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Role of ERK3 in Regulating RhoGDI1-PAKs Signaling Axis

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Role of ERK3 in Regulating RhoGDI1-PAKs Signaling Axis

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Hitham Abdulrahman Aldharee ENTITLED Role of ERK3 in Regulating RhoGDI1-PAKs Signaling axis BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Extracellular signal-regulated kinase 3 (ERK3) is an atypical protein kinase of the mitogen-activated protein kinase (MAPK) family. In comparison to well-investigated ERK1/2 (classical) MAPKs, much less has been discovered about ERK3 signaling and its cellular functions. Recent studies have shown that ERK3 is overexpressed in various types of cancers such as lung cancer and breast cancer and that ERK3 promotes cancer cell migration and invasion. How ERK3 regulates cancer cell motility and invasiveness, however, is still largely unknown. RhoGTPases, including Rho, Cdc42 and Rac1, play critical roles in regulating cell motility and invasiveness through activating downstream effectors such as p21-activated kinases (PAKs). Rho GTPase-GDP dissociation inhibitor 1 (RhoGDI1) inhibits RhoGTPases' activity by binding to GDP-bound GTPases and preventing their activation. Cdc42 and Rac1 are well-known activators of group I PAKs, including PAK1, PAK2 and PAK3. They activate group I PAKs through binding to the latter's auto-inhibitory domains and exposing the auto-phosphorylation residues. Interestingly, group I PAKs were shown to interact with and phosphorylate ERK3 at S189 of the SEG activation motif. In addition, proteomic analyses of ERK3-interacting proteins by Yeast-Two-Hybrid screening (Y2H) and Immunoprecipitation-Mass spectrometry (IP-MS) revealed that ERK3 interacts with RhoGDI1. These findings raise an intriguing hypothesis that ERK3 regulates RhoGDI1/RhoGTPases/PAKs signaling pathway, thereby

promoting cancer cell migration. To test this hypothesis, first, we confirmed the interactions of ERK3 with RhoGDI1, PAKs and Rho GTPases by co-immunoprecipitation and co-immunofluorescent staining. Interestingly, co-immunofluorescence data showed that ERK3 co-localizes with RhoGDI1, PAK1 and Cdc42 at the leading edge of the plasma membrane, suggesting that ERK3 might regulate PAKs' activation. Indeed, we found that in both A549 lung cancer cell and MDA-MB231 breast cancer cells, ERK3 greatly upregulates group I PAKs' activating phosphorylations at Ser144/Ser141 and Thr423/Thr402, but has no effect on PAKs' expression levels. We then sought to elucidate how ERK3 promotes PAKs' phosphorylations (activation). By performing Cdc42/Rac1 activation assay we found that ERK3 upregulates the cellular levels of the active forms of Cdc42 and Rac1 (GTP-Cdc42 and GTP-Rac1), which accounts for the increase of PAKs' activity. As RhoGDI1 inhibits the activation of Cdc42 and Rac1 via direct binding, we investigated the effect of ERK3 on their interactions. While exogenous overexpression of ERK3 did not show clear effect, expression of ERK3 kinase dead mutant (a potential dominant negative form of ERK3) greatly increased the interaction of RhoGDI1 with Rac, suggesting a potential role of ERK3 in facilitating the dissociation of RhoGDI1 off Rac. Mechanistically, our preliminary data of in vitro kinase assay suggests that ERK3 might phosphorylates RhoGDI1, which may decrease RhoGDI1's binding with Rac1/Cdc42. Lastly, we found that ERK3-induced cancer cell migration was almost abolished by the inhibitors of group I PAKs. In conclusion, these results clearly suggest that ERK3 promotes cancer cell migration through upregulating group I PAKs signaling.

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Acknowledgement

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I. Introduction

i. Mitogen-Activated Protein Kinase Pathways

Mitogen-Activated Protein Kinases (MAPKs) are serine/threonine kinases that transduce several extracellular signals into intracellular responses (Coulombe and Meloche, 2007; Bind et al., 2004). MAPKs have been shown to play significant roles in regulating multiple cellular events, such as cell proliferation, differentiation, apoptosis, metabolism and motility (Dhillon et al., 2007). Six groups of MAPKs have been identified in mammals, including extracellular signal–regulated kinases (ERK)-1/2, ERK3/4, ERK5, ERK7/8, c-Jun N-terminal Kinases (JNK)-1/2/3 and the p38 isoforms α,β,γ, δ (Dhillon et al., 2007). Based on the activation process and the regulatory mechanisms, MAPKs are classified into classical (conventional) or non-classical (atypical) kinases (Figure 1, Coulombe and Meloche et al., 2007). The Conventional MAPKs group consists of four subgroups: ERK1/2, p38s, JNKs and ERK5. These MAPKs are activated by mitogenic signals through a cascade that involves MAPK kinase kinase (MAPKK) and MAPK kinase (Figure 2) that turns in activating MAPKs through phosphorylating a conserved T-X-Y phosphorylation motif (Kostenko et al., 2012) (Figure1). Importantly, dual threonine/ tyrosine phosphorylations in T-X-Y motif are critical for MAPKs' activation (Coulombe and Meloche et al., 2007).The atypical MAPKs group includes four members: ERK3, ERK4, ERK7 and Nemo-like kinase (NLK). In contrast to classical MAPKs, atypical MAPKs have a single phosphor acceptor residue and possess glycine or glutamic acid residues instead of tyrosine in their phosphorylation motif. Interestingly, this group of

MAPKs cannot be activated by the mitogenic signals for conventional MAPKs. In comparison with the well-established conventional MAPKs signaling cascade, much less is known about atypical MAPKs signaling and functions (Coulombe and Meloche, et al., 2007).

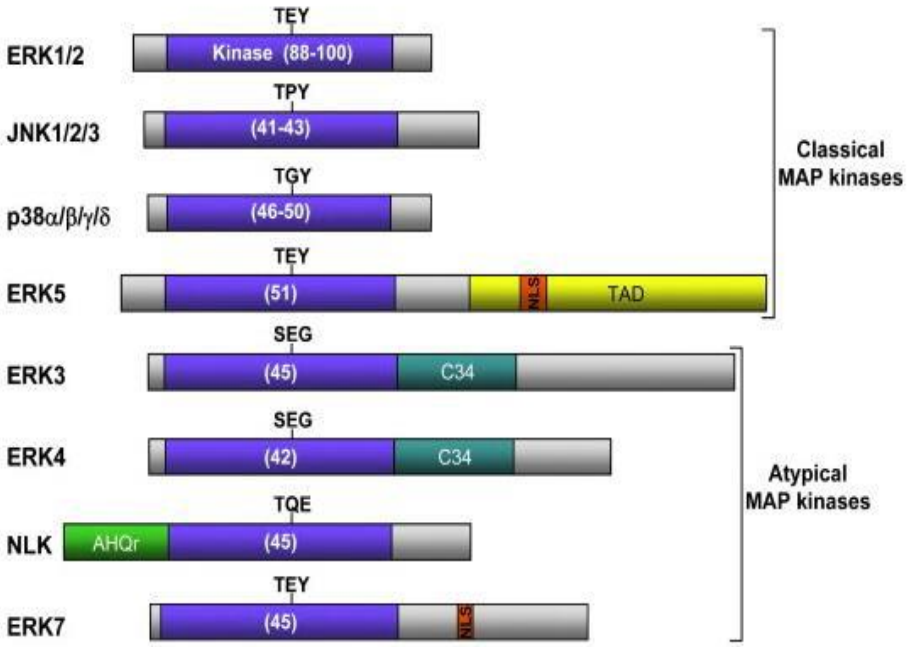


Figure 1: Schematic representation of human MAPKs structure.

The composition of MAP kinases is shown as N- and C-terminal extensions of variable lengths surrounding a kinase domain (in blue). NLS: nuclear localization sequence; TAD, transactivation domain; C34: conserved region in ERK3/4; AHQr: alanine (A), histidine (H) and glutamine (Q) rich domain. Numbers in the kinase domains indicate the percentage of homology each MAPK has in its kinase domain relative to that of ERK1 (Coulombe and Meloche et al., 2007).

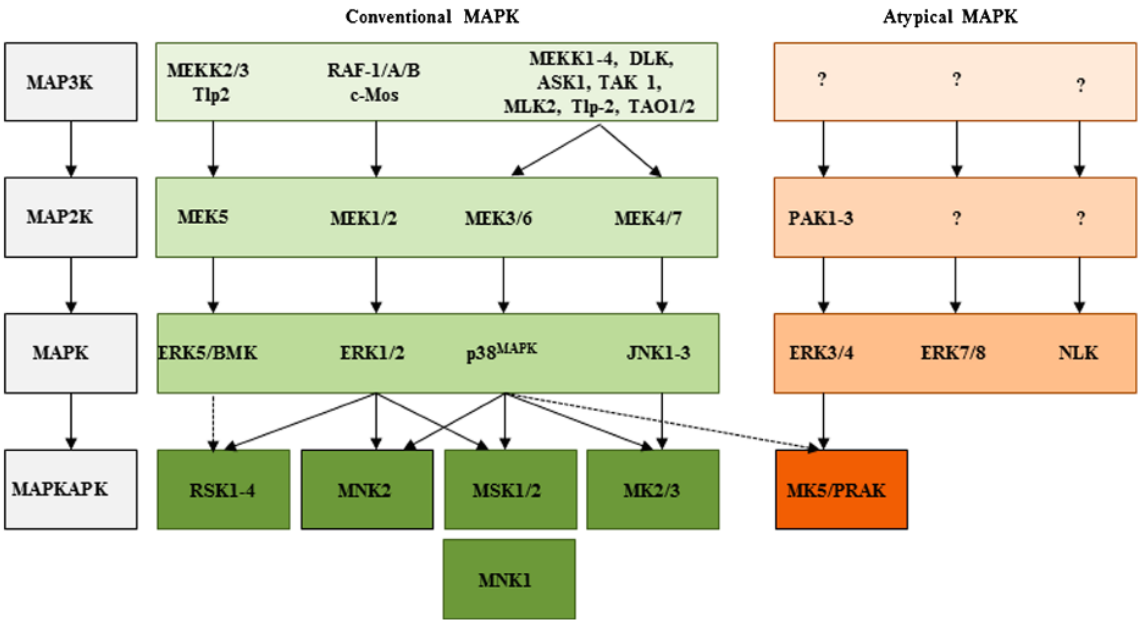


Figure 2: Schematic presentation of distinct MAPK signaling pathways in human.

ii. ERK3

Extracellular Regulated Kinase 3 (ERK3) is a member of a typical MAPK (Coulombe and Meloche et al., 2007). ERK3 gene was cloned in the early 1990s using an ERK1 derived probe (Boulton, et al., 1991). ERK3 protein contains 721 amino acids, but shows a molecular mass of 100 kDa possibly due to posttranslational modifications. ERK3 protein highly unstable with a half-life of 30 minutes (Turgeon et al., 2000). It has been shown that ERK3 mRNA is expressed ubiquitously in all mammalian tissues with the highest expression in the brain, skeletal muscle and gastrointestinal tract (Turgeon et al., 2000). ERK3 protein structure is composed of a kinase domain at the amino terminus followed with a central C34 (conserved in ERK3/4) domain and a unique very long carboxy terminal domain that is not shown in classical MAPKs (Figure 1, Coulombe and Meloche et al., 2007). Interestingly, on the contrary to conventional MAPKs such as ERK1/2, ERK3

possesses a single Ser-Glu-Gly (SEG) phospho-acceptor motif in its activation loop, instead of the Thr-X-Tyr (TXY). ERK3 and ERK4 have the so-called C34 domain, whose biochemical and cellular functions are unclear. The c-terminus of ERK3 is hyperphosphorylated during mitosis, which is important for ERK3 protein stability (Tanguay, Rodier, and Meloche. 2010). However, more studies need to be done to explore the importance of the unique C- terminal domain in ERK3 that is not conserved in classical MAPKs and ERK4 (Figure 1, Coulombe and Meloche et al., 2007).

Little is known about the activation of ERK3 kinase. Thus far, no upstream cellular stimulus has been identified for ERK3 activation. S189 within the SEG activation motif of ERK3 is known to be phosphorylated by ERK3 itself (Coulombe and Meloche et al., 2007) (Cheng et al., 1996) and by group I p21-activated kinases (PAK1, 2, and 3) (Mota-Peynado, et al., 2011; Déléris, et al., 2011), which presumably enhances ERK3 kinase activity. Besides S189 phosphorylation, ERK3 is regulated on gene expression and protein stability (Coulombe and Meloche et al., 2007). ERK3 expression is shown to be upregulated by BRAF in melanoma (Hoeflich et al., 2006) and by BMI1 (via derepressing ERK3 targeted by let7i miRNA) in head and neck cancers (Elkhadragy et al., 2017). ERK3's expression level and functions are also regulated by protein stability through ubiquitination-dependent proteasome degradation pathway (Coulombe et al., 2003, 2004). In addition, ERK3 protein has been shown to be localized in the nucleus and cytoplasm and at the leading edge of the plasma membrane of migrating cells (Bind et al., 2004; Julien et al., 2003; Schumacher et al., 2004). ERK3 can be translocated from the nucleus to the cytoplasm and its nuclear export is mediated by CRM1 (Déléris, et al., 2008)

iii. Physiological functions of ERK3

Various physiological roles, including development and immune functions, have been revealed for EKR3 in mammalian cells. Neonate mice with homozygous ERK3 gene disruption (ERK3 $^{-/-}$) die soon after birth due to lung maturation defect and acute respiratory system malfunction, suggesting that ERK3 is important for lung maturation and function (Klinger S *et al*., 2009). In addition, ERK3 $^{-/-}$ mice had reduced weights of fetal liver, lung and heart.

ERK3 also has been reported to regulate T-cell activation and development. Two Studies (Marquis et al., 2014; Sirois et al., 2015) reported that T cells showed a reduced level in activation and proliferation in ERK3-deficient condition, accompanied by abnormal cytokines secretion. These findings suggest that ERK3 is involved in regulating TCR-induced T cell activation. In addition, insulin secretion in β-islet cells was decreased by ERK3 depletion (Anhe et al., 2006). Moreover, ERK3 is induced by pro-inflammatory cytokines and promotes endothelial cells migration and proliferation through regulating SRC-3/SP-1-mediated VEGFR2 expression (Wang et al., 2014). Furthermore, ERK3 interacts with hCdc14A (a member of the dual-specificity phosphatase family) and is localized to mitotic spindles during mitosis and may regulate cells division and differentiation (Hansen et al., 2008).

ERK3/ERK4 were identified as substrates of group I p21-activated kinases (PAKs). PAKs (1/2/3) directly phosphorylate ERK3 and ERK4 at S189 and S186 residues, respectively in their activation loop (Déléris, et al., 2011). Phosphorylations of ERK3 and ERK4 at this residue enhances their interactions with MAPK-activated protein kinase-5 (MK5), leading to MK5's kinase activation and phosphorylation at Thr182 (Seternes OM

et al., 2004; Schumacher S et al., 2004). It remains controversial whether or not ERK3 directly phosphorylates MK5 at Thr182. The study by Seternes et al claimed that MK5 is a direct substrate of ERK3, whereas the study by Schumacher S et al. demonstrated that ERK3 (or ERK4) increased MK5's T182 phosphorylation by promoting MK's autophosphorylation on this residue.

iv. ERK3's roles in cancers

The conventional MAPKs have been well studied in cancer cells and shown to play critical roles in a variety of processes, including proliferation, survival and migration/invasion (Kostenko, Dumitriu, and Moens 2012).The involvement of atypical MAPKs in tumorigenesis has just emerged recently. Accumulating studies have demonstrated the importance of ERK3 signaling in cancers. ERK3 expression level has been reported to be upregulated in multiple types of cancers, including lung cancer (Long et al., 2012), oral squamous cell carcinoma (Rai R *et al*., 2004) and gastric cancer tissues (Kostenko S *et al.*, 2012). At cellular levels, ERK3 promotes migration and invasion of lung cancer cells and breast cancer cells (Long et al 2012; Almahdi et al, 2015). In breast cancer cells, ERK3 localizes mainly in the nucleus, but also at the leading edge of the plasma membrane. Of particular interest, ERK3 was shown to localize in the lamellipodia structure which is known as cell protrusion structure and critical for cancer cell migration (Almahdi et al, 2015). In the same study, ERK3 overexpression was shown to alter cell morphology by regulating cell adhesion and spreading. Taken together, these findings suggest that ERK3 plays important roles in cell morphology and migration.

ERK3 also promotes cancer cell invasion. A study by (Long et al 2012) showed that ERK3 directly binds to steroid receptor co-activator (SRC-3) and phosphorylates S857

residue which is essential for SRC3 to interact with the ETS transcription factor PEA3 and form the ERK3/SRC-3/PEA3 complex. As a consequence, the expression of matrix metalloproteinases (MMPs) are upregulated via this transcriptional complex (SRC-3/PEA3), which turns in promoting cancer cells invasiveness.

v. PAKs

The p21-activated kinases (PAKs) are serine/threonine kinases that participate in various cellular events (Dan et al., 2001; Knaus and Bokoch, 1998). They are conserved in yeast and Drosophila (Bokoch et al., 2003). PAKs consist of 545 amino acid with a molecular mass of 65-74 kDa. In 1994, PAKs were discovered and identified as small GTPases binding proteins when scientists were looking for Rac and Cdc42 targets (Manser et al., 1994). The p21 small GTPases (Rac/Cdc42) were later found to be PAKs' bona fide activators (Rudel and Bokoch 1997; Bokoch et al., 1998). Based on their protein structures and the regulatory mechanisms, mammalian PAKs are divided into two groups, group I (PAK1, PAK2, and PAK3) and group II (PAK4, PAK5, and PAK6) (Sells et al., 1997; Manser et al., 1997).

Group I PAKs (PAK1, PAK2, and PAK3) are mainly expressed in brain, muscle, and spleen in mammalian tissues. They possess a p21-binding domain (PBD) or Cdc42/Rac interacting domain (CRID) that overlaps with the auto-inhibitory domain (AID) near the N-terminal region (Figure 3, Manser and Zhao. 2005). Right after the PBD domain is the lipid binding motif where phospholipids such as phosphatidic acid (PA) can bind and activate these kinases (Abramovici et al., 2009). In addition, a SH3 binding site which is known as a binding domain for PAK-interacting exchange factor (PIX) is conserved in the

regulatory domain. The C-terminal catalytic domain is 90 % identical in PAK1, PAK2, and PAK3 (Nicholas et al., 2014).

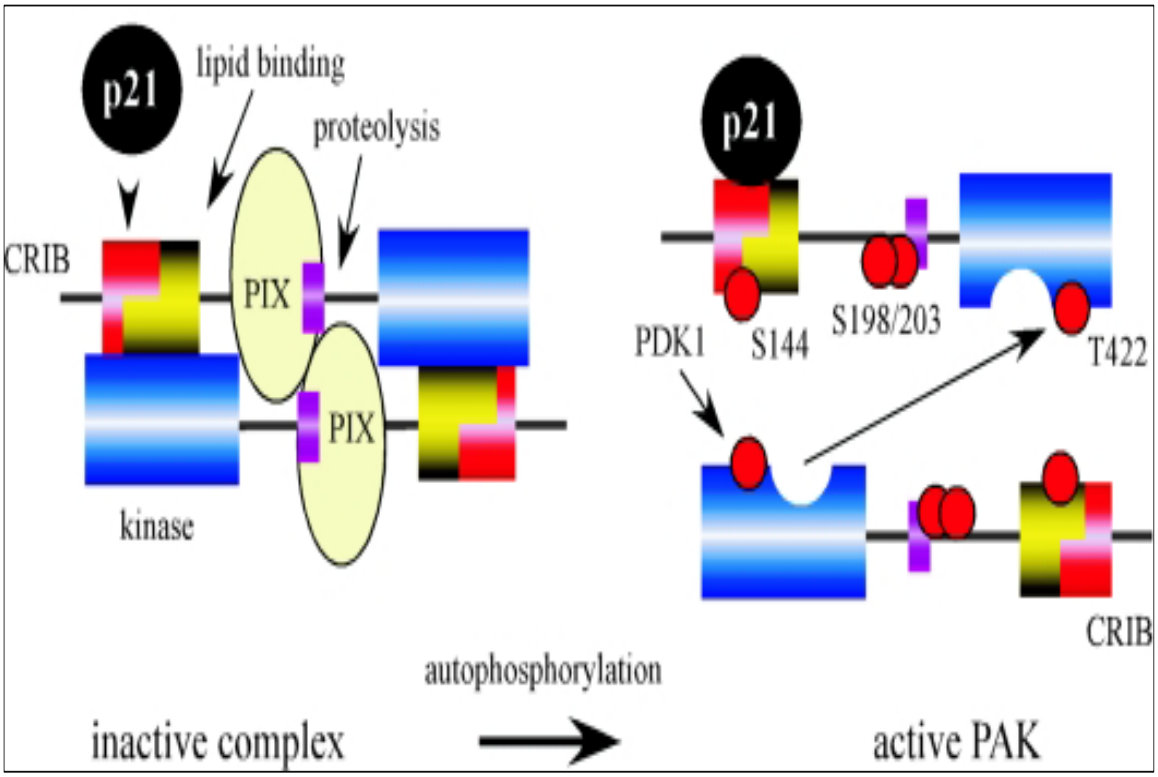


Figure 3: Structure of group I (PAK1, PAK2, PAK3) p21-activated kinases.

Group I PAKs have multiple auto- and trans-phosphorylation residues which are located in both the regulatory domain and the catalytic domain (Figure 3). These phosphorylation sites play roles in protein-protein interaction and kinases activation through relieving the auto-inhibition of PAKs. PAK1/PAK3 possess S144 (S141 for PAK2) auto-phosphorylation residue in their regulatory domains that is critical for kinase activation (Parrini et al., 2002; Pirruccello et al., 2006). Thr 423 of PAK1/PAK3 (Thr 420 for PAK2) is a trans-phosphorylation residue that is located in the catalytic domain and

participates in PAKs' activation through preventing dimerization (Parrini et al., 2002; Pirruccello et al., 2006). It is important to point out that this is the only phosphorylation site identified in the catalytic domain of PAK1/2/3 (Parrini et al., 2002; Pirruccello et al., 2006). Several other autophosphorylation sites in the N-terminal regulatory region, such as Ser199, Ser204, Ser192 and Ser197, have been reported to be involved in PAKs' interactions with other proteins and the kinase activity (Manser et al., 1997; Gatti et al., 1999).

vi. Regulation of Group I PAKs Activation

PAKs' activation is regulated by the modification of group I PAKs' activation loop and the subsequent conformational change of protein structure. Inactive group I PAKs exist as homodimers in cells. The dimerization causes trans-inhibition conformation in which the auto-inhibitory domain (AID) in the amino terminal region of one molecule binds to and inhibits the catalytic domain of the other (Figure 3, Lei et al., 2000, Manser and Zhao. 2005). Under trans-inhibition conformation, the phosphorylation residues in both the regulatory domain and the catalytic domain are not exposed and thus unable to be phosphorylated for activating PAKs.

PAKs can be activated by the binding of the active form of small GTPases (GTP-Rac/ GTP-Cdc42) to CRID. Binding of Rho-GTPases disrupts the PAKs' dimerization and activates PAKs through exposing the auto- and trans-phosphorylation residues (S144 and Thr 423 for PAK1/3 and S141 and Thr 420 for PAK2). The phosphorylations of S141 and S144 in the regulatory domain partially activate kinases, followed by the subsequent phosphorylations of Thr420 and Thr423 residues in the catalytic domain and the full activation of PAKs activation. GTPases-dependent group I PAKs' activation occurs in

responses to growth factors, such as EGF and VEGF, that activate Rac and Cdc42 through a guanine nucleotide exchange factor (GEF) (Gatti et al., 1999; Bagheri et al., 2000; Chong et al., 2001; Parrini et al., 2002; Pirruccello et al., 2006).

Besides activation via small GTPases, GTPase-independent mechanisms have been reported for activating PAKs. Pyruvate dehydrogenase kinase 1 (PDK1) phosphorylates Thr 423 of PAK1/3 (Thr 420 of PAK2) and activates these PAKs (King et al., 2000). Group I PAKs also can be activated when phospholipids, such as phosphatidic acid (PA), bind to the lipid binding motif and cause conformational change that disturbs kinases' dimerization (Abramovici et al., 2009). Sphingolipids were also shown to activate group I PAKs though a similar mechanism (King et al., 2000). Moreover, Cyclin-dependent kinase 5 (Cdk5) and Cdc2 phosphorylate Thr212, which disrupts PAKs' homodimerization and enhances kinases' activation (Thiel et al., 2002). On the other hand, group I PAKs are negatively regulated via several mechanisms. For instance, the binding of Pak-interacting protein (PIP) to CRID/PBD inhibits PAKs activation and signaling (Xia et al., 2001).

vii. Physiological Roles of Group I PAKs

PAKs are involved in regulating a wide variety of cellular events such as cytoskeletal remodeling, cell motility, proliferation and cell survival. A pioneering study (Sells et al., 1997) shows that PAK1 promoted the formation of lamellipodia and filopodia structures which are important for cells to migrate. In the same study, PAK1 was shown to also localize at focal adhesions and control stress fiber formation. P21- activated kinases (PAK1, 2, 3) have several downstream targets that control cytoskeletal remodeling and cell motility, such as merlin, filamin, myosin light chain kinase, and LIM kinase (Rane and Minden. 2014).

Group I PAKs are involved in the regulation of MAPK signaling pathways. PAKs are known to activate ERK1/2 in response to growth factors and extracellular matrix signals (Beeser et al., 2005; Park et al., 2007). As mentioned above, PAK1/2/3 were recently found to also phosphorylate ERK3 and ERK4 on their activation loop at S189 and S186 residues, respectively, which turns in activating MK5, an downstream effector of ERK3 and ERK4 (Déléris, et al., 2011). As to the role in cell survival, PAK1 negatively regulates pro-apoptotic proteins dynein light chain 1 (DLC1) and BimL, by which PAK1 downregulates apoptosis (Vadlamudi et al., 2004). Furthermore, cell cycle progression is promoted when PAK1 activates the transcription factor NFkappaB and upregulates transcriptional level of cycling D1 (Dadke et al., 2003).

Owning to their essential roles in regulating cell morphology, motility and survival, group I PAKs play different biological roles in regulating development and metabolism. For example, PAK2 knock-out mice have embryonic lethal abnormalities suggesting that it plays critical function in embryogenesis (Qu et al., 2011; Arias and Chernoff 2008). While PAK1 knockout mice show no clear developmental defect, PAK1 level was shown to be downregulated in type 2 diabetic mice and may play an important role in glucose homeostasis (Wang et al., 2011). In addition, group I PAKs are important for heart muscle development and functions (Egom et al ., 2010; Ai et al ., 2011).

viii. Group I PAKs Roles in Cancer

The implications of group I PAKs (especially PAK1) have been well studied and identified as oncogenic proteins in multiple types of cancers. They promote cancer cell migration, proliferation, differentiation, as well as tumor growth and metastasis. Group I PAKs have been shown to be overexpressed and/or highly activated in cancers (Liu et al, 2016).

In breast cancer, about 50 % of breast tumors show hyperactivated and/or highly expressed PAK1 (Maroto et al, 2008). Furthermore, cancer cell migration, survival and colony formation are promoted by PAK1 through regulating the Wnt, Erk and Akt pathways (Ye DZ and Field J, 2012). In melanoma, PAK1 expression level is positively regulated by BRAF point mutation (V600E), a major cause of this type of cancer (Maldonado et al., 2003). In addition, group I PAKs are upregulated in non-small cell lung cancer (NSCLC) patients with metastasis as a result of altered epidermal-growth factor receptor (EGFR) signaling (Liu et al., 2016). EGFR signaling stimulates the activation of Rho-GTPases (such as Rac/Cdc42) which are activators of PAKs. Rho GTPases activate group I PAKs, thereby promoting NSCLC migration and proliferation. The combination of Group I PAKs inhibitor (IPA3) and EGFR-ITK (Gefitinib) greatly inhibited cancer cell proliferation and tumor growth. These findings suggest that group I p21-activated kinases (PAK1, PAK2, and PAK3) promote lung tumor formation and metastasis (Liu et al., 2016).

ix. Rho Family GTPases (Rho GTPases)

Rho GTPases comprise a family of low molecular weight switches that are involved in regulating several cellular aspects such as cell migration, proliferation, and cytoskeletal remolding (Ridley and Hall 1992; Ridley et al., 1992; Raftopoulou and Hall 2004). Rho sub-family belongs to Ras superfamily which consists of five sub-families (Boureux et al., 2007) Rho family of GTPases was first discovered and identified in Aplysia ((Madaul and Axel, 1985). Later on, Rho genes were uncovered in yeast, Drosophila, rat and human. Rho sub-family consists of small GTPases including Rho, Rac and Cdc42. Rac1 and Cdc42 were discovered in 1989 and 1990, respectively (Didsbury et al., 1989; Munemitsu et al., 1990). Rho GTPases share several common properties, such as their molecular weight (about 21 kDa), containing a GTPase, and the presence of the C-terminal domain which facilitates the localization of Rho proteins at the plasma membrane (Madaul and Axel, 1985).

Rho GTPases' activation is regulated through three main regulators: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Madaul and Axel, 1985). The action of (GEF) is required to catalyze the exchanging of the cytosolic GDP-bound inactive state of small GTPases to the GTP-bound active state. The activation process occurs at the plasma membrane as a response to various receptors signaling such as epidermal growth factor receptor (EGFR) and G-protein coupled receptor (GPCR). On the contrary, these GTPases are inactivated by the action of (GAPs) which catalyzes the hydrolysis of GTP-bound active form to GDP-bound inactive form. Lastly, GDIs are known to negatively regulate small GTPases' activation through binding to GDP-bound GTPases and preventing

GTPases' plasma membrane translocation and activation that is executed by GEFs (Figure 3, Huveneers and Danen, 2009). Three isoforms of RhoGDI have been identified: the ubiquitously expressed isoform 1 (RhoGDI1) (Fukumoto et al., 1990), RhoGDI2 and RhoGDI3 which are expressed manly in brain and lung (Adra et al., 1997; Zalcman et al., 1996).

RhoGDIs regulate the shuttling of small GTPases (Cdc42 and Rac) between the cytosol and the plasma membrane, which is critical for GTPases' activation/inactivation cycling catalyzed by GEF and GAP (Adra et al., 1997; Sasaki et al., 1998; Olofsson 1998; Golovanov 2001) Thus, the dissociation rate of the GTPases from RhoGDI tightly controls the activation of GTPases and the downstream PAKs. Interestingly, the association of RhoGDIs with Rho-GTPases is regulated by PAKs and other kinases (DerMardirossian et al. 2004) .For instance, PAK1 phosphorylates RhoGDI1 at two essential residues (S101 and S174) both *in vitro* and *in vivo*, altering its conformation and promoting the dissociation of Rac1 (DerMardirossian et al. 2004). In addition, Src kinase phosphorylates RhoGDI at Tyr156 residue, which significantly reduces the re-association of RhoA, Cdc42 and Rac1 with RhoGDI (DerMardirossian et al. 2006).

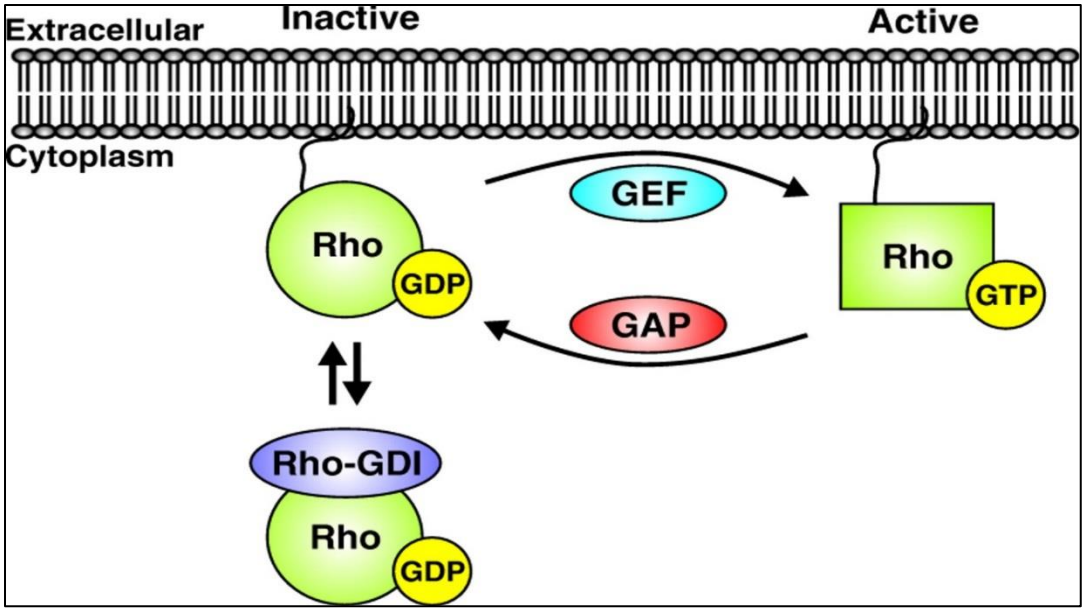


Figure 4: Activation/Inactivation cycle of Rho-GTPases

x. Hypothesis and specific aims

ERK3 promotes both migration and invasion of cancer cells. While ERK3 is known to phosphorylate SRC-3 and upregulate SRC-3-mediated expression of MMPs that are important for cell invasion, how ERK3 regulates cell morphology and motility is unclear. Two recent studies showed that ERK3 interact with PAKs, the master regulators of cell morphology and motility (Déléris, et al., 2011; Almahdi et al., 2015). In addition, both Yeast two hybrid (Y2H) screening (Vinayagam et al., 2011) and immunoprecipitation-mass spectrometry (IP-MS) analysis (unpublished data from our laboratory) identified RhoGDI1 as an interacting partner of ERK3 protein. Given that RhoGDI1 acts as an inhibitor of RhoGTPases and the downstream PAKs, these findings raise an intriguing hypothesis that ERK3 upregulates group I PAKs' activation through targeting RhoGDI1/RhoGTPases signaling axis, thereby promoting cancer cell motility. Precisely, this study aimed to identify a downstream target (pathway) of ERK3 by which it promotes cancer cell migration. Three aims are proposed to test the hypothesis: **(1)** To determine the role of ERK3 in regulating group I PAKs' activity (phosphorylations); **(2)** To determine the underlying mechanisms by which ERK3 regulates PAKs' activity; **(3)** To determine whether ERK3 and Group I PAKs cooperatively regulate cell migration.

II. Materials and Methods

i. Cell culture

Human cervical cancer cell line Hela, breast cancer cell line MDA-MB231 and lung cancer cell line A549 were purchased from *American Type Culture Collection* (ATCC). A549 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. HeLa and MDA-MB213 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS. All the culture media and supplements were purchased from Life Technologies/Invitrogen.

ii. DNA constructs

To generate pSG5-RhoGDI1-HA, first, full length RhoGDI1 cDNA was amplified from pCMV-XL5-RhoGDI1 (kindly provided by Dr. Julian Cambronero's Lab) using the following primers containing a *KpnI restriction* site: 5'GGGGTACCA TGGCTGAGCAGGAGCCCACA-3' and 5'GGGGTACCTCAGTCCTTCCAGTCC TTCTTG-3'. pSG5-RhoGDI1-HA was then generated by ligating RhoGDI1 fragment into pSG5-KHMA2M1-HA following *KpnI* digestion. To generate pET-28b (+) RhoGDI1-His, full length RhoGDI1 cDNA was amplified from pCMV-XL5-RhoGDI1 using the following primers containing *ECORI* sites: 5'CGGAATTCGATGGCTGAGCAGGAGCCCAC-3' and 5'CGGAATTCTCAGTC TCCAGTCCTTCTTG-3'. pET-28b (+) RhoGDI1-His was then generated by ligating RhoGDI1 fragment into pET-28b (+)-His following *ECOR I* digestion.

iii. Proteins expression and purification

Full length ERK3 kinase was expressed and purified in Sf9 insect cells as described previously (Seterns et al., 2004). RhoGDI1-His was expressed in E. coli (BL21) following the transformation with pET-28b (+) RhoGDI1-His plasmid, then purified using Nickel-NTA His-tagged proteins purification kit (Millipore Sigma, USA) following the manufacturer's protocol.

iv. Plasmids and siRNAs transfections

Transient transfections with plasmids were performed using Lipofectamine 3000 Reagent (Invitrogen/ThermoFisher Scientific, Carlsbad, CA, USA) or FuGENE HD transfection reagent (Active motiv, USA). siRNA transfections were performed using Dharma-FECT Transfection Reagent (Dharmacon, Lafayette, CO, USA), following the manufacturer's instructions. The silencer select siRNA targeting human ERK3 and the Silencer Negative Control #1 were purchased from Ambion/ThermoFisher Scientific (Waltham, MA, USA). Unless specifically indicated, cells were harvested for various analyses 48 hours after transfection.

v. Generation of breast cancer cell pools stably expressing shRNAs by lentiviral transduction

MDA-MD231 cell line with stable knockdown of endogenous ERK3 by lentiviral transduction of a short hairpin RNA (shRNA) specifically targeting ERK3 mRNA (shERK3). Stable cell pools expressing the non-targeting shRNA (shGIPZ) were used as a control. The pseudo-lentiviruses were produced in 293T cells by co-expression of constructs expressing shRNA and Trans-Lentiviral packaging plasmid mix (Open Biosystems). Pseudoviral particles were harvested 48 hrs post-transfection and

concentrated using PEG-it virus precipitation solution (System Biosciences) by following the manufacturer's instructions. Cells were transduced with prepared virus in the presence of polybrene (5 μg/ml). Two days post- transduction, cells were split and selected by puromycin (1 μg/ml) for 10 days. Knockdown of the targeted genes' expression was verified by Western blotting analysis.

vi. Western blotting

Cells were lysed with EBC lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 1 mM complete protease inhibitors [Roche Diagnostics] and 1 mM Phosphatase Inhibitor Cocktail III [Sigma-Aldrich]). Protein lysates were mixed with 5X SDS sample buffer and boiled then resolved on 12% SDS-PAGE gels. Western blotting was performed by first blocking nitrocellulose membranes with 5% non-fat milk in PBS-T buffer for 30minutes, followed by overnight incubation with the primary antibodies at 4°C and 1 hour incubation with appropriate secondary antibody at room temperature. The Western blot was visualized by chemiluminescence (Thermo Scientific). The following primary antibodies were used in Western blotting: anti-ERK3 (Abcam), anti-p-ERK3 (S189) (generated in our lab), anti-p- PAK1/3 (Thr423)/PAK2 (Thr402) (Cell Signaling Technology), anti-p- PAK1/3 (Ser144)/PAK2 (Ser141) (Cell Signaling Technology), anti-PAK1, 2, 3 (Cell Signaling Technology), anti-Rac1, 2, 3 (Cell Signaling Technology), anti-RhoGDI1 (Cell Signaling Technology), anti-Cdc42 (Cell Signaling Technology) and anti–β-actin (Sigma).

vii. Immunofluorescence

HeLa and A549 cells were transiently transfected with pSG5-ERK3-HA, pcDNA-ERK3-MYC, pcDNA-PAK1-MYC, pcDNA-Cdc42-MYC, or pcDNA-RhoGDI. Two days later, cells were analyzed by immunofluorescent staining using primary mouse anti- myc antibody (Cell Signaling Technology), rabbit anti-RhoGDI (Cell Signaling Technology), or rabbit anti-ERK3 (Abcam) and fluorescein-conjugated goat-anti-mouse or goat-anti-rabbit secondary antibody. Cell nucleus was labelled by DAPI (1μg/ml) staining of DNA. Images were captured with a Leica CTR 6000 Microscope (Leica Microsystems) and analyzed using ImagePro 6.2 software (Media Cybernetics).

viii. Two-chamber transwell cell migration assay

Cell migration was analyzed using a modified 2-chamber transwell system (BD Biosciences), following the manufacturer's instructions. Cells were detached by trypsin-EDTA, washed once with 1× PBS, and then resuspended in serum-free medium. 0.6 ml of complete culture media with 10% FBS was added to each bottom well. A549 Cells were added in each transwell insert and allowed to migrate for 17 hours in a 37°C cell incubator. Cells in the upper surface of the transwell were removed using cotton swabs. Migrated cells attached on the undersurface were fixed with 4% paraformaldehyde for 10 minutes and stained with crystal violet solution (0.5% in water) for 10 minutes. Migrated cells were then photographed and counted under a microscope at 50 X magnification.

ix. Cdc42/Rac1 Activation Assay

Cells were lysed with cell lysis buffer (provided with the kit). Cell lysates were incubated with agarose beads bound with the p21-GTPase binding domain of PAKs (PAK-PBD) for 1 h at 4°C for pulling down GTP-bound Cdc42 and Rac1 using Cdc42/Rac1 Activation Assay kit (Cytoskeleton, Inc) following the manufacturer's instructions. GTP-Rac1 or GTP-Cdc42 was detected by Western blotting using the anti-Rac1 or anti-Cdc42 antibody provided in the kit.

x. Immunoprecipitation

Cells were lysed with EBC lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 1 mM Complete protease inhibitors [Roche Diagnostics], 10 mM NaF, 1 mM sodium orthovanadate, and 1 mM Phosphatase Inhibitor Cocktail III [Sigma-Aldrich]). One mg of total protein lysate of A549 cells was used for each immunoprecipitation using 2 μg of ERK3 antibody (Bethyl Laboratories) or normal rabbit IgG (Santa Cruz Biotechnology). The supernatant was precleared with 30 μl protein A/G agarose beads for 40 minutes at 4°C with constant rotation. The samples were then incubated with the desired Ab for 1.5 hours, followed by the addition of 30 μl protein A/G agarose beads for additional 1 hour. The beads were washed 3 times (10 minutes per wash) with lysis buffer. Proteins were boiled off the beads in 2× SDS sample buffer and resolved on 12% SDS-PAGE gel. 2% of the amount of protein supernatant for immunoprecipitation was loaded as the input control. Western blotting was performed as described previously.

xi. *In vitro* ERK3 kinase assay

The *in vitro* kinase assay was carried out in 40 mM Tris HCl (pH 7.5), 10 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol, and 5 mM β-glycero-phosphate. Each reaction contained 100 ng of purified ERK3 or ERK3KD and 500 ng of the purified protein substrate RhoGDI1-His, 5 μCi ^{32}P-ATP (Perkin Elmer) and 25 μM cold ATP in a total volume of 30 μl. The reaction was carried out at 30 °C for 30 minutes and then stopped by adding 5X SDS sample buffer and boiling the samples. Proteins were resolved by SDS-PAGE gel, stained with Coomassie Brilliant Blue (Bio-Rad) and visualized by autoradiography.

xii. Statistics

Results are expressed as mean ± SEM. Statistical significance was determined by a 2-tailed Student's *t test*. A P value of less than 0.05 was considered statistically significant.

III. Results

i. ERK3 interacts with RhoGDI1 and forms a complex with small-GTPases and PAK1 in A549 cells.

Cellular signals are relayed through protein-protein interactions which transduce extracellular signals into intracellular events. We identified RhoGDI1 as an interacting partner of ERK3 both from our own IP-MS analysis of ERK3 interacting proteins and in a published Yeast-two-hybrid screening dataset (Vinayagam et al., 2011) that was deposited in BioGRID (Biological General Repository for Interaction Databases). Therefore, we wanted to validate the interaction between ERK3 and RhoGDI1 in cells. First, we analyzed the interaction between these two proteins at the endogenous level. Endogenous ERK3 in A549 lung cancer cells was immunoprecipitated using rabbit anti-ERK3 Ab and the presence of RhoGDI1 in the immune-complex was analysed by Western blotting. Indeed, RhoGDI1 was shown to be co-immunoprecipitated with ERK3 in A549 cells (Figure 5A). We then wanted to determine the effect of ERK3's kinase activity on its interaction with RhoGDI1. RhoGDI1-HA was transiently expressed in stable A549 cells that were transduced with lentivirus vector CDH, CDH-ERK3-WT, or CDH-ERK3-KD. The interaction between RhoGDI1 and ERK3 was analysed by pulling down RhoGDI1 using anti-HA conjugated beads, followed by Western blotting. Interestingly, increased interaction between RhoGDI1 and ERK3 was detected under the condition of overexpression of wild type ERK3 in comparison with CDH empty vector (Figure 5B). In contrast, no ERK3 was pulled down together with RhoGDI1 in cells with overexpression

of kinase dead ERK3 (ERK3KD). These results suggest that ERK3-RhoGDI1 interaction is dependent on ERK3 kinase activity.

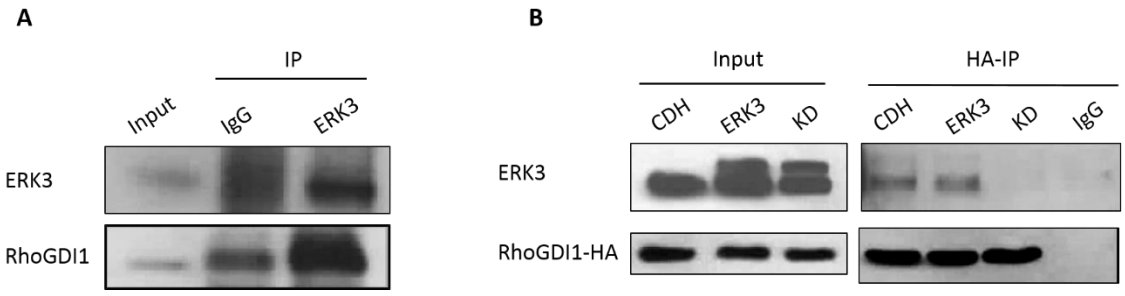


Figure 5: ERK3 interacts with RhoGDI1 in a kinase-dependent mechanism in A549 cells. (A) Co-IP and Western blotting analyse of the interaction of endogenous ERK3 with RhoGDI1. IP was done with a mouse anti-ERK3 antibody, followed by immunoblotting with either a rabbit anti-ERK3 antibody or an anti-RhoGDI1 antibody. 2% of cell lysate was loaded as input. (B) Co-IP/Western blotting analyses of overexpressed ERK3 and RhoGDI1 proteins. pSG5-RhoGDI1-HA was transiently transfected into stable A549 cells that were transduced with lentiviruses either expressing the CDH empty vector, wild type ERK3 or kinase dead ERK3 (KD). IP was done using Anti-HA coated agarose beads followed with immunoblotting with rabbit anti-ERK3 and anti-RhoGDI1 antibodies.

ii. ERK3 co-localizes with RhoGDI1, PAK1 and Cdc42 in Hela and A549 cells.

ERK3 was shown to localize in the nucleus and the cytoplasm (Déléris et al., 2008) and at the leading edge of the plasma membrane (Almahdi et al, 2015). The sub-cellular distribution of ERK3 can provide hints for explaining how ERK3 participates in different cellular events such as cell migration. Thus, we wanted to investigate whether there is co-localization of ERK3 with RhoGDI1/cdc42 GTPase/PAK1 complex that play critical roles in regulating cell motility. For this purpose, both Hela cells and A549 cells were co-transfected with pSG5-ERK3-HA plus pcDNA-Cdc42-MYC, pSG5-ERK3-HA plus pcDNA-PAK1-MYC, or pcDNA-ERK3-MYC plus pSG5-RhoGDI1-HA plasmids. The subcellular co-localization of these proteins was examined by immunofluorescence microscopy (Figure 6). In line with previous studies, ERK3 was seen to localize in the nucleus, the cytoplasm and the plasma membrane of transfected cells. Interestingly, ERK3 co-localizes with Cdc42, PAK1 and RhoGDI1 at the leading edge of the plasma membrane (pointed by arrows) in both HeLa (Figure 6A) and A549 (Figure 6B). Of particular note, Cdc42 overexpression induced the formation of filopodia in both cell lines, where Cdc42 and ERK3 co-localize, suggesting that ERK3 and Cdc42 may cooperatively regulate cytoskeletal structure and cell migration (the left panels, Figure 6A and 6B).

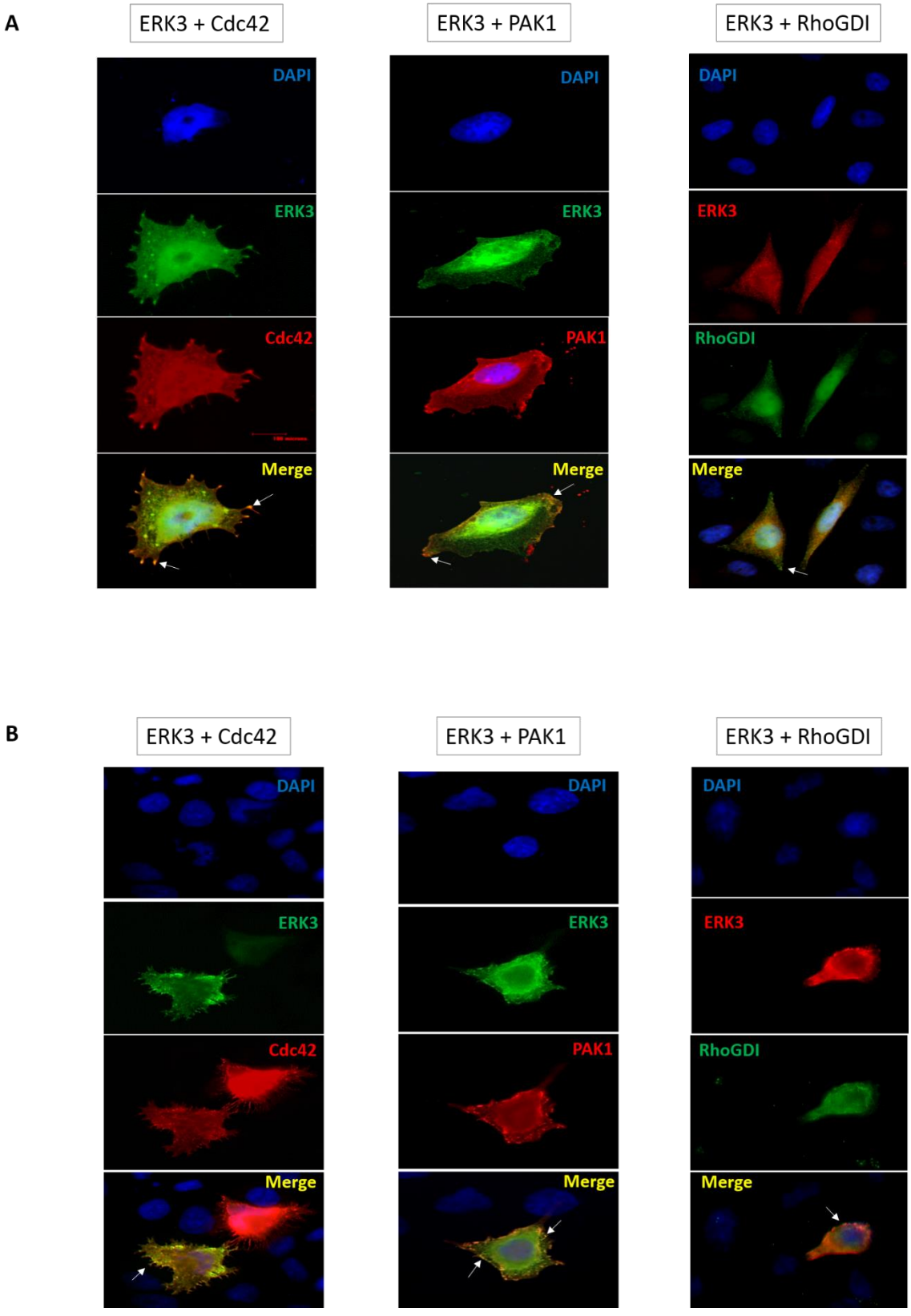

A
ERK3 + Cdc42
ERK3 + PAK1
ERK3 + RhoGDI
DAPI
ERK3
Cdc42
PAK1
RhoGDI
Merge
B
ERK3 + Cdc42
ERK3 + PAK1
ERK3 + RhoGDI
DAPI
ERK3
Cdc42
PAK1
RhoGDI
Merge

Figure 6: Immunofluorescent analyses of the subcellular distribution and co-localization of exogenously expressed ERK3, PAK1, Cdc42 and RhoGDI1 proteins. HeLa (Figure 6A) and A549 (Figure 6B) cells were transiently co-transfected with pSG5-ERK3-HA plus pcDNA-Cdc42-MYC (panels on the left), pSG5-ERK3-HA plus pcDNA-PAK1-MYC (panels in the middle), or pcDNA-ERK3-MYC plus pSG5-RhoGDI1-HA plasmids (panels on the right). Two days after transfection, cells were immuno-stained with an anti-Myc Ab for the detection of Myc-tagged cdc42, PAK1 and ERK3 proteins, with an anti-ERK3 Ab for detecting HA-ERK3 protein (green), or an anti-RhoGDI1 (green) Ab for the detection of HA-RhoGDI1. Cell nucleus was marked by DAPI staining (Blue). Pictures were taken under 63X magnification.

iii. ERK3 positively regulates group I PAKs' activation (phosphorylations) in A549 cells.

Group I PAKs phosphorylate ERK3 and ERK4 at the SEG activation motif and upregulates their kinase activities and cellular functions (Déléris, et al., 2011). Owing to the fact that ERK3 interacts with RhoGDI1, Cdc42 GTPase and PAK1 at the leading edge of the plasma membrane, we were interested in investigating whether ERK3 upregulates group I PAKs' activity as a positive feedback. We either transiently knocked down endogenous ERK3 using siERK3 (Figure 7A) or overexpressed ERK3 by transfecting pSG5-ERK3-HA plasmid (Figure 7B) in A549 cells. We found that ERK3 knockdown greatly decreased phosphorylations of PAK1/3 at Ser144 and Thr423 and PAK2 at Ser141 and Thr402, but caused little change at total levels of PAKs (Figure 7A). ERK3 overexpression increased phosphorylations of PAK1/2/3 at Ser144 and Ser141, but had no clear effects on the phosphorylations at Thr423/Thr402 and total protein levels of PAKs (Figure 7B). Overall, these results suggest that ERK3 upregulates group I PAKs' phosphorylations (indicators of kinase activation) but has little effect on their expression levels.

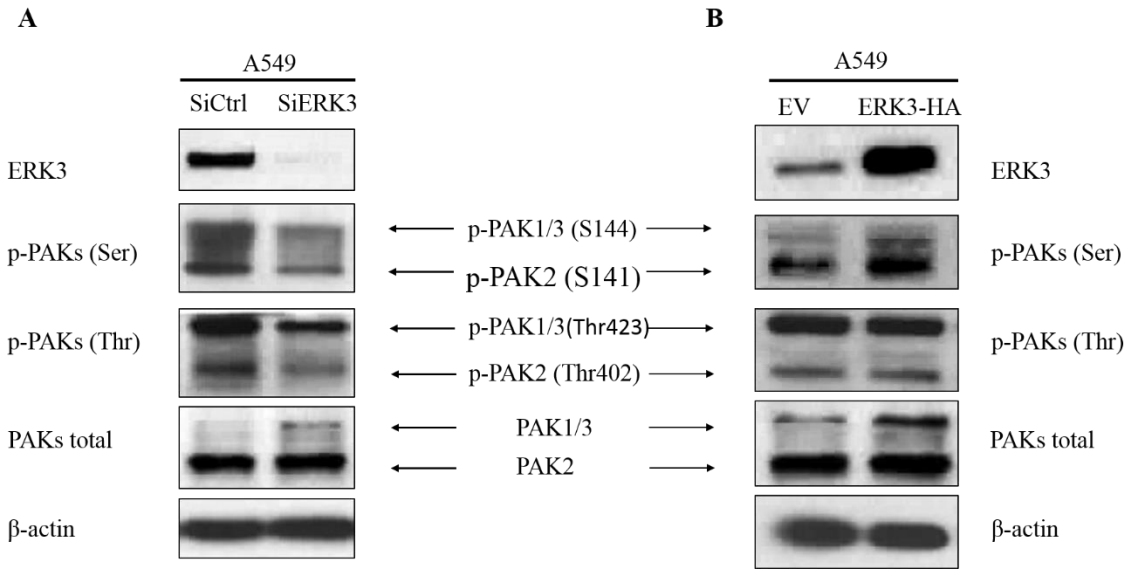


Figure 7: ERK3 upregulates group I PAKs' activation (phosphorylations) in A549 cells. (A) A549 Cells were transiently transfected with either 20 nM non-targeting control siRNA (SiCtrl) or ERK3 siRNA (SiERK3). (B) A549 Cells were transiently transfected with either pSG5-HA empty vector (EV) or pSG5-ERK3-HA construct. For both conditions, cells were harvested 48 hours post-transfections. Phosphorylations and total protein expression levels of PAKs1/2/3 were analyzed by Western blotting using anti-phospho-PAK1/3(Thr423)/PAK2(Thr402), anti-phosho-PAK1/3(Ser144)/PAK2(Ser141) and an antibody recognizing PAK1/2/3, respectively. β-Actin was probed to serve as a loading control.

iv. ERK3 positively regulates group I PAKs activation (phosphorylations) in MDA-MB 231 cells.

After we found that ERK3 upregulates group I PAKs' phosphorylation in lung cancer cells, we were interested in confirming this finding in another type of cancer cells. Given our research focus on the interplay of ERK3 and PAK signaling in cancer cell migration, we chose to use MDA-MB231, a highly migrated and invasive breast cancer cells line in which group I PAKs was shown to be activated by phosphatidic acid (PA) (Abramovici et al., 2009). Endogenous ERK3 was knocked down by stably expressing shRNA specifically targeting ERK3 (Figure 8 ERK3 immunoblot, shERK3 versus shGIPZ). Similar to what we found in A549 cells (Figure 7), ERK3 depletion greatly reduced PAKs' phosphorylations on these activating serine/threonine residues under conditions with or without PA stimulation in MDA-MB 231 cells. These results suggest that the positive regulation of group I PAKs' phosphorylations/activation by ERK3 occurs in different types of cancer cells.

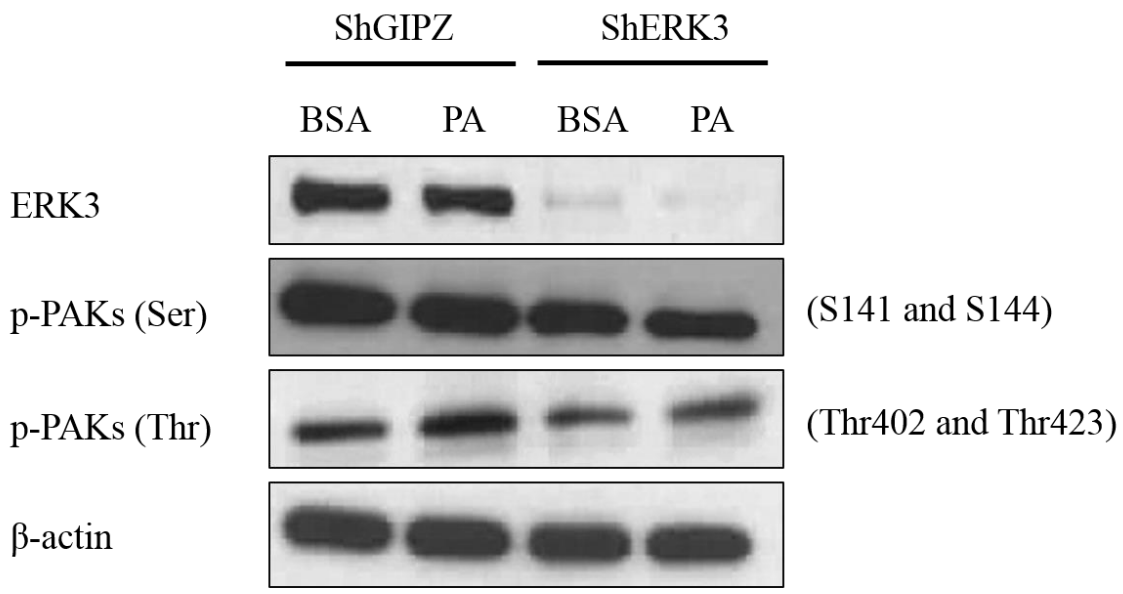


Figure 8: ERK3 upregulates group I PAKs' phosphorylations in MDA-MB 231 cells. MDA-MB 231 cell line with stable knockdown of ERK3 (ShERK3) and the control cell line (ShGIPZ: non-targeting shRNA) were treated with 200μM /ml of Phosphatidic acid (PA) or the BSA vehicle control for 25 minutes. Cells were then harvested and analyzed using western blotting for group I PAKs phosphorylation using anti-p-PAK1/3(Thr423)/PAK2(Thr402) and anti-p-PAK1/3 (Ser144)/PAK2 (Ser141), respectively. β-Actin was probed to serve as a loading control.

v. ERK3 affects the interactions of RhoGDI1 with PAK1 and Rac1/2/3.

To be activated by the action of GEF, Rho GTPases including Racs1/2/3 need to first dissociate from RhoGDI1 (DerMardirossian et al. 2004). In addition, PAKs can interact with and phosphorylate RhoGDI1, which promotes the dissociation of RhoGDI1 from Racs1/2/3 (DerMardirossian et al. 2004). As such, we wanted to investigate whether ERK3 affects the interactions of RhoGDI1 with Rac1/2/3 and PAKs, by which ERK3 upregulates PAKs' activation/phosphorylations. RhoGDI1 protein was overexpressed in A549 stable cell pools, followed by immunoprecipitation of RhoGDI1 and Western blotting analysis of the associated proteins (Figure 9). We found that while ERK3 overexpression had little effect on the interaction of RhoGDI1 with Rac1/2/3 (compare Rac Blot in ERK3 lane versus that of CDH control lane in RhoGDI1 IP panel), it greatly reduced the interaction of RhoGDI1 with PAK1 (compare PAK1 Blot in ERK3 lane versus that of CDH control lane in RhoGDI1 IP panel). Interestingly, kinase-dead ERK3 (ERK3-KD) increased RhoGDI1's interaction with Rac1/2/3 but decreased its interaction with PAK1. These results demonstrate that EKR3 affects the association/dissociation of RhoGDI1 with Racs and PAK and that its kinase activity might be important in this regulation.

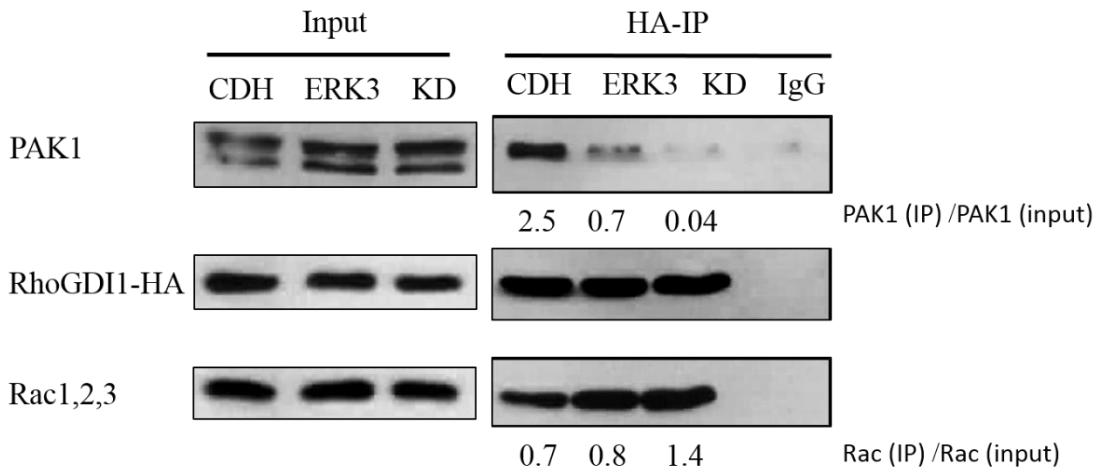


Figure 9: ERK3 affects the interactions of RhoGDI1 with PAK1 and Rac1/2/3 in A549 cells. pSG5-RhoGDI1-HA was transiently transfected into A549 stable cell pools that were transduced with lentiviruses either expressing the CDH empty vector, wild type ERK3 or kinase dead ERK3 (KD). IP was done using Anti-HA coated agarose beads, followed by immunoblotting with antibodies against PAK1, RhoGDI1 and Rac1/2/3. Densitometry of the PAK1 and Rac1/2/3 bands was performed. Numbers are the ratios of immnoprecipitated PAK1 (PAK1(IP)/PAK1 input and the immunoprecipitated Rac (Rac (IP)/Rac input.

vi. ERK3 upregulates the cellular levels of GTP-bound active Cdc42 and Rac1.

As GTP-bound Cdc42 and Rac1 GTPases are group I PAKs' activators (Gatti et al., 1998; Bagheri et al., 2000), we tested whether ERK3 promotes the activation of group I PAKs via increasing the activities of Cdc42 and Rac1. First, we wanted to test this point under the normal culture conditions (culture media with 10% FBS). As shown in Figure 10A, ERK3 knockdown indeed decreased the level of the GTP-bound active forms of both Cdc42 and Rac1. We then examined the effect of ERK3 on the activation of Cdc42 and Rac1 upon the stimulation with EGF. EGF greatly stimulated the activation of both Cdc42 and Rac1, as demonstrated by the remarkable increase of cellular levels of GTP-cdc42 and GTP-Rac1 (EGF-Sictrl versus PBS-SiCtrl, Figure 10B). Knockdown of ERK3 decreased the levels of GTP-Cdc42 and GTP-Rac1 (EGF-SiERK3 versus EGF-SiCtrl, Figure 10B). These findings suggest that ERK3 positively regulate the activation of Cdc42 and RAC1 in A549 cells, which accounts for the increase of group I PAKs' activation (phosphorylations).

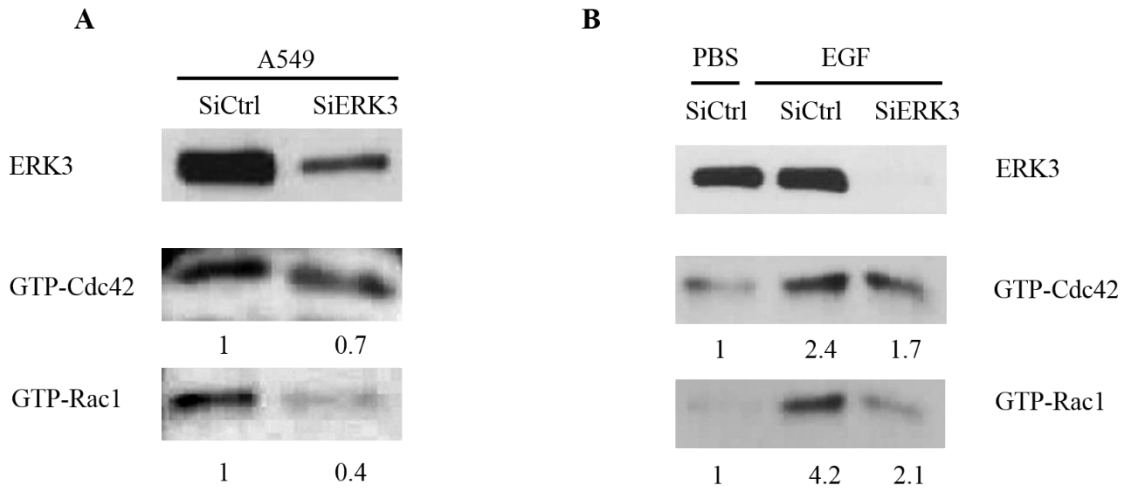


Figure 10: Knockdown of ERK3 decreases the cellular levels of GTP-bound Cdc42/RAC1 in A549 cells. (A) Cells were transiently transfected with either 20 nM non-targeting control siRNA (SiCtrl) or ERK3 siRNA (SiERK3). 2 days later, cells were harvested and lysed. GTP-bound Cdc42 and Rac1 were pulled down following the procedures as described in GTP-bound Cdc42/RAC1 activation assay in Materials and Methods, followed by Western blotting using each specific antibody. Knockdown of ERK3 was verified by Western blot analysis of the total cell lysate. Numbers below the protein bands are the quantification of protein levels by densitometry analysis. The band density in "siCtrl" was set as 1. (B) A549 cells were transfected with either SiCtrl or SiERK3 siRNAs. Two days post transfection, cells were serum starved for 24 hours and then treated with 50 ng /µl EGF for 10 minutes. Cellular levels of GTP-bound Cdc42 and Rac1 were then measured by GTP-bound Cdc42/RAC1 activation assay, followed by Western blotting. Numbers below the protein bands are the quantification of protein levels by

densitometry analysis. The band density in “PBS-siCtrl” was set as 1 and other are relative to it.

vii. Effects of ERK3 on RhoGDI1's expression level in A549 cells.

We have found that ERK3 interacts and co-localizes with RhoGDI1 in lung cancer cells and upregulates the activities of Cdc42 and Rac1. Therefore, we wondered whether ERK3 regulates the expression level of RhoGDI1, thereby regulating the cellular level of active small GTPases. We found that neither ERK3 knockdown (Figure 11A) nor over-expression (Figure 11B) affected the expression level of RhoGDI1, suggesting that ERK3 promotes Cdc42/Rac1 activation not through the downregulation of RhoGDI1's expression level.

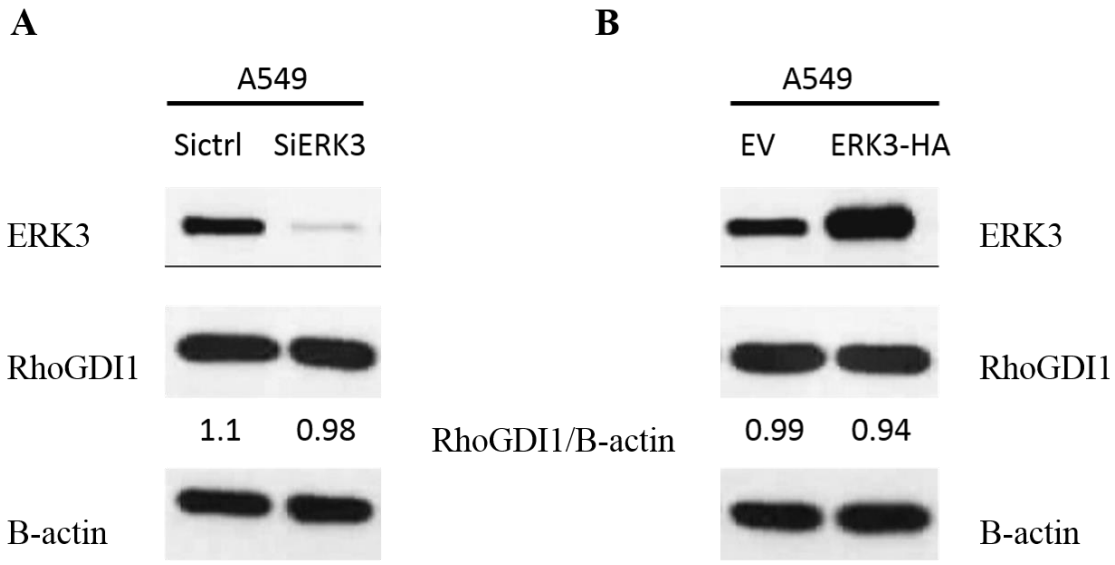


Figure 11: Effects of ERK3 on RhoGDI1's expression level in A549 cells. (A) Cells were transiently transfected with either 20 nM non-targeting control siRNA (SiCtrl) or ERK3 siRNA (SiERK3). (B) Cells were transiently transfected with either 1 µg pSG5-HA (empty vector) or pSG5-ERK3-HA. In both conditions, Cells were harvested and lysed 48 hours after transfection, and then analyzed by Western blotting for RhoGDI1 expression using a RhoGDI1 specific antibody. β-Actin was probed to serve as a loading control. Densitometry of the bands was performed and the ratio of RhoGDI1 to β -actin is displayed in the figure.

viii. ERK3 may phosphorylate RhoGDI1 *in vitro*.

It has been shown that phosphorylations of RhoGDI1 promotes its dissociation off RhoGTPases and/or lowers the re-binding affinity of RhoGDI1 to RhoGTPases, leading to the increased activation of Cdc42 and Rac1 (DerMardirossian et al. 2004). We thus tested the possibility of ERK3 phosphorylating RhoGDI1. We performed in vitro ERK3 kinase reaction using the purified RhoGDI1 (Figure 12A) as the substrate. In comparison with no kinase control or the addition of kinase dead ERK3, the phosphorylation of RhoGDI1 was increased by incubating with wild type ERK3 protein (autoradiograph of Figure 12B), suggesting that RhoGDI1 is a potential substrate of ERK3 kinase.

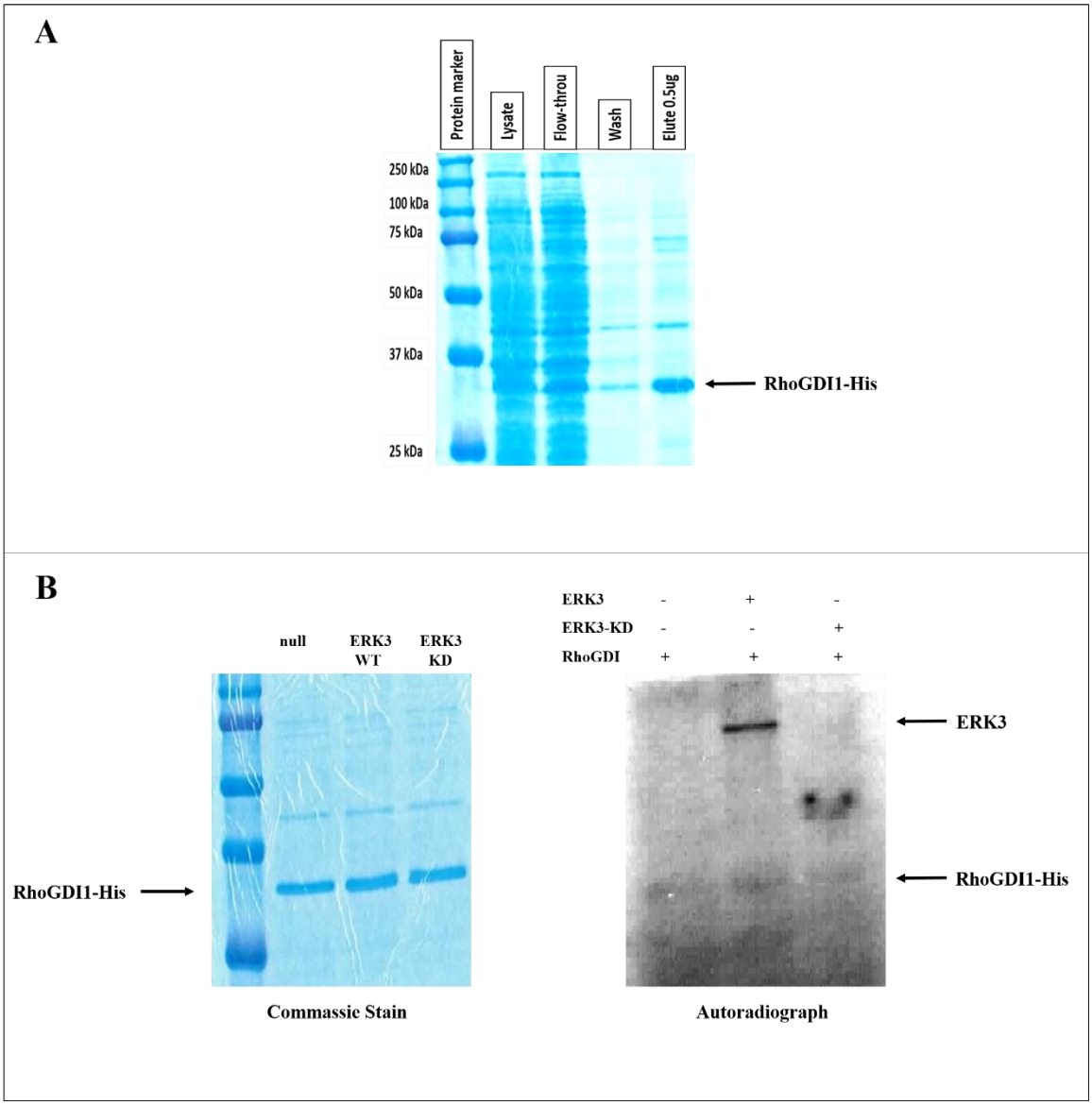
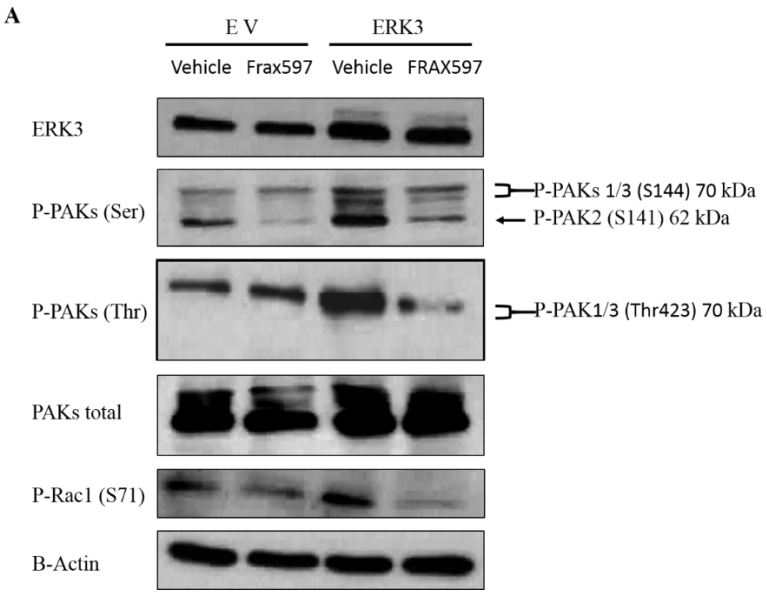

A
Protein marker
Lysate
Flow-throu
Wash
Elute 0.5ug
250 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
RhoGDI1-His
B
null
ERK3 WT
ERK3 KD
RhoGDI1-His
Commassie Stain
ERK3 - + -
ERK3-KD - - +
RhoGDI + + +
ERK3
RhoGDI1-His
Autoradiograph

Figure 12: In vitro ERK3 kinase assay withRhoGDI1 as the substrate. (A) RhoGDI1 with a His tag was expressed in E. coli (BL21) cells, purified using nickel agarose beads and analyzed by Coomassie staining on the SDS-PAGE gel. Lysate: total bacterial cell lysate. Flow-through: the fraction of flowing through the nickel beads; Wash: the wash buffer after the beads washing; Eluate: the proteins eluted off the beads at the final step of purification. (B) In vitro kinase assay was performed by incubating 500 ng of purified RhoGDI1-His protein (as a substrate) with 100 ng of purified wild type ERK3 protein (ERK3 WT) or kinase-dead ERK3 mutant protein (ERK3-KD). Coomassie staining of the proteins is shown in the left panel and autoradiograph of protein phosphorylations is shown in the right panel.

ix. ERK3 promotes lung cancer cell migration through promoting group I PAKs' activity in A549 cells.

ERK3 promotes cancer cell migration (Almahdi et al, 2015; Long et al 2012,). How ERK3 promote cancer cell motility, however, is still unclear. Aforementioned results raise an interesting idea that ERK3 may regulate cell migration through group I PAKs. To determine whether ERK3 acts as an activator of group I PAKs, we examined the effects of ERK3 overexpression on the phoshorylations of Rac1 and PAKs in A549 cells with or without the treatment of FRAX597, an inhibitor of group I PAKs. As expected, ERK3 overexpression greatly increased phosphorylations of Rac1 and PAKs (ERK3/vehicle versus EV/vehicle, Figure 13A). Importantly, FRAX597 treatment abolished the increase of phosphorylations of Rac1 and PAKs stimulated by ERK3 overexpression (ERK3/FRAX597 versus ERK3/vehicle, Figure 13A), confirming that ERK3 indeed promotes PAKs' kinase activity. We then tested the roles of ERK3 and PAKs in A549 cell migration. In line with its role in regulating Rac1/PAKs' activity (Figure 13A), ERK3 overexpression greatly increased A549 cell migration and this stimulating effect was abolished by the treatment with FRAX597 (Figure 13B). These results clearly suggest that ERK3 promotes A549 cell migration through activating PAKs.


A
E V
ERK3
Vehicle
Frax597
Vehicle
FRAX597
ERK3
P-PAKs (Ser)
P-PAKs 1/3 (S144) 70 kDa
P-PAK2 (S141) 62 kDa
P-PAKs (Thr)
P-PAK1/3 (Thr423) 70 kDa
PAKs total
P-Rac1 (S71)
B-Actin

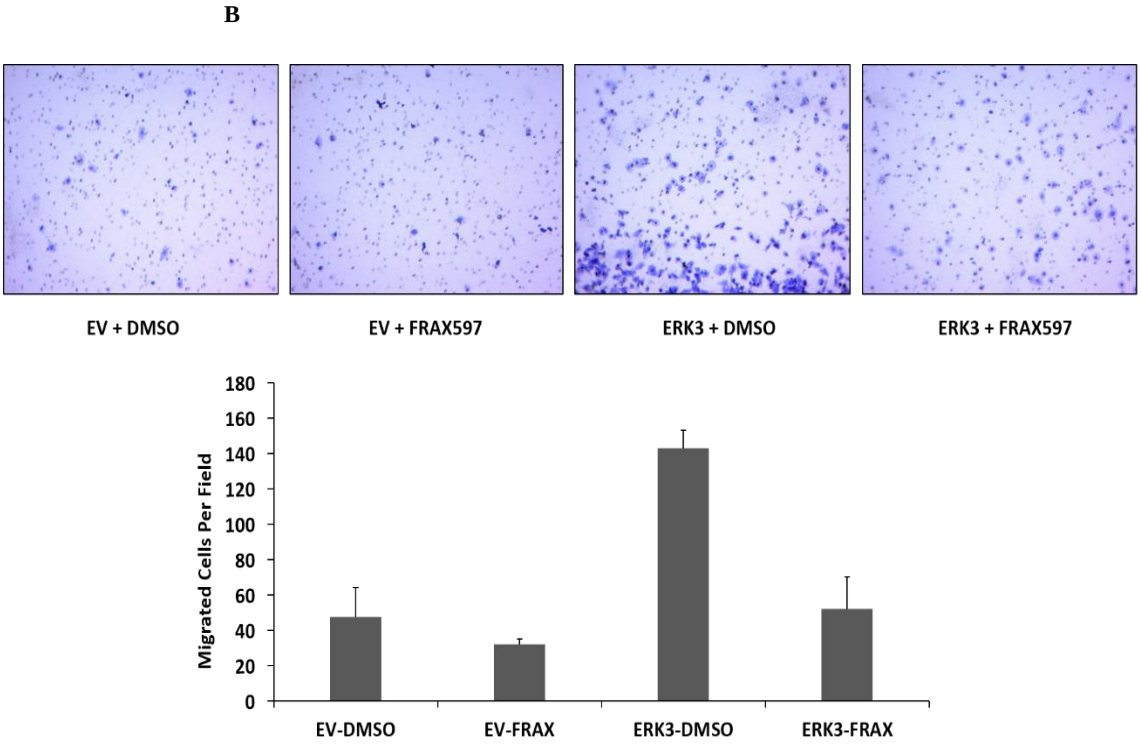

B
EV + DMSO
EV + FRAX597
ERK3 + DMSO
ERK3 + FRAX597
Migrated Cells Per Field
180
160
140
120
100
80
60
40
20
0
EV-DMSO
EV-FRAX
ERK3-DMSO
ERK3-FRAX

Figure 13: ERK3 promotes lung cancer cell migration through upregulating group I PAKs' activity. A549 cells were transfected with pcDNA-MYC (empty vector: EV) or pcDNA-ERK3-MYC. 48 hours after transfection, cells were then treated with 400 nM of either DMSO or FRAX 597 (group I PAKs inhibitor) for 16 hours. (A) Western blot analyses of phosphorylations of PAKs (p-PAKs) and Rac1 (p-Rac1) and protein expression levels. (B) Cell migration assay using a modified two-chamber transwell system. Representative images of migrated cells with crystal violet staining are shown in the upper panels. Quantified cell migration is presented as number of migrated cells per field (shown in the lower panel).Values in bar graph represent mean ± S.D.

IV. Discussion

Extracellular signal-regulated kinase 3 (ERK3) is a member of the atypical mitogen activated protein kinase (MAPK) subfamily. ERK3 possesses a single SEG phospho-acceptor motif in its activation loop, instead of the TXY motif conserved in the classic MAPKs, such as ERK1/2 (Coulombe and Meloche, 2007; Bind et al., 2004). In comparison with the well-established ERK1/2 signaling cascade, much less is known about ERK3 signaling. It has been shown that ERK3 is found to be overexpressed in multiple types of cancer such as lung cancer (Long et al., 2012) and breast cancer (Almahdi et al., 2015). Moreover, ERK3 has been shown to promote cancer cell migration and invasion (Long et al., 2012; Almahdi et al., 2015). RhoGTPases, including Rho, Cdc42 and RAC1, are important factors regulating cell morphology and motility (Ridley and Hall 1992; Ridley et al, 1992; Raftopoulou and Hall 2004). RhoGDIs are Rho GTPase-GDP dissociation inhibitor. They inhibit Rho-GTPases' activities by rendering Rho-GTPases at GDP-bound inactive status (Adra et al., 1997; Sasaki et al., 1998; Olofsson 1998; Golovanov 2001). Cdc42 and RAC1, also known as p21 GTPases, activate group I PAKs (p21 GTPase activated protein kinases) (PAK1, PAK2, PAK3) by binding to PAKs' auto-inhibitory domains and relieving PAKs' auto-inhibition (Gatti et al., 1998; Bagheri et al., 2000; Chong et al., 2001; Parrini et al., 2002; Pirruccello et al., 2006). Group I PAKs were recently shown to phosphorylate ERK3 at S189 of the SEG activation motif (Déléris, et al., 2011). In addition, proteomic analyses of ERK3-interacting proteins by Yeast-Two-Hybrid screening (Y2H) and Immunoprecipitation-Mass spectrometry (IP-MS) revealed

that ERK3 interacts with RhoGDI1 (Vinayagam et al., 2011). Taken together, these findings suggest that ERK3 may be involved in RhoGDI1/ Rho GTPases/PAKs signaling, by which it promotes cell morphology and migration.

In this study, we first verified the interactions of ERK3 with the RhoGDI1/Rho GTPases/PAKs signaling complex. Co-immunoprecipitation assays demonstrated that ERK3 and RhoGDI1 physically interact with each other at both endogenous and exogenous conditions in A549 lung cancer cells. We then went further to investigate at which cellular parts this interaction takes place and how it can influence cancer cell motility and morphology. Previous studies have shown that ERK3, RhoGDI1, group I PAKs and Rho GTPases are located both in the cytoplasm and at the plasma membrane and play roles in controlling cell morphology and movement. Along with this, our immunofluorescence data show that ERK3 co-localizes with RhoGDI1 and PAK1 in the cytoplasm and at the leading edge of the plasma membrane. Moreover, ERK3 shows distinct co-localization with Cdc42 in the filopodia structure of HeLa and A549 cells. The sub-cellular distribution of these co-localized proteins provides strong indications that ERK3 is involved in RhoGDI1/ RhoGTPases/PAKs signaling and cooperatively control cancer cell morphology and migration. Indeed, we then found that ERK3 upregulates the cellular levels of the active forms of Cdc42 and Rac1 (GTP-Cdc42 and GTP-Rac1). In line with this, ERK3 promotes the activating phosphorylations (activities) of group I PAKs presumably through increasing the binding of active GTPases to PAKs. Interestingly, we further found that ERK3-induced cancer migration was almost abolished by the inhibitors of group I PAKs. In conclusion, these results clearly suggest that ERK3 promotes cancer cell migration through upregulating group I PAKs signaling.

Group I PAKs' full activation is regulated by the phosphorylations at Ser144/Ser141 and Thr423/Thr402 (Parrini et al., 2002; Pirruccello et al., 2006). To elucidate the role of ERK3 on group I PAKs' activation, we determined the phosphorylation status of these residues under the conditions of either depletion of endogenous EKR3 or exogenously overexpressing ERK3. We found that ERK3 positively regulates PAKs' phosphorylations at Ser144/Ser141 and Thr423/Thr402 with minor changes on total PAKs' levels. Overall, these findings do suggest that ERK3 promotes group I PAKs' activation, but does not affect no PAK 1/2 /3's expression. Furthermore, we confirmed these results in another type of MDA-MB231 breast cancer cells, indicating that the upregulation of group I PAKs' activation by ERK3 occurs in different types of cancers.

GTP-bound active forms of Rho GTPases bind to and activate group I PAKs. In order to bind to PAKs, Cdc42/Rac1 must first be dissociated from RhoGDI1 for the subsequent activation by GEF. Phosphorylations of RhoGDI1 alter its conformation and promote its dissociation from GDP-Cdc42 and GDP-Rac1. For example, RhoGDI1 is known to be phosphorylated by PAK1 at S101 and S174, which promotes the dissociation of GDP-Cdc42 and GDP-Rac1 off RhoGDI1 (DerMardirossian et al. 2004). As ERK3 interacts with RhoGDI1 and upregulate the cellular level of the active form of Rho GTPases, we thus postulated that ERK3 may phosphorylate RhoGDI1 leading to the release of GDP-Cdc42 and GDP-Rac1 and their subsequent activation. On one hand, this idea was supported by our preliminary data showing that RhoGDI1 appears to be phosphorylated by ERK3 in vitro. It is certainly important to further confirm RhoGDI1's phosphorylation by ERK3 and to identify the specific phosphorylation site(s). On the other

hand, we found that overexpression of wild type ERK3 had little effect on the interactions of Rac1/2/3 with RhoGDI1, whereas overexpression of catalytically inactive ERK3 (ERK3KD) greatly increased (by 2 folds) the interaction of Rac1/2/3 with RhoGDI1. A possible explanation for this is that as A549 cells has high level of endogenous wild type ERK3, ERK3's role in regulating the interaction of RhoGDI1 with Rac1/2/3 might not be affected by additional exogenous expression of wild type ERK3; however, ERK3KD could act as a dominant negative mutant of wild type ERK3 and brings about great change. Surprisingly, both ERK3 and ERK3KD greatly reduced the interaction of RhoGDI1 with PAK1. While we currently do not know whether this is related to the activation of PAKs, it is important to determine the effects of ERK3KD on the activities of Cdc42/Rac1 and PAKs.

ERK3 localizes in the nucleus and the cytoplasm (Déléris et al., 2008) and at the plasma membrane (Almahdi et al., 2015). While it is known that ERK3 nuclear export to the cytoplasm is mediated by CRM1 (Déléris, et al., 2008), it is unclear how ERK3 is translocated to the plasma membrane. RhoGTPases Rac1 and Cdc42 can transport proteins in the nucleus and/or cytoplasm (e.g., nuclear protein SET) to the plasma membrane to promote cancer cell migration (Klooster et al., 2007; Lerida et al., 2015). Interestingly, our current study shows that ERK3 co-localizes with Cdc42 and PAK1 at the leading edge of the plasma membrane. Taken all these together, we speculate that RhoGTPases mediate ERK3's translocation to the plasma meterman to promote cancer cell motility. We will be testing this idea in our future work.

In summary, our present study shows that ERK3 interacts with RhoGDI1/RhoGTPases/PAKs signaling complex at the leading edge of plasma membrane, activates Cdc42 and RAC1 and subsequently group I PAKs, thereby promoting cancer cell migration. For future work, we plan to elucidate: 1) how EKR3 is translocated to the leading edge of the plasma membrane; 2) the underlying mechanisms by which ERK3 activates RhoGTPases; 3) the role of ERK3 and PAKs cooperatively promoting tumor formation and metastasis.

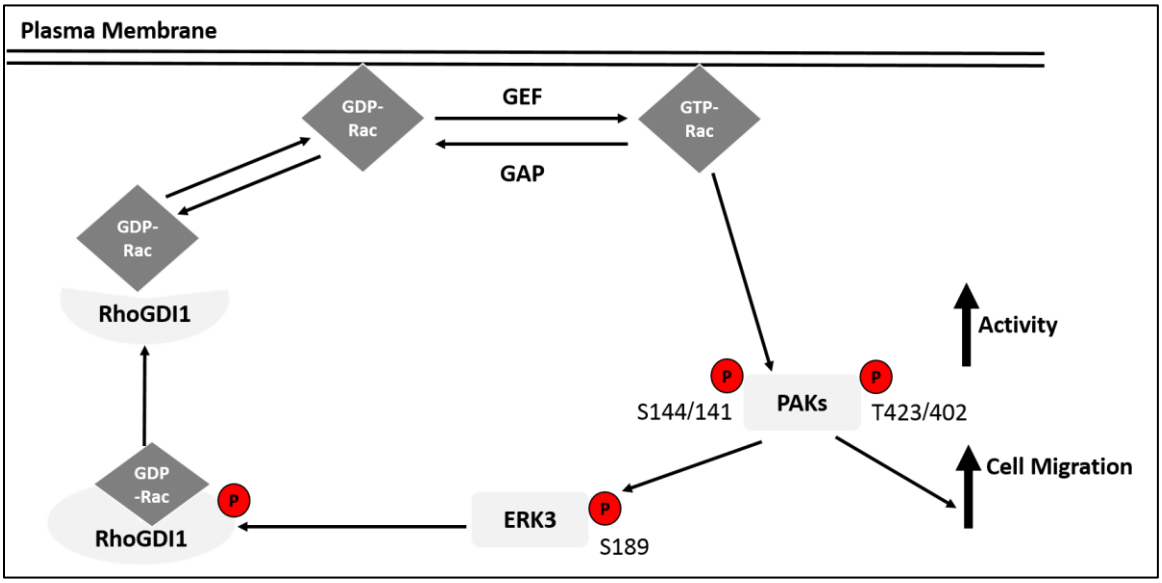


Figure 14: A working model of how ERK3 promotes cancer cell migration through upregulating group I PAKs' activity.

V. References

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