrations of antibodies. After washing six times with TTBS, each wash lasting three minutes, membranes were exposed to 10 mL secondary antibody solution and shaken for one hour at room temperature with a 1:10,000 concentration of antibody to T-TBS, once again, a dilution dependent on concentrations of antibodies. After six more three minute T-TBS washes, the membrane was exposed to a mixture of 500 µL Western Lightning Plus-ECL Oxidizing Reagent Plus and 500 µL Western Lightning Plus-ECL Enhanced Luminol Reagent Plus (Perkin Elmer, Shelton, CT) for one minute. Membranes were wrapped in a plastic sheath, air was removed, and using an autoradiography cassette (Fisher Scientific, Pittsburgh, PA), Blue Devil film (Genesee Scientific, San Diego, CA) was placed on top of the membrane and was developed with a Kodak photo processor.

2.5 DNA Transfection

293FT cells were obtained and plated on cell plates to reach approximately 60% confluence. Desired quantities of DNA were obtained, typically 3 µg of total DNA, and mixed with 500 µL of serum free medium (D-MEM) while 5 µL turbofect (Fermentas, Glen Burnie, MD) was also mixed with 500 µL serum free medium. Both were allowed to incubate inside of a sterile cell culture bench for five minutes at room temperature. These solutions were then mixed and allowed to incubate for an additional ten minutes. Upon removal of medium from cell cultures, the turbofect containing DNA solutions were added
to the cell plates drop-wise and allowed to incubate at 37°C in 90% air, 10% CO₂, and humid conditions for ten minutes before adding 10 mL of serum containing medium. Cells were incubated with DNA overnight (for 12 hours) at 37°C. After incubation, medium was removed and 10 mL of fresh serum containing medium was added to cells. Cultures were additionally incubated for 24 hours before cells were lysed.

2.6 Immunoprecipitation

Upon cell lysis, 50 µL of total cell lysates were saved for western blot and the remaining lysate, typically 5 mg of total protein volume, was used for immunoprecipitation. 20 µL per sample of GammaBind G Sepharose beads (GE Healthcare, Piscataway, NJ) for antibody purification were obtained. The GammaBind G beads were then vigorously washed twice with lysis buffer. To wash, 500 µL of lysis buffer was added to the beads, centrifuged at a 16,100 relative centrifuge force (rcf) for one minute at 4°C, supernatant was removed, another 500 µL of lysis buffer was added, beads were centrifuged for another minute, supernatant was removed, and then beads were re-suspended in 100 µL of lysis buffer. This lysis buffer solution containing beads was then added to the total cell lysates, and samples were incubated at 4°C for one hour in order to pre-clear the lysates and prevent any non-specific binding between proteins and beads. After the one hour incubation period, samples were centrifuged at 16,100 rcf for one minute at 4°C, pellets were discarded, and supernatants were isolated. To each sample of pre-cleared lysates, 3 µL of antibody (200 µg/mL) was added to each sample, allowing incubation at 4°C overnight to pull-down the target protein. The next day, 30 µL per
sample of pre-washed GammaBind G beads were added to the total cell lysates containing the primary antibody and the samples were allowed to incubate at 4°C for one hour. Samples were then centrifuged at 16,100 rcf for one minute at 4°C. Supernatants were then decanted and the pellets were isolated and washed twice with lysis buffer. Sufficient quantities of 5x protein loading buffer were added to dilute the entire sample to a 1x concentration. The bead and loading buffer containing samples were then heated to 100°C for five minutes and immediately incubated on ice in preparation for SDS-PAGE gel electrophoresis.

2.7 Yeast Two-Hybrid Assay

A yeast-two hybrid screening was performed using a pre-transformed 11-day mouse embryo Matchmaker cDNA library containing a pACT2-Gal4 activation domain vector (Clontech, Mountain View, CA). A dominant negative mutant form of full-length human ERK6AA was used as bait, fused in frame with the Gal4 DNA binding domain from a pGB3 yeast expression vector, derived from a modified pGBKT7 vector (Clontech, Mountain View, CA). AH109 yeast strains transformed with the ERK6 bait were selected by growth in the absence of tryptophan, which were further subjected for screenings according to the Matchmaker III protocol. The ERK6 transformed yeast was allowed to mate overnight at 30°C with pre-transformed yeasts bearing the 11-day embryo cDNA library onto quadruple dropout media lacking tryptophan, leucine, histidine, and adenine, conditions which are considered appropriate for the selection of high affinity interacting partners. Mating efficiency was then determined by plating cells onto either single or
double dropout media, lacking tryptophan, leucine, or both tryptophan and leucine. After mating, yeast was incubated for two weeks in the quadruple dropout media. Growing colonies were then verified for X-α-Galactosidase expression in X-α Gal containing quadruple dropout plates. The pACT2 plasmid library from blue colonies was then isolated. Plasmids containing the cDNA of putative ERK6 binding proteins were re-transformed into AH109 yeast containing pGB3-ERK6AA bait or pGB3 fused with ERK5AA as a negative control. Transformed cells were then selected by growing the yeast on double dropout media lacking tryptophan and leucine. The yeast was then re-plated onto quadruple dropout media containing X-α Gal to test the specificity of occurring interactions. Plasmids exhibiting a blue color, which provided elements for growth under highly stringent conditions, were further isolated and their sequences were confirmed. These clones were further cloned into mammalian expression vectors for further studies.

2.8 In vitro Kinase Assay

HA-tagged ERK2 and ERK6 MAP kinase activity, obtained from transfected MDCK and NIH3T3 cell lysates, was measured upon immunoprecipitation with anti-HA antibody (Covance, Princeton, NJ) using myelin basic protein (MBP) (Sigma, St. Louis, MO) as a substrate. Briefly, 24 hours after transfection, cells were incubated with serum free medium for two hours with ERK6 and overnight with ERK2. After serum starvation, cells were stimulated with the addition of 10% bovine calf serum medium for 15 minutes with ERK6 and five minutes with ERK2. Cells were then washed with cold PBS and lysed with 500 mL of MAP kinase lysis buffer composed of 20 mM HEPES pH of 7.5, 1% NP-
40 lysis buffer, 10 mM EGTA, 20 mM β-glycerophosphate, 1 mM NaVO₃, 1 mM dithiothreitol (DTT), 25 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mg of leupeptin and aprotinin per mL of buffer. Cleared lysates containing the HA-tagged kinases were then immunoprecipitated with anti-HA monoclonal antibody, incubating lysates at 4°C for one hour. Immunocomplexes were recovered by adding 20 mL of PBS suspended Gamma bind G™ sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Beads were washed once with PBS containing 1% NP-40 and 2 mM vanadate, once with 100 mM Tris pH 7.5 and 0.5 M LiCl, and once with a kinase reaction buffer containing 12.5 mM MOPS pH7.5, 12.5 mM glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM Na₃VO₄. Samples were then re-suspended in 30 mL of a kinase reaction buffer containing 1 mCi of ³²P-ATP per reaction and 20 mM of unlabeled ATP along with 2 mg of MBP. Samples were incubated at 30°C for 30 minutes, where the reaction was terminated by adding 5x Laemmli SDS sample dilution buffer. After this incubation, products were resolved in a 12%, or 15% for MBP, SDS-PAGE gel. Phosphorylation of MBP was then observed by placing film on top of the dried gels and observing relative intensities with Phosphorimager (Molecular Dynamics). Expression levels of HA-tagged ERK2 and ERK6 were then assessed by western blot analysis with the application of anti-HA monoclonal antibody, HA.11 (Covance, Princeton, NJ).

### 2.9 Focus Formation Assay

NIH3T3 cells were allowed to grow to 60% confluency in 6 cm cell plates. Cells were then co-transfected with 2 µg of plasmid DNA or empty control vectors using the
calcium phosphate transfection method. These cell plates were divided into two groups, those examining ERK2 and Scribble, and those examining ERK6 and Scribble. In the series of plates examining ERK2 and Scribble, cell plates were transfected with H-RasV12 (a constitutive ERK2 activator) in one plate and H-RasV12 along with Scribble in another plate. In the other series of plates examining ERK6 and Scribble, cell plates were transfected with Rit79L (a constitutive ERK6 activator) in one plate and Rit79L along with Scribble in another plate. Control vector transfected cells and Scribble transfected cells, in which total DNA transfection volume was adjusted with the empty vector, served as controls. A summary of the performed transfection may be seen in Table 2.1. To perform the focus formation assay, the cell plate’s medium was altered to D-MEM containing supplemental 5% calf serum with 2 mM L-glutamine. Fresh medium was periodically replaced every three to four days for three weeks, allowing for the formation of foci. Upon foci formation, plates were washed with cold 1x PBS, fixed with methanol, and enough 0.5% crystal violet solution in 25% methanol was added to cover the bottom of the plate to stain foci. Cells were incubated with crystal violet for ten minutes at room temperature, and plates were carefully rinsed with distilled water to remove excess crystal violet. Plates were allowed to air dry at room temperature, after which foci were counted and quantified, allowing for the comparison amongst sets of transfected groups.

<table>
<thead>
<tr>
<th>Cell Plate Samples</th>
<th>Control</th>
<th>Scribble</th>
<th>H-RasV12</th>
<th>H-RasV12 + Scribble</th>
<th>Rit79L</th>
<th>Rit79L + Scribble</th>
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</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
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<td></td>
<td></td>
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<tr>
<td>Control Vector</td>
<td>2 µg</td>
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</table>
Table 2.1. Transfection Volumes of Focus Formation Assay Samples. 2 µg of total DNA volume was used per plate of cells. Samples transfected with one plasmid DNA received 2 µg of DNA. Samples co-transfected with two plasmids received 1 µg of each DNA plasmid.

2.10 Immunostaining

Glass coverslips were sterilized and placed in 12-well plates. 5 x 10³ cells per well of HeLa cells were then plated out and incubated in 10% SS and 1% penicillin-streptomycin (500 mL of D-MEM with 50 mL of SS and 5 mL of penicillin-streptomycin) containing medium and plated onto the coverslips, allowing sufficient times for cell cultures to reach 50% confluency. Cells were then washed with 1x PBS and fixed in 1 mL of cold methanol for 20 minutes. Cells were then made permeable via incubation with PBS-T (1x PBS with 1% Triton-X) for five minutes at room temperature. Cell cultures were blocked for one hour at room temperature with blocking buffer (PBST with 5% BSA). Upon removal of blocking buffer, cells were incubated overnight at 4°C with a 1:200 dilution of primary antibodies in blocking buffer. After this incubation period, cells were washed six times for five minutes per wash. FITC-conjugated secondary antibody solutions (Vector Laboratories, Burlingside, CA) at 1:500 dilutions along with 1:2000 dilutions of 1 µg/mL DAPI were incubated with cells for one hour at room temperature. After washing another six times for five minutes per wash, coverslips were mounted on microscope slides with Vectashield (Vector Laboratories, Burlingside, CA). Slide imaging
was performed with a Zeiss Axiovert 200 inverted fluorescence microscope (Zeiss, Thronwood, NY), and images were recorded.

2.11 Polymerase Chain Reaction (PCR) for the Amplification of Scribble Domains

Full length Scribble DNA, with a concentration of 500 ng/mL, was obtained from the Kazusa DNA institute through their HUGE (HUman larGE protein database) project (RIKEN, Kazusa, Japan) and diluted 1:1000 with distilled de-ionized water (dDW). The PCR reaction was then performed following the manufacturer’s protocol for the Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). In brief, starting concentrations of components used were as follows: 5 µL of 10x PCR Buffer, 1 µL of 10 mM dNTP mixture, 1.5 µL 50 mM MgCl₂, 1 µL of 10 µM sense primer, 1 µL of 10 µM anti-sense primer, 1 µL of dilute Scribble (template) DNA, 0.2 µL of Platinum Taq DNA Polymerase, and 39.3 µL dDW. Primer sequences are described in Tables 2, 3, and 4. These were mixed into a sterile 0.5 mL tube for a single 50 µL reaction. Components were initially incubated at 94°C for five minutes to completely denature the template and activate the Taq polymerase, followed by 35 cycles of PCR with a denaturing reaction at 94°C for 30 seconds, an annealing reaction at 55°C for 30 seconds, and an extending reaction at 72°C for three minutes. Upon completion of all 35 cycles, the reaction tubes were maintained at 4°C (or stored at -20°C) until samples were run on a 1% DNA agarose gel by electrophoresis. Band sizes were semi-quantified with DNA ladders and desired DNA was excised and extracted, as described in the following section.
### Table 2.2: Primers Used to Subclone Scribble Segments

<table>
<thead>
<tr>
<th>Scribble Segments</th>
<th>Base Pairs</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>902</td>
<td>ScBam1</td>
<td>ScNot1</td>
</tr>
<tr>
<td>B</td>
<td>1022</td>
<td>ScBam3</td>
<td>ScNot2</td>
</tr>
<tr>
<td>C</td>
<td>1408</td>
<td>ScBam4</td>
<td>ScNot3</td>
</tr>
<tr>
<td>D</td>
<td>1402</td>
<td>ScBam5</td>
<td>ScNot4</td>
</tr>
</tbody>
</table>

### Table 2.3: Primers Used to Clone Scribble

<table>
<thead>
<tr>
<th>Scribble A</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>ATAGGATCCAGCGACAACGAGTCCAGGGG</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGCCGCCACGTCACAGTGCAG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scribble B</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>ATAGGATCCCGGGAACCGCCTGCAGAGTCTG</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGCCACGTCCAGACCAGTCAGGCAG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scribble C</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>ATAGGATCCGTTTCGTTCGACAGGGCGCAAT</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGCTCCAGCTGCACCCGCACTCGCCGCGT</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scribble D</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>ATAGGATCCCTCGAGTGTTGACGGCGACACC</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGCTTCGGGCTGTGCTGCTGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>ERK2ΔC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>ATAGGATCCATGGCGGCGGCGGCGGCG</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGCCTCTTTCAAAAAATAGTTTTTGTAG</td>
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</tr>
<tr>
<td>ERK6ΔC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>ATAGGATCCATAGCTCTCCGCG</td>
<td></td>
</tr>
<tr>
<td>Anti-sense</td>
<td>ATAGCGGCCGAGCTGCGGAGCTGAGCT</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4: Primers Used to Clone ERK2 and ERK6**

2.12 Restriction Endonuclease Digestion

Eukaryotic expression vectors pEF1-His A, B, and C (Invitrogen, Carlsbad, CA) was obtained. 2 µg of vector DNA was added to a microcentrifuge tube containing 3 µL of 10x Buffer B, 0.5 µL of BamHI, 0.5 µL NotI, and nuclease water to make a final volume of 30 µL in order to dilute the 10x Buffer B to a final concentration of 1x. Tubes were incubated for one hour at 37°C. Digested vectors were stored at -20°C until inserts were prepared and ready for the following DNA purification step.

2.13 Agarose Gel Electrophoresis

0.8% or 1% DNA agarose gels were prepared with 0.4 grams or 0.5 grams, respectively, of molecular grade Agarose powder (Bioline, Taunton, MA) with 50 mL of 1x TAE (242 grams of Tris base, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M
EDTA pH 8.0 per 1000 mL of distilled de-ionized water), depending on DNA fragment sizes (0.8% gels were used with larger fragment sizes and 1% gels with smaller fragment sizes). Samples were run simultaneously with a DNA marker, either Quick-Load PCR Marker (New England Biolabs, Ipswich, MA) or HyperLadder I (Bioline, Taunton, MA), depending on DNA fragment sizes. Samples and ladders were run for approximately 30 minutes at 100V, or until fragment bands had sufficiently separated during electrophoresis. DNA bands of interest were excised and isolated in preparation for DNA extraction. The QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) was used to purify DNA from agarose gel. Excised gel containing DNA samples were initially weighed and isolated in a 1.5 mL microcentrifuge tube. Three volumes of Buffer QXI were added per one volume of gel for DNA fragments with sizes ranging from 100 bp to 4 kb. 10 µL of re-suspended QIAEX II was then added to each tube and samples were vortexed. Tubes were incubated at 50°C for ten minutes, where samples were vortexed every two minutes to ensure that QIAEX II remained suspended in solution throughout the incubation period. After centrifuging samples for 30 seconds at 16,100 rcf, supernatants were discarded and pellets were washed with 500 µL of Buffer QXI, vortexing to mix the solution. After centrifuging samples for 30 seconds at 16,100 rcf, supernatants were discarded and pellets were washed twice with 500 µL of Buffer PE, vortexing to mix the solution and centrifuging again in between washes. After supernatants were removed, pellets were air-dried until they turned white. To elute DNA, 20 µL of distilled de-ionized water was added to each sample and the pellet was re-suspended by vortexing. After centrifuging at 16,100 rcf for another 30
seconds, cleared supernatants were transferred into a clean microcentrifuge tube, which now contained the purified DNA.

2.14 DNA Ligation

T4 DNA ligase and 5x ligation buffer (Invitrogen, Carlsbad, CA) were obtained and thawed. In a microcentrifuge tube, 2 µL of ligation buffer, 2 µL of the digested eukaryotic expression vector, 2 µL of the digested eukaryotic expression vector of interest, 1 µL of T4 ligase, and 3 µL of de-ionized water were added. Samples were then incubated overnight at 16°C, an optimum temperature to prevent self-ligation of digested DNA fragments.

2.15 Spectrophotometric Calculation of Nucleic Acid and Protein Concentrations

Whole cell lysates (for protein) or nucleic acid solutions were obtained. Samples were placed in a NanoDrop ND-1000 Spectrophotometer after using nuclease free water to blank the measurements, establishing a clean background. Absorbances at 260 and 280 nm were read and concentrations of samples were acquired and recorded for further applications.

2.16 E. Coli Transformation

Alpha-select chemically competent E. coli (Bioline, Randolph, MA) were removed from storage conditions of -80°C and were immediately placed on ice and allowed to thaw.
Desired quantities of target DNA sequences, for example, 10 ng of circular pCEF-His-tagged Scribble DNA, were added to 1.5 mL sterile microcentrifuge tubes containing *E. coli*, as stirring the contents inside the tube was avoided. Samples were incubated on ice for approximately five minutes. Shortly after, the tubes were placed in a 42°C water bath for 30 seconds to heat shock the *E. coli* and incorporate target DNA into the cells. Tubes were then placed back on ice for five minutes to allow *E. coli* to retain the plasmid DNA. 500 µL of warm SOC medium (2% tryptone, 0.5% yeast extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, and 10 mM MgSO₄) was also added to the tubes. Cells were then placed in an incubator and shaken at 200 rpm for ten minutes at 37°C. Tubes were centrifuged and the supernatants containing excess medium were decanted. 50 µL of *E. coli* were then streaked on LB (Luria-Bertani) agar plates containing 50 µg/mL ampicillin and were incubated at 37°C overnight, allowing colonies to grow. The following morning, a single cell colony was isolated from the agar plates by gentle picking with a plastic tip and was inoculated to tubes containing 3 mL of LB broth containing 1% ampicillin. Tubes were then placed in an incubator at 37°C and were shaken for approximately ten hours.

### 2.17 Small Scale DNA Preparation

Samples containing DNA transformed *E. coli* were obtained after a ten hour shaking period. 1.5 mL of these samples were transferred into microcentrifuge tubes, centrifuged at 16,100 rcf for 30 seconds, and pellets were then prepared for miniprep DNA purification using the Wizard Plus SV Minipreps DNA Purification System (Promega,
Madison, WI), following the manufacturer’s protocol. In brief, after centrifugation and supernatant removal, pellets were resuspended in 250 µL of Cell Resuspension Solution, followed by a five minute incubation period at room temperature. 250 µL of Cell Lysis Solution was then added to each sample, inverting the tubes four times, followed by another incubation period at room temperature. 10 µL of Alkaline Protease Solution was then added to samples, inverting tubes four times, and once again incubating samples at room temperature for five minutes. 350 µL of Neutralization solution was then added to the samples, tubes were inverted four times, and samples were centrifuged at 16,100 rcf for ten minutes. Supernatants were carefully removed and added to the appropriate spin columns, followed by centrifugation at 16,100 rcf for one minute, discarding the flow through upon completion. Finally, 750 µL of Wash Solution with ethanol was added to the spin column, immediately centrifuging samples at 16,100 rcf for two minutes. Column contents were then transferred to sterile microcentrifuge tubes and 100 µL of 90°C nuclease free water was added on top of the column tubes. DNA was eluted by centrifuging samples at 16,100 rcf for one minute. DNA samples were then stored at -20°C for following applications.
Results

3.1 Yeast Two-Hybrid Assay

Little information is available regarding ERK6 signaling and interactions. Most information known about this kinase has been determined through primary analysis of other p38 stress activated isoforms, as these proteins share approximately 60% amino acid homology. Known upstream activators include MEK3 and MEK6. Therefore, a yeast two-hybrid assay was performed to identify any possible associating partners, using full length ERK6 as bait. These screenings were performed three times with a human brain cDNA library as a pool of possible associating proteins. MEK3, a known upstream regulatory kinase, was identified in all three screenings. Through these screenings, Scribble, a cell polarity regulator, was identified as an ERK6 associating protein. Further research established that the Scribble amino acid sequence between AA580-870 was contained within the binding region amongst Scribble and ERK6. This interesting association between these two previously unidentified binding partners prompted us to further explore these interactions. Therefore, a full length Scribble plasmid was obtained from the HUGE (HUman larGE protein database) Project of the Kazusa DNA Institute.
Figure 3.1. A MatchMaker Yeast Two-Hybrid System: Scribble Binds to ERK6. The top panel demonstrates yeast colonies streaked on a plate containing all necessary supplemental nutrients required for yeast and negative clone growth, which occurs without any protein-protein interactions on this plate. The bottom panel demonstrates an agar containing minimal nutrients in which the bait must interact with a binding partner to activate nutrition compensation in the plasmid sequence in order for growth to occur. If nutrition compensation occurs, identified by one letter abbreviations, construct colony growth is expected. ERK5 served as the negative control, with no colony formation present. LT = Leucine/Tryptophan; AHLT = Adenine/Histidine/Leucine/Tryptophan.
3.2 Scribble as a Dual Kinase Binding Partner

The unexpected results obtained from the yeast two-hybrid assay suggested that Scribble, containing 16 LRR and four PDZ domains, possesses the ability to bind to ERK6. It is well known that MAP kinases have the ability to form signaling complexes by associating with other scaffolding proteins, allowing for the spatiotemporal regulation of signaling. Some scaffolding proteins can bind more than one kinase, playing a role as a multivalent regulator. Therefore, we wanted to confirm if Scribble binds to any MAP kinases other than ERK6. To test this possibility, immunoprecipitation assays (IP) were performed with 293FT cells by co-transfecting several major HA-tagged MAP kinases, including all human p38 homologues, along with His-tagged Scribble. HA-tagged MAP kinases included ERK1, ERK2, JNK, p38α, p38β, p38γ (ERK6), p38δ, and ERK5. Upon transfection, cell lysates were obtained and protein complexes were pulled down with anti-HA antibody and western blot analysis was performed with either anti-His or anti-Scribble antibodies. IP results clearly confirmed the ability of Scribble to bind with ERK6, and surprisingly, also showed a binding affinity for ERK2, however, not as pronounced as ERK6 binding affinity (there was about a 20% affinity in ERK2 compared to that of ERK6, as determined by western blot). It is interesting to note that ERK1 and ERK2 share 88% amino acid sequence homology, yet IP results showed that Scribble has the ability to bind to ERK2 but not ERK1.
Figure 3.2. MAP Kinase and Scribble Immunoprecipitation Analysis. 293FT cells were co-transfected with HA-tagged MAP kinases and His-tagged Scribble, as indicated above. **Top panel:** Western blot analysis of total cell extract (TCE) with anti-HA antibody confirmed the presence of HA-tagged kinases. **Bottom panel:** Immunoprecipitation analysis revealed the presence of Scribble in cell lysates containing co-transfected ERK2 and ERK6 with Scribble. The HA-tagged kinases were pulled down out of solution and Scribble expression was checked, indicating an affinity of Scribble for both ERK2 and ERK6.
3.3 Identification of Binding Domains Between Scribble and MAP Kinases

Scribble is a relatively large protein with a molecular weight of 210 kD and containing a sequence of over 1,500 amino acids. Immunoprecipitation results revealed the ability of Scribble to bind with ERK2 and ERK6. To further unravel these relationships in a molecular context, we initially wanted to determine what specific regions of the amino acid sequence of Scribble are required for the formation of these Scribble-kinase associations. Therefore, a DNA domain search was performed for Scribble using the web based scansite program (http://scansite.mit.edu). This program search revealed that Scribble contains two major functional domains, known as LRR and PDZ domains, which are encompassed by amino acid sequences 1 to 314 and 670 to 1126, respectively. After the domain search, we divided Scribble into four independent segments. We then attached an AU1-tag to the N-terminus of Scribble with a pCEF-AU1 eukaryotic expression vector containing a basic structure as indicated in Figure 3A. Figure 3B presents a scheme of the four different expression vectors that were constructed following database search results. These four expression vectors were named Scribble A, B, C, and D, and starting from the N-terminus, contained amino acid sequences from 1 – 314, 315 – 669, 670 – 1126, and 1127 - 1551, each independently expressing an AU1-tag. A total of five vectors were constructed, four of which contained specific regions of the full-length sequence, with the fifth vector containing the entire Scribble sequence as an original, full-length positive control.
Figure 3.3A. Basic Structure of pCEF Eukaryotic Expression Vectors. AU1-tags were inserted immediately after the promoter to allow for the identification of proteins. The specific pCEF-AU1 eukaryotic expression vector used for experimentation contained an EF1 promoter.
Figure 3.3B. Four Constructs with Representative Segments of Full-length Scribble.
The top image is a depiction of a simplified, full-length Scribble transcript. Scribble A, B, C, and D represent the eukaryotic expression vectors containing specific domains of full length Scribble. Scribble A, B, C, and D denote amino acid sequences 1 to 314, 315 to 669, 670 to 1126, and 1127 to 1551, respectively. All five of the full length and partial construct vectors contained AU1-tags at the N-terminus to allow for identification during IP experiments.
3.4 Immunoprecipitation Analysis with Partial Scribble Constructs

Using AU1-tagged eukaryotic expression vectors, specific regions of the full length Scribble transcript required for binding with ERK2 and ERK6 could be identified, helping to shed light on mechanisms of interaction between these proteins. 293FT cells were transfected with the full-length Scribble vector and the four independent constructs together with either Green Fluorescence Protein (GFP; the negative control), HA-ERK2, or HA-ERK6. After cells were transfected with DNA, cells were cultured overnight and protein expression was confirmed by checking GFP expression using a fluorescent microscope. Cells were then lysed and total cell extracts were pulled down with an anti-HA antibody. Western blot analysis with an anti-AU1 antibody revealed that both LRR and PDZ domains are utilized for binding with ERK2 and ERK6 MAP kinases.
Figure 3.4. Partial Scribble Constructs and MAP Kinase Immunoprecipitation Analysis. Co-transfected 293FT cells were lysed, and kinase binding protein complexes were pulled down with anti-HA antibody. Subsequent western blot membranes were then probed with an anti-AU1 antibody to detect the presence of Scribble. **Top panel:** In cells co-transfected with ERK2 and ERK6, Scribble A, C, and the full length vector were successfully immunoprecipitated, indicating that the two partial transcripts containing the LRR and PDZ domains were able to bind to the two MAP kinases, as compared to the negative control co-transfected with GFP. **Middle panel:** Expression of HA-tagged kinases was examined in total cell extracts to confirm expression of transfected ERK2 and ERK6, which was verified. **Bottom panel:** Total cell extracts were probed with an anti-AU1 antibody to confirm expression of full length and partial Scribble transcripts, validated in all samples.
3.5 Immunoprecipitation Analysis with Deletion Mutants

Immunoprecipitation results revealed that Scribble has the ability to bind to both ERK2 and ERK6 MAP kinases, and that LRR and PDZ domains are necessary for this association to occur. The next step was to try to further clarify the mechanisms of interaction occurring between this cell polarity regulator and these MAP kinases. Thus, a DNA functional domain search was performed using the web based scansite program (http://scansite.mit.edu), for ERK2 and ERK6. The results indicated that only the ERK6 MAP kinase contained a Class 1 PDZ binding motif at the C-terminus, consisting of five amino acids. Surprisingly, ERK2 does not contain a class specific PDZ motif. We still need to further elucidate this binding mechanism in future studies. To confirm the functionality and role in protein-protein interactions of these PDZ binding motifs, eukaryotic expression vectors were constructed with a PCR based subcloning technique for both ERK2 and ERK6 with ten amino acids deleted from the C-terminus, excluding the PDZ motif from ERK6.

Co-transfections of 293FT cells were performed with HA-tagged ERK2 and ERK6 partial deletion constructs, designated as such with ΔC, along with full length, His-tagged Scribble. Full length HA-tagged ERK2 and ERK6 were also co-transfected as a positive control. After cells were lysed, total cell extracts were pulled down out with an anti-HA antibody, and western blot analysis was performed with anti-His antibody. Results indicated that the ten amino acid containing PDZ motifs from the C-terminus are required for kinase binding to Scribble. Of note, expression of the HA-tagged ERK2 deletion mutant was significantly less than that of the other HA-tagged kinases. Further
experimental trials of this IP assay divulged a decreased relative detection volume of ERK2 when compared to that of ERK6, where there was approximately a 20% affinity in ERK2 compared to that of ERK6.
Figure 3.5. MAP Kinase Deletion Mutants and Scribble Immunoprecipitation Analysis. 293 FT cells were co-transfected with HA-tagged MAP kinases and full length, His-tagged Scribble. Protein complexes were pulled down with anti-HA antibody and western blot was performed with anti-His antibody. **Top panel:** Immunoprecipitation analysis revealed the presence of His-tagged Scribble in cell lysates containing co-transfected ERK2 and ERK6 with Scribble. The HA-tagged kinases were pulled down out of solution and Scribble expression was checked, indicating an affinity of Scribble for both ERK2 and ERK6, but not to the C-terminus deletion mutations. **Middle panel:** Western blot analysis of total cell extract with anti-His antibody confirmed the presence of transfected Scribble in cells. **Bottom panel:** Western blot analysis of total cell extract with anti-HA antibody confirmed the presence of transfected kinases in cells.
3.6 Analysis of Scribble Function: *In vitro* Kinase Assay

Several general regions of Scribble and both ERK2 and ERK6 had now been identified which were important for binding between this cell polarity regulator and both MAP kinases. However, no biological or biochemical information was available regarding the function of binding between these proteins. Therefore, kinase assays were performed in MDCK and NIH3T3 cells to determine the primary role of MAP kinase and Scribble associations, and to further determine, from a biochemical standpoint, if these associations are negatively or positively regulating these two MAP kinases. These assays were performed in the absence and presence of serum to confirm that Scribble alone does not have any kinase activating potential. The three samples used were ERK1, ERK2, and ERK6, with ERK1 serving as a negative control since we had previously found that Scribble is unable to bind to ERK1, which is highly homologous to ERK2.

Unphosphorylated myelin basic protein (MBP) was used as the substrate to determine MAP kinase activity and the effects that Scribble has on kinase activity. Results revealed that Scribble expression alone was insufficient to influence MBP phosphorylation status, hence, kinase activity, as determined by ERK1 activity in both cell lines. However, in both MDCK and NIH3T3 cells, ERK2 and ERK6 samples had decreased kinase activity in the presence of Scribble, as determined by a reduction in phosphorylated MBP expression. This further suggested that Scribble acts as an inhibitor of kinase activity in both MDCK and NIH3T3 cells. However, the amount of reduced MBP phosphorylation was dependent on which cell line was used. Specifically, the effects of Scribble on kinase activity were much more profound in MDCK cells than NIH3T3. In addition, these results implied that
the physical interaction between Scribble and ERK2/ERK6 was crucial to the kinase inhibiting functions of Scribble.
Figure 3.6. Kinase Assay: Scribble Effects on MAP Kinase Activity. Two different cell lines, MDCK and NIH3T3 cells, were used in these kinase assays to ensure accuracy of results. Assays were performed in the presence and absence of Scribble and serum, denoted with “+” and “−”, respectively, to verify that Scribble alone does not have any
kinase activating potential. ERK1, an ERK2 homologue, was used as a negative control as previous immunoprecipitation results conveyed its inability to bind with Scribble. 

**A:** Isolated ERK2 and ERK6 from MDCK cells exhibited downregulated kinase activity in the presence of Scribble, as determined by MBP phosphorylation status. 

**B:** Isolated ERK2 and ERK6 from NIH3T3 cells also exhibited downregulated kinase activity in the presence of Scribble, as determined by MBP phosphorylation status.
3.7 Scribble as a Tumor Suppressor: Focus Formation Assay

A primary biological function of Scribble on these two kinases had now been partially resolved. These kinase assays indicated that Scribble functions as an inhibitor of ERK2 and ERK6 kinase activity. ERK2 and ERK6 are described as proteins involved in biochemical pathways playing a role in cellular growth, survival, and response to stress stimuli. Consequently, after we found that Scribble downregulates activity in both kinases, we further reasoned that with upregulated ERK2 and ERK6 activities, induced by Ras and Rit, respectively, we could further shed light on the role of Scribble and kinase interactions. Previously performed focus formation assays (FFAs), using NIH3T3 cells, showed that over-expression of H-RasV12 or RitQ79L can induce focus formation at least by activating ERK2 and ERK6 kinase activities, respectively. Therefore, FFAs were performed with NIH3T3 cells in order to determine how Scribble can influence the formation of cell foci. pCEF-H-RasV12 and pCEF-RitQ79L vectors, specific constitutive activators of ERK2 and ERK6, respectively, were transfected into cells in the presence and absence of Scribble. Cell cultures were also transfected with an empty vector, a control to observe normal cell growth, or Scribble, in the absence of any constitutive MAP kinase activators, to determine if over-expression of Scribble could affect focus forming abilities.

Subsequent cell cultures had one profound implication: Scribble acts as a tumor suppressor, as deduced by inhibition of focus formation. Cells transfected with the empty vector or Scribble exhibited no focus formation, suggesting that Scribble over-expression alone does not promote focus formation. On the other hand, compared to the control, cells transfected with H-RasV12 and RitQ79L exhibited a remarkably large and significant
increase in the number of foci present. These results suggested that uncontrolled Ras and Rit activation, and consequently constitutive ERK2 and ERK6 signaling activation, played a substantial role in focus formation. Moreover, cells co-expressed with H-RasV12 and RitQ79L in the presence of Scribble over-expression demonstrated a dramatic reduction in the number of foci on the cell plates.
Figure 3.7A. Focus Formation Assays: Scribble and Constitutively Active Kinase Signaling. These cells were co-transfected with respective plasmid DNA, as indicated above, allowing for cultures to grow for three weeks following transfection by replacing medium every three to four days. Cell cultures were then fixed and stained to clearly visualize foci.
Figure 3.7B. Quantified Focus Formation Assay Data. Experiments were performed in triplicate and the numbers of foci on all plates were counted twice and averaged. Standard deviations of quantified samples are included.
3.8 Scribble and MAP Kinase Distribution: Immunostaining

With the identification of a physical interaction between Scribble and both ERK2 and ERK6, along with the clarification of the biological function of this association, we also wanted to determine where in the cell these protein interactions occurred. It is known that endogenous Scribble localizes at adherens junctions as part of the Scribble polarity module. In addition, several MAP kinases have been found to localize at the inner leaflet of the membrane as part of a scaffolding complex until activation occurs, which causes MAP kinase nuclear translocation and activation of transcription factors.

To determine how these interacting proteins co-localize together inside the cell and how MAP kinases affect intracellular Scribble distribution, HeLa cells were cultured and transfected with an empty vector, serving as the control, ERK1, and ERK6, all in the presence and absence of His-tagged Scribble, in addition to a sample of cells transfected with Scribble alone. Cells were incubated with an anti-Scribble antibody, which was further visualized with green fluorescein isothiocyanate, or FITC, staining, and cell nuclei were stained with 4′,6-diamidino-2-phenylindole, or DAPI. Results confirmed that endogenous Scribble is localized to the plasma membrane and further suggested that ERK1 and ERK6 do not affect the distribution pattern of Scribble. In cells containing transfected Scribble, staining indicated that over-expressed Scribble, in addition to plasma membrane localization, was distributed throughout cytosolic portions of the cell. Of note, in ERK1 and Scribble transfected cells, Scribble appeared to locate to peri-nuclear regions in addition to plasma membrane localization, an observation that must be further examined and confirmed.
Figure 3.8A. Control Vector Transfected Cells and Scribble Distribution. HeLa cells were transfected with a control vector. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Figure 3.8B. ERK6 Transfected Cells and Scribble Distribution. HeLa cells were transfected with HA-tagged ERK6 plasmids. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Figure 3.8C. Scribble Transfected Cells and Scribble Distribution. HeLa cells were transfected with His-tagged Scribble plasmids. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Figure 3.8D. Control Vector and Scribble Co-transfected Cells and Scribble Distribution. HeLa cells were co-transfected with both control vector and His-tagged Scribble plasmids. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Figure 3.8E. ERK1 and Scribble Co-transfected Cells and Scribble Distribution. HeLa cells were co-transfected with both HA-tagged ERK1 and His-tagged Scribble plasmids. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Figure 3.8F. **ERK6 and Scribble Co-transfected Cells and Scribble Distribution.** HeLa cells were co-transfected with both HA-tagged ERK6 and His-tagged Scribble plasmids. Cell samples were incubated with either αScribble antibody, DAPI, and αgoat/FITC conjugate antibodies or DAPI with αgoat/FITC conjugate antibodies in the absence of αScribble to serve as a control. Scribble localization is observed with green staining.
Discussion

4.1 MAP Kinases: ERK2 and p38γ

Mitogen activated protein kinases (MAP kinases) are a family of serine/threonine specific kinases that respond to a wide variety of upstream stimuli including growth factors, mitogens, proinflammatory cytokines, osmotic stress, and heat shock. MAP kinase sub-families are categorized according to biochemical function, sources of activation, and downstream effectors. These kinases play significant roles in regulating cellular activities such as proliferation, differentiation, survival, and apoptosis (Garrington, 1999). However, precise analysis of the complex and interdependent pathways intrinsic to these signaling networks is a difficult task due to the limited availability of kinase inhibitors specific to multiple pathways.

Extracellular signal-regulated kinase-2, or ERK2, is one of the most well-studied, canonical MAP kinases, and is widely activated by growth factors and biochemical cascades involving the small GTPase, Ras, and MEK, ultimately allowing for the transduction of activated proliferation signals. p38γ (ERK6), on the other hand, is a poorly understood member of the p38 stress activated family of stress activated protein (SAP) kinases, which is due, in large part, to the lack of specific pharmacological inhibitors. Our previous research focused on better understanding the biochemical aspect of the ERK6 signaling pathway, \textit{in vitro}. Our group successfully identified an upstream activator of
ERK6, namely, Ras-like protein in tissues, or Rit, by exclusively activating oncogenic ERK6 signaling with a constitutively active form of Rit. Major proteins involved in ERK2 and ERK6 signaling may be seen in Figure 4.1.

**Figure 4.1. ERK2 and ERK6 Signaling.** The major proteins involved in ERK2 and ERK6 activation may be seen here. Signaling between B-Raf and MEK3 and MEK6 remains unknown and is currently being investigated.

### 4.2 ERK6 and Rit

Rit was recently identified as a novel branch of the Ras superfamily, and is found highly expressed in many embryonic and adult tissues (Sakabe, 2002). Rit shares more than 50% amino acid homology with Ras, and is closely related to Rin (Ras-like protein in neurons) and Ric (Ras-related protein which interacts with calmodulin) proteins, which are
evolutionarily conserved sub-families of Ras G-proteins that are highly expressed in brain tissues (Andres, 2006). Our previous studies involved determining downstream effects of Rit, where NIH3T3 cells transfected with constitutively active Rit plasmids resulted in the development of multiple foci. At the time, no signaling pathway involved in the Rit-induced effects was known until successive experimentation identified the involvement of ERK6. ERK6 is the least studied protein of the four known isoforms in the p38 family of MAP kinases. Our previous reports suggested that Rit is a specific activator of ERK6, whereas no other MAP kinases, including ERK1, ERK2, JNKs, p38s, and ERK5, are activated.

4.3 ERK6 and Associating Proteins

Scaffolding proteins play a significant role in biochemical functions by allocating specific proteins and regulating their activities in a spatiotemporal manner. MAP kinases have been found to tether with such scaffolding proteins until activation occurs, in which kinases are then able to reach downstream effectors and induce biological output, for example, cellular growth and differentiation. Therefore, we hypothesized that such binding partners and scaffolding proteins may exist specific to ERK6, regulating Rit-MAP kinase signaling. Yeast two-hybrid screenings, using ERK6 as bait, revealed the ability of ERK6 to bind to MEK3, a previously acknowledged upstream activator, as well as Scribble, a cell polarity regulator previously not known to interact with ERK6. Loss of function Scribble mutations were originally identified as promoters of tumorigenesis in certain epithelial cancers and have since been an active area of cancer research. Recent
analysis of Scribble function has demonstrated that Scribble LRR domains are necessary for regulation of cell proliferation and cell polarity, whereas PDZ domains are dispensable for epithelium formation but enhance LRR localization to septate junctions, providing a comprehensive control of proliferation (Zeitler, 2004).

4.4 MAP Kinases and Scaffolding Proteins

Intracellular signal transduction occurs via cellular response to specific extracellular incidents through a series of molecular events that connect the environment and cell surface to the nucleus and modification of downstream gene expression patterns, regulating molecular activities in the cell through the elicitation of secondary changes. These molecular events are highly dependent on several intricate combinations of factors including intracellular location, concentration of molecules present, and activation or inhibition of signaling constituents. Such inhibition of signaling activity may occur through, for example, scaffolding proteins that bind and regulate protein function, such as MAP kinase phosphorylation events. Several models of MAP kinase regulation via scaffolding proteins have been identified and studied, such as KSR1 and MP1. KSR1 is a protein that interacts with and regulates Raf and ERK signaling by conditionally potentiating and attenuating intensity and duration of cascade activation (McKay, 2009). MP1 is another scaffolding protein that binds MEK1, an ERK1 activator, regulating MAP kinase signaling (Sharma, 2005). Interestingly, MP1 is specific to ERK1 and not ERK2, two highly homologous kinases sharing 88% amino acid homology. These are two representative and well studied examples of multiple scaffolding proteins that have now
been identified as regulators of such MAP kinases as ERKs, p38 isoforms, and Jun kinases, some of which regulate one specific MAP kinase, whereas several others are dual-specific scaffolding proteins, as indicated in Table 1.1 of the Introduction.

Yeast two-hybrid screenings and immunoprecipitation analysis suggested that Scribble not only can bind ERK2 and ERK6, but has the selectivity to distinguish between highly homologous canonical MAP kinases, ERK1 and ERK2. In the past, molecular functions of ERK1 and ERK2 in biological systems were recognized merely as redundant and interchangeable, with no outstanding biological distinction between the two kinases. Both ERK1 and ERK2 are expressed in almost all tissues but display varying levels depending on the tissue (Pags, 2004). The hypothesis that ERK1 acts in a complex manner by attenuating or fine-tuning ERK2 activity is supported by two lines of evidence. First, ERK2 activation was significantly upregulated, determined by phosphorylation status and immediate-early gene transcription, in ERK1-deficient murine fibroblasts and neurons (Mazzucchelli, 2002; Pags, 1999). Second, ERK2 signaling in ERK1-deficient mice was enhanced, a phenomenon linked to improvement of certain forms of learning and memory (Mazzucchelli, 2002). Furthermore, ERK1-deficient cells exhibit enhanced ERK2 activation with no increase in ERK2 protein expression, which is believed to occur due to competition between both kinases in binding to MEK, an upstream activator (Vantaggiato, 2006). As inactive ERK1 can affect certain observable phenotypes, such as oncogenic transformation in NIH3T3 cells, it is suggested that ERK1 displaces ERK2 from MEK, rather than regulating downstream effectors, a hypothesis supported by an increase in MEK-ERK2 complex formation in ERK1-deficient fibroblasts (Vantaggiato, 2006).
Importantly, it has been shown that, relative to kinase partners, optimal concentrations of scaffolding proteins are required to maximize signaling output, where ratio alterations of involved proteins can influence threshold properties of the system as well as influence non-linear cellular responses (Kholodenko, 2006; Levchenko, 2000). Recent research, such as our own, has and will continue to elucidate biological roles, functions, mechanisms, and interactions specific to individual kinases as new technologies, such as gene knockouts and siRNA and shRNA based gene knockdown, continue to progress.

4.5 **Scribble: A Multivalent Scaffolding Protein**

Scribble is a protein that has been identified as a cell polarity regulator, where in conjunction with Dlg and Lgl, form the Scribble polarity module, which plays a role in establishing apicobasal polarity in epithelial cells. As previously described, Scribble locates at adherens junctions in mammalian epithelial cells, and regulated co-localization of these three proteins is necessary for the maintenance of normal cellular activity. Scribble contains 16 LRR domains and four PDZ domains, which are necessary for organized protein-protein interactions. This was initially observed through structure-function analysis in *Drosophila* which revealed the necessity of the second PDZ motif for the dictation of Dlg localization (Humbert, 2008). However, in addition to functioning as a polarity regulator, Scribble has shown the ability to bind to ERK2 and ERK6, inhibiting their kinase activities and potentially serving as a scaffold for these kinases.

Studies have has shown that many proteins are able to perform several intracellular tasks, such as Axin. Axin is a scaffold protein containing several domains that enable
execution of two primary functions: acting as a negative regulator of Wnt signaling and facilitating Jun kinase activation, all the while operating as a scaffold for many signaling molecules. Axin appears to differentially assemble, forming two individual but dependent complexes, one specific to Wnt signaling and the other specific to Jun kinase signaling (Zhang, 2001). This type of dual function activity may be observed in other proteins as well, such as ZO-1, -2, and -3 zonula occludens proteins, peripheral proteins which locate at junctional sites, such as adherens junctions (Bauer, 2010). Recent evidence has accumulated suggesting that these ZO proteins serve two primary functions: exertion of functions related to structural barrier mechanisms and involvement in signal transduction and transcriptional modulation, accomplished through the association of regulatory molecules, including adapter proteins, signaling molecules, and growth factors, moderating cell cycle progression (Bauer, 2010). Scribble, another multi-domain protein, may also perform several cellular tasks in a similar manner. As previously mentioned, the second PDZ motif is required by Scribble for Dlg binding, yet no other specific Scribble domains have been identified in any known specific protein-protein interactions. Our results suggested that PDZ and LRR domains were required for binding between Scribble and both ERK2 and ERK6, however, none of the four PDZ or 16 LRR domains were exclusively identified as mandatory components in forming associations with either kinase. Thus, it may be possible that Scribble forms individual but dependent complexes, one specific to interactions involving the establishment and maintenance of apicobasal polarity, the other specific to the negative regulation of ERK2 and ERK6 signaling. Zeitler et al., reported that, in Drosophila, Scribble LRR domains play a role in the regulation of cell
proliferation and cell polarity, whereas PDZ domains are dispensable for epithelium formation but enhance LRR localization to septate junctions. This important relationship could suggest that, although binding between Scribble and MAP kinases may occur through both LRR and PDZ domains, Scribble may localize to adherens junctions via LRR domains, and may tether kinases via PDZ domains, co-localizing multi-protein complexes at adherens junctions until upstream signaling molecules induce activation, and subsequently, kinase dissociation and stimulation of downstream effectors. This hypothesis is supported by recent research that reveals the binding of AF6, a Ras interacting protein, and Eph-related receptor tyrosine kinases, involved in the developing nervous system, via PDZ domains. This relationship allows for the mediation of Ras signaling, where AF6 may function as a scaffold protein, localizing receptors to specific regions within the cytoplasmic membrane, or linking them to defined signal transduction pathways (Hock, 1998).

4.6 Scribble and MAP Kinases

Immunoprecipitation studies revealed the importance of LRR and PDZ domains in the Scribble protein necessary for binding ERK2 and ERK6. Moreover, results from deletion mutant studies disclosed the importance of the ten amino acid sequence at the C-terminus of both ERK2 and ERK6, containing a PDZ binding domain necessary for binding to Scribble. However, further analysis must be performed to determine the precise regulatory mechanisms of these interactions. Insight into this mechanism may be provided by the differences in the affinity of Scribble for both kinases, as we consistently observed
that higher levels of Scribble were immunoprecipitated with ERK6 than ERK2. This may be explained by the activation status of both kinases, as determined by the presence of phosphate groups, which may alter the three dimensional conformation of the proteins, favoring a physical association with ERK6 over ERK2. Conducting further immunoprecipitation analysis with co-transfection of arbitrary cells with constitutively active upstream proteins, such as H-RasV12 and RitQ79L, and Scribble, may enhance our current understanding of the physical association occurring between these proteins, as the continual presence of phosphate groups may alter the affinity of Scribble with both kinases. Similarly, the basic structure of each kinase, in the absence of phosphate induced activation, may allow for the formation of a tighter association between proteins, analogous to the specificity that an enzyme has for its substrate.

Another explanation for this difference in Scribble affinity between ERK2 and ERK6 may be explained by a possible indirect binding of ERK2 to Scribble through ERK6. Tang et al., previously reported that K-Ras activates ERK6 by upregulating expression, independent of phosphorylation, but stimulates p38α through phosphorylation. In turn, this upregulated ERK6 expression promotes Ras transformation and phosphorylated p38α inhibits Ras transformation. Furthermore, the formation of an ERK6-ERK complex is critical in the induction of K-Ras transforming activity. Similar to the formation of an ERK6-ERK complex, perhaps ERK2 and ERK6 have the ability to form a protein complex, and in the presence of upregulated expression of both kinases, preferential binding occurs between Scribble and ERK6, whereas ERK2 indirectly binds to Scribble through ERK6.
This phenomenon may also be explained in the context of signaling modification, in which differences in upstream signal intensities instigate variable associating cycles between both kinases, where ERK6 binding domains are more accessible, and therefore are more likely to bind with Scribble. Additionally, Levchenko et al., reported that optimal concentrations of scaffolding proteins are required to maximize signaling output, where ratio alterations of involved proteins can influence threshold properties of the system. It may be possible that this preferential binding of Scribble to ERK6 over ERK2 may be due to the concentrations of interacting proteins, and adjusting protein concentrations may alter this observation, increasing the affinity of Scribble to ERK2.

4.7 Possible Role of Scribble Interactions with ERK2 and ERK6

Focus formation assays reconfirmed that NIH3T3 cells expressing constitutively active upstream positive regulators of ERK2 and ERK6, such as H-RasV12 and RitQ79L, respectively, displayed high focus forming ability. However, in cells co-expressing Scribble in addition to constitutively active upstream regulators, focus forming ability was significantly inhibited. As focus forming ability denotes tumorigenicity, our observations suggested the functional role of Scribble as a cellular tumor suppressor. The in vitro kinase assays further revealed that the association between Scribble and both kinases was indeed mandatory for the downregulation of kinase activity. As we expected, Scribble was unable to inhibit ERK1 kinase activity, a protein that Scribble was incapable of binding to. Thus, in combination with the implications obtained from previous in vitro kinase assay results, it was concluded that Scribble inhibits oncogenicity as long as tumor forming
events occur through the induction of ERK2 and ERK6 signaling. Of note, the observed inhibitory effects of Scribble on focus formation were more prominent in those cell cultures expressing constitutive ERK6 signaling with RitQ79L than those expressing constitutive ERK2 signaling with H-RasV12. We presumed that this inhibition of ERK2 and ERK6 activity is most likely due to the consequence of Scribble to bind, sequester, and isolate both kinases, inhibiting the transduction of upstream activation to downstream effectors.

Another plausible role of this physical interaction may be determined when identifying individual roles of the proteins. The canonical MAP kinases, ERK1 and ERK2, have been viewed as homologues containing overlapping functions, until recently. These widely accepted conjectures have made identification of individual functions rather difficult. An ERK2 study in murine models illustrated that ERK2 knockout mice are embryonic lethal, as ERK1 activities were insufficient to compensate for those activities of ERK2 (Yao, 2003). Interestingly, ERK1 knockout mice were generated via homologous recombination in embryonic stem cells resulted in normal sized, viable, and fertile mice, as functions performed by ERK2 appeared to be sufficient to compensate for those functions lost by ERK1. These ERK1 knockout mice only displayed minor anomalies in thymocytes, resulting in immature development (Pages, 1999). In addition, recent findings revealed that ERK2 activation plays a large role in carcinogenesis (Vantaggiato, 2006). This was confirmed by our focus formation assays, as Scribble appeared to inhibit the tumorigenesis promoting activities of ERK2. As Scribble has the ability to distinguish between ERK1 and ERK2, the formation of this Scribble association with ERK2 and
ERK6 may serve to minimize redundant signaling that may ultimately lead to tumor formation. Additionally, new functions for these two homologous kinases as signal transducers have emerged in the last few years. Pags et al., reported that ERK1 acts in a complex manner by attenuating or fine tuning ERK2 activity. Perhaps then, in a similar manner, specific downstream activation of ERK2 and ERK6 effectors is contingent upon orchestrated signaling between both kinases.

ERK6 (p38γ) studies have revealed several important relationships about the functions of this kinase. Sabio et al., previously reported that ERK6 knockout mice, under specific pathogen free and stress free conditions, exhibited normal viability, fertility, and appearance, with no obvious health problems. However, this observation is inconclusive, as all functional roles of ERK6 have not yet been fully elucidated, and specific systemic molecular deficits may results in different phenotypes. Furthermore, these studies, involving analysis of p38 proteins and their phosphorylation activities, suggested that a functional redundancy exists in highly homologous protein families (Sabio, 2005). This redundancy may be explained, in part, by the importance of the p38 signaling cascade, where several homologous proteins are required to ensure that indispensable signaling events for cell viability occur and downstream effectors are appropriately regulated. In addition, MEK3 and MEK6 are generally categorized as common upstream activators of ERK6. However, evidence advocates that a specificity and selectivity of MEK3 and MEK6 exist, namely, that MEK3 appears to favor phosphorylation of p38α and β, while MEK6 phosphorylates all p38 family members equally well (Pearson, 2001). This bias
presumptuously hints at subtle differences in the discrimination of ERK6 activation, giving rise to different outputs in signaling and eventually in biological phenotypes.

Biochemical signaling events, processes fundamental to cell survival, are predominantly governed by the regulation of timing and location of signaling molecules in cells. MAP kinases typically induce downstream effects via translocation from cytosolic regions into the nucleus, a process that occurs at rates dependent upon phosphorylation status of the kinases (Marchi, 2008). Yet, there is currently a lack of clear and unambiguous information regarding intracellular kinase localization and distribution. Scribble may act as a scaffolding protein for ERK2 and ERK6 depending upon kinase activation status, a topic that must be further examined in following experiments. It is known that, as kinases are responsible for transducing extracellular signals, they must be on “stand by”, localized in a particular cytosolic region and ready to translocate to the nucleus to achieve its desired effects, but the actual status of “stand by” has not been well documented so far. The presence of a scaffolding molecule could account for maintaining kinases in a state of readiness. ERK6 is a unique kinase in the sense that, in transfected cardiac myocytes, unlike p38α and β, which in the presence of Leptomycin B, a nuclear export inhibitor, remained highly concentrated in cardiac myocyte nuclei, ERK6 remained concentrated in cytosolic regions, hypothesized to be due to interaction with cytoplasmic substrates (Court, 2002). We speculate that this phenomenon can be explained through the interactions with Scribble, preventing its entry into the nucleus until appropriate conditions exist, allowing ERK6 activation and nuclear translocation. However, it is known that
overexpression of proteins, like that of ERK6 in transfected cardiac myocytes, can force an artificial localization.

Diversities and specificities in kinase signaling pathways exist to maintain cellular homeostasis, allowing for orchestrated cellular function. Deregulation of MAP kinase signaling and Scribble mutations may both result in induction of impaired cellular activities, abnormal cell proliferation, and ultimately carcinogenesis. Providing insight into mechanisms and signaling interactions between Scribble, a cell polarity regulator, and both ERK2 and ERK6 may allow for the development of specific pharmacomimetics and/or biological inhibitors to restore normal cellular function and prevent tumorigenesis.
Literature Cited
Literature Cited


VITA

Steven Christofakis was born in New Orleans, Louisiana on December 10, 1984, to Paul and Peggy Christofakis. He has lived in Richmond, VA since the age of three. He attended the University of Mary Washington in Fredericksburg, VA, graduating with a B.S. in Biology in 2007. He went on to complete his M.S. in Physiology at the VCU School of Medicine in the Spring of 2010 and, beginning in the Fall of 2010, will attend the NYU College of Dentistry as part of the class of 2014.