Functional characterization of cancer-related mutations of ERK3

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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ABSTRACT

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Extracellular signal-regulated kinase 3 (ERK3) is an atypical member of the mitogen-activated protein kinase (MAPK) family. Recent studies have shown that ERK3 is highly upregulated in multiple cancers, such as lung cancer and colon cancer. Importantly, ERK3 promotes cancer cell migration and invasion by phosphorylating steroid receptor activator 3 (SRC-3), hence upregulating pro-invasive matrix metalloproteinase genes. While the link between ERK3 and cancers has been recognized, little is known about ERK3 mutations in cancer progression. In this study, we have investigated ERK3 mutations on arginine 64 (arginine 64 mutated to cysteine or histidine, R64C or R64H) and leucine 290 (leucine 290 mutated to proline or valine, L290P or L290V) that are found in cancers of lung, large intestine and skin (COSMIC database). Interestingly, both R64 and L290 residues are located in the kinase domain of ERK3 and are conserved in all ERK isoforms (ERKs1-4). In order to characterize these mutations, we generated expression vectors coating plasmids of ERK3 and each ERK3 constructs containing point mutation, and then overexpressed them in HeLa cells and lung cancer cell lines with or without stable knockdown of endogenous ERK3. Notably, we found that all of these cancer-related mutations lead to reduction of ERK3 phosphorylation at S189 within the activation loop. To study the functional impact of these ERK3 mutations on
cancer cell invasiveness, we expressed each of these mutants or the wild type (WT) ERK3 in HeLa cells and lung cancer cell lines with or without stable knockdown of endogenous ERK3 and performed trans-well migration and invasion assays. Interestingly, we found that in comparison with WT ERK3, both L290P and L290V mutations significantly increased ERK3’s ability in promoting cell migration and invasion, whereas R64C and R64H mutations resulted in a decrease in cell migration. Given that all these cancer-related mutations led to reduction of ERK3 phosphorylation at S189, these results suggest that S189 phosphorylation within the activation loop is not associated with ERK3’s function in promoting cancer cell motility. To elucidate the underlying mechanism by which ERK3 L290 mutants increase cancer cell invasiveness, we examined the kinase activity of WT and mutant ERK3 in vitro by assessing the phosphorylation of SRC-3, a substrate for ERK3. We found that ERK3 L290P and L290V mutants have similar kinase activity to that of WT ERK3. ERK3 protein is known to shuttle between the nucleus and the cytoplasm, which may alter its function. Interestingly, by immunofluorescent staining, we found that both L290P and L290V mutations greatly increased the cytoplasmic localization of ERK3 proteins, whereas WT ERK3 is mostly nuclear. In conclusion, this study demonstrates that cancer-related L290P and L290V mutations lead to increased ability of ERK3 to promote cancer cell migration and invasion, possibly through their increased cytoplasmic localization.
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I. Introduction

i. Mitogen-Activated Protein Kinases Pathways

Mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases that transduce various extracellular physical and chemical stimuli, such as mitogens, hormones and stress, into intracellular responses (Coulombe and Meloche, 2007; Bind et al., 2004). They play central roles in pathways that regulate fundamental cellular processes, including differentiation, migration, growth, apoptosis, gene expression and proliferation (Dhillon et al., 2007). The cellular outcomes produced by MAPKs depend on the kinetics and balance of activation and inactivation, the location of the kinases and substrate availability (Raman et al., 2007). In mammals, there are six groups of MAPKs: 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).

MAPKs can be classified into classical (conventional) or non-classical (atypical) MAPKs based on structural and regulatory characteristics (Figure 1, Coulombe and Meloche et al., 2007). Conventional MAPKs include ERK1/2, p38s, JNKs, and ERK5. The pathway to activation of conventional MAPKs comprises a three-tiered cascade of kinase activity (Figure 2), in which a MAPK kinase kinase (MAP3K, e.g., RAFs) phosphorylates and activates a MAPK kinase (MAP2K, e.g., MEK1/2), which in turn phosphorylates the MAPK on a conserved T-X-Y site leading to its activation (Kostenko et al., 2012) (Figure 1). The T-X-Y site is called the activation motif as dual phosphorylations of the T-X-Y site by MAP2K are critical for MAPKs’ activation (Coulombe and Meloche et al., 2007).
The atypical MAPKs include ERK3, ERK4, ERK7 and Nemo-like kinase (NLK). Atypical MAPKs have a glycine or glutamic acid residue in their activation loop instead of the tyrosine for conventional MAPKs. For example, ERK3/4 have a single phospho-acceptor site in their activation loop comprised of S-E-G residues. Atypical MAPKs do not follow the three-kinase module as the conventional MAPKs. In comparison with the ample knowledge of conventional MAPKs, much less is understood regarding the activation signals and upstream regulators of atypical MAPKs (Coulombe and Meloche, et al., 2007).

**Figure 1: Schematic representation of human MAPKs structure.**

MAP kinases are composed of a kinase domain (in blue) flanked with N- and C-terminal extensions of varying lengths. TAD, transactivation domain; NLS: nuclear localization sequence; C34: conserved region in ERK3/4; AHQr, alanine (A), histidine (H) and glutamine (Q) rich domainNumbers 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).
ii. ERK3

Extracellular signal-regulated kinase 3 (ERK3), also known as MAPK6, was identified using an ERK1 probe in the early 1990s (Boulton, et al., 1991). ERK3 mRNA is expressed in most mammalian tissues, mainly in brain, skeletal muscle and the gastrointestinal tract (Turgeon, et al., 2000). ERK3 protein contains 721 amino acids with a molecular weight of about 100 kDa (Turgeon et al., 2000). It is comprised of a kinase domain at its N-terminus, a C34 domain (conserved in ERK3 and ERK4) in the middle, and a C-terminus extension (Figure 1, Coulombe and Meloche et al., 2007).

ERK3 is 45% identical to ERK1/2 in the kinase catalytic domain on the amino acid level. However, ERK3 has a single phospho-acceptor site (S189) in its activation loop
E-G motif, in place of the highly conserved T–X–Y motif found in conventional MAPKs. ERK3 was reported to be phosphorylated \textit{in vivo} on this single phospho-acceptor site in resting cells. Phosphorylation of ERK3 S189 residue can occur \textit{in trans} by an upstream kinase, or \textit{in cis} by auto-phosphorylation (Coulombe and Meloche et al., 2007) (Cheng, et al., 1996). P21-activated kinases (PAKs) have been found to phosphorylate ERK3 at S189 residue (Mota-Peynado, et al., 2011; Déléris, et al., 2011).

The C34 domain is found in both ERK3 and ERK4 and its function is virtually unknown. Differing from most of other MAPKs including ERK4, ERK3 has a unique long C-terminal extension. The functions of this characteristic C-terminus extension are poorly understood. ERK3’s C-terminus was shown to hyperphosphorylated by Cdk1 at multiple residues and these phosphorylations increases ERK3 protein stability during cell mitosis (Tanguay, et al., 2010). The regulation of the C-terminal extension on ERK3’s kinase activity and cellular functions remain to be explored. ERK3 is located in both the cytoplasmic and nuclear compartments in proliferating cells (Bind et al., 2004; Julien et al., 2003; Schumacher et al., 2004). In addition, ERK3 is distinct from other MAPKs as it is a highly unstable protein with a half-life of 30 min in proliferating cells. Its activity and function are regulated by protein turnover (Coulombe et al., 2003, 2004).
iii. Physiological role of ERK3

ERK3 plays an important role in fetal growth and lung maturation as ERK3-deficient mice exhibited intrauterine growth restriction during pregnancy and early neonatal death due to lung maturation defect (Klinger et al., 2009). ERK3 has also been shown to be involved in insulin secretion (Anhe et al., 2006). Suppression of ERK3 expression inhibited glucose-stimulated insulin secretion in pancreatic β cells (Anhe et al., 2006). Furthermore, ERK3 contributes to endothelial cell migration and proliferation by upregulating vascular endothelial growth factor receptor 2 (VEGFR2) expression (Wang et al., 2014).

ERK3 is involved in cell-cycle progression and cellular differentiation. When Cdk1 phosphorylates ERK3 in the C-terminal extension, ERK3 is stabilized during mitosis due to phosphorylation of four C-terminal residues (Ser684, Ser688, Thr698 and Ser705), which ultimately results in accumulation of ERK3 protein (Tanguay et al., 2010). What exact role ERK3 plays during mitosis, however, is unclear.

ERK3 also plays a critical role in regulating the immune system. It has been shown that ERK3-deficient T-cells demonstrate decreased cell proliferation and cytokine secretion, suggesting that ERK3 supports T-cell activation to promote differentiation and proliferation. ERK3 impacts T-cell responses through sustaining CD4+ CD8+ T-cell survival and is involved in thymic positive selection (M Marquis et al., 2014; Sirois et al., 2015).

MAPK-activated protein kinase-5 (MK5) is an important physical and functional partner of ERK3 (Perander, et al., 2016). ERK3 interacts with MK5, in which S189 plays an important and positive regulatory role (Seternes OM et al., 2004; Schumacher S et al., 2016).
Phosphorylation of S189 in ERK3’s activation loop was found to be required for the formation of stable complex with MK5 (DÉLÉRIS et al., 2008; Perander et al., 2016). While ERK3 certainly stimulates MK5’s phosphorylation at Thr182, it is controversial whether MK5 is a direct substrate of ERK3 kinase. One study showed that ERK3 can phosphorylate MK5 on Thr182 (Seternes OM et al., 2004), whereas another study demonstrated that ERK3 stimulates MK5’s auto-phosphorylation on Thr182 through physical interaction, leading to full activation of MK5 kinase (Schumacher S et al., 2004). The latter study further suggested that the activation of MK5 is independent of ERK3 enzymatic activity, but is dependent on its own catalytic activity and a region in the C-terminal extension of ERK3 (Schumacher et al., 2004). Nevertheless, the ERK3/MK5 partnership has been shown to be critical for their protein stability and subcellular localization, mouse embryonic development (Schumacher et al., 2004) and dendritic spine formation in mouse hippocampal neurons (Brand, et al., 2012).

iv. ERK3 in cancers

While the involvement of the conventional MAPKs in tumorigenesis has been well studied, much less is known about the atypical MAPKs. Recent studies have shown that ERK3 expression is upregulated in multiple human cancers, including lung cancer (Bhattacharjee et al., 2001), gastric cancer (Liang et al., 2005), breast cancer (Drabsch et al., 2011) and melanoma (Nambiar et al., 2005). In line with its upregulated expression in cancers, ERK3 has been shown to play critical roles in promoting cancer cell migration and invasion. The importance of ERK3 in cancer cell invasiveness was first revealed in lung cancer cells (Long et al., 2012). Overexpression of ERK3 significantly increased lung
cancer cells’ migration and invasion and, conversely, ERK3 knockdown greatly decreased cell invasiveness. In addition, knockdown of ERK3 greatly decreased the ability of H1299 lung cancer cells to form tumor nodules when implanted into mouse-tails, demonstrating that ERK3 is essential for the invasiveness of lung cancer cells in vivo (Long et al., 2012). Mechanistically, ERK3 was demonstrated to increase the invasiveness of lung cancer cells by interacting with and phosphorylating steroid receptor co-activator (SRC3) at serine 857 (S857). Phosphorylation at S857 is critical for the interaction of SRC-3 with the ETS transcription factor PEA3. The ERK3/SRC-3/PEA3 complex upregulates the gene expression of the pro-invasive matrix metalloproteinases (MMPs).

Similarly, a study by another group showed that over-expression of ERK3 in breast cancer cells altered cellular morphology and increased cell migratory ability (R Al-Mahdi et al., 2015). Their data showed that as cells initiated adhesion to the matrix, they increased levels of ERK3 at the cell periphery that drove changes in cell morphology and influenced migratory behavior.

Furthermore, a recent study demonstrated a novel role for ERK3 in elevating cancer cells’ DNA damage response and chemo-resistance to topoisomerase-2 inhibitors. ERK3 promotes Tyrosyl DNA phosphodiesterase 2 (TDP2) activity by phosphorylating TDP2 at S60 (Bian et al., 2016).
v. Mutations of protein kinases in cancer

Cancers result from genetic alterations, including those causing the dysregulations of protein kinase signaling pathways. Protein kinases are a group of frequently mutated genes in cancers and are involved in various molecular events that are essential for the development and progression of cancers. Protein kinases could act as proto-oncogenes or tumor suppressors. Owning to their critical roles in cancer growth and progression, protein kinases have emerged as important targets for cancer therapy and numerous kinase inhibitors have been approved for cancer treatment.

Protein kinase pathways can be altered or mutated by multiple mechanisms, such as gene copy number changes (gain or loss), mutations (including deletions, chromosomal translocation and point mutations) and epigenetic changes in gene promoters. For example, human epidermal growth factor receptor 2 (HER2) gene is amplified in around 30% of human breast cancer cases (Zahi Mitri et al., 2012). HER2 gene amplification leads to protein overexpression and hyperactivation of ERBB kinase signaling. Many studies show that overexpression of ErbB2 interrupts normal cellular regulations and promotes cell transformation and breast tumor growth and metastasis (Zahi Mitri et al., 2012). Chromosomal translocation and the resultant gene fusion is another common type of genetic alternation in cancers. For example, the oncogenic BCR-ABL fusion protein kinase is generated by the fusion of BCR and ABL kinase gene following the chromosomal translocation between chromosomes 9 and 22. BCR-ABL causes chronic myelogenous leukemia (CML) (Ruibao Ren et al., 2012). Moreover, many protein kinases are altered by point mutations in their functional domains. For example, multiple cancer-related point mutations were identified in the kinase domain of PI3KCA, the catalytic subunit of PI3K
Protein kinase signaling pathways can be altered by the mutations of the upstream and/or downstream regulators of protein kinases as well. In fact, for MAPK signaling pathways, RAS mutations are the most common oncogenes in humans, with a mutation incidence of up to 30% in human cancers. Likewise, mutations in RAFs are also frequent, especially in melanoma. However, MAPK/ERK mutations are considered to be rarer and, to date, ERK mutations have not been considered to be the direct cause of human cancer (Sergiy Kostenko et al., 2012). Although ERK3 was shown to play an important role in cancer, the effect of ERK3 mutations on cancer invasiveness has not been studied.

In an attempt to identify ERK3 gene mutations in cancers, we searched the catalogue of somatic mutations (COSMIC) in cancers, a comprehensive online database with a large number of collections with a focus on gene mutations in cancers. By reviewing this database, a number of point mutations in ERK3 were found to exist in multiple cancers including those of lung, ovary and skin cancer tissue (Table 1). Of note, mutations at two amino acids in ERK3 (arginine at location 64 and leucine at location 290) have been identified in different tumor tissues, including those of lung, intestine, and colon. Interestingly, these two sites mutated in ERK3 are located in the kinase domain, and are conserved in ERKs1-4 (Figure 3), suggesting an important role for these amino acids.
Table 1: ERK3 mutations in cancers

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total samples tested</th>
<th>Total mutated samples</th>
<th>Mutation frequency</th>
<th>Specific ERK3 mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large intestine</td>
<td>789</td>
<td>22</td>
<td>2.79%</td>
<td>L290V, L290P, R64C, R64H</td>
</tr>
<tr>
<td>Lung</td>
<td>1775</td>
<td>14</td>
<td>0.79%</td>
<td>L290V, L290P, R64C</td>
</tr>
<tr>
<td>Skin</td>
<td>701</td>
<td>10</td>
<td>1.43%</td>
<td>L290V</td>
</tr>
</tbody>
</table>

Figure 3: Alignment of the sequences of ERK1, ERK2, ERK3 and ERK4 proteins demonstrates that both R64 and L290 (highlighted) of ERK3 are conserved in ERKs
vi. Hypothesis and specific aims:

Based upon the background information aforementioned, we hypothesize that ERK3 point mutations (R64 and L290) in its kinase domain alter ERK3 kinase activity and its functions in cancer cells. The objective of this study was to investigate the effects of these point mutations on ERK3 kinase activity and cellular functions. There are two specific aims: 1) To determine the effects of ERK3 mutations on ERK3 phosphorylation in the activation loop and its kinase activity; 2) to determine the effects of ERK3 mutations in regulating ERK3’s cellular functions, including migration and invasion.
II. Materials and Methods

i. Cell culture and transfection

HeLa (human cervical cancer) cell, 293T (human embryonic kidney) cell line and lung cancer cell lines, including H1299 and A549, were purchased from ATCC. H1299 and A549 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. HeLa and 293T 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. Plasmids were transfected into the cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. Unless specifically indicated, cells were harvested for various analyses 48 hours after transfection.

ii. Expression plasmids

The mammalian expression plasmid of ERK3 with a HA tag at the N-terminus (PSG5-HA-ERK3) was used for generating ERK3 mutant plasmids by site-directed mutagenesis using the QuickChange Kit (Stratagene). The cancer-related point mutations of ERK3 at arginine 64 (including R64C and R64H) and at leucine 290 (including L290P and L290V) were generated. In addition, ERK3R64A and ERK3L209A were created for serving as a mutant control that might have loss of function. pSG5-HA-ERK3KD expressing a kinase dead (KD) mutant was also used in the experiments. Two critical lysine
residues K49 and K50 in the ATP-binding site domain were mutated to alanine in pSG5-HA-ERK3KD. Furthermore, we used an ERK3 mutant plasmid (pSG5HA-ERK3S189A) in which the S189 phospho-acceptor site in the activation loop of ERK3 was mutated to alanine (S189A). All ERK3 mutant plasmids generated were verified by sequencing.

For lentiviral expression of ERK3 proteins with mutations at L290, pCDH-ERK3-Myc6-L290A, pCDH-ERK3-Myc6-L290P and pCDH-Myc6-ERK3-L290V were generated by site mutagenesis using pCDH-Myc6-ERK3 as the template, a lentiviral plasmid expressing ERK3 with 6 Myc tags at the N-terminus (Long et al., 2012). All plasmids with the expected mutations were verified by DNA sequencing.

iii. Generation of stable cell pools expressing shRNAs by lentiviral transduction

We generated HeLa & H1299 stable cell pools with stable knockdown of endogenous ERK3 by lentiviral transduction of a short hairpin RNA (shRNA) specifically targeting the 3’UTR of ERK3 mRNA (shERK3). As this shERK3 only targets a non-coding region of ERK3, we were able to over-express the coding sequence of ERK3 by transfecting the stable cells with ERK3 plasmids. Stable cell pools expressing the non-targeting shRNA (shGIPZ) were used as controls. 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 using Western blotting analysis.

iv. **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 10% SDS-PAGE gels. Western blotting was performed by first blocking nitrocellulose membranes with 5% non-fat milk in PBS-T buffer for 30 minutes, 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 by our lab), and anti–β-actin (Sigma).

v. **Two-chamber transwell cell migration and invasion 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. Cells were added in each transwell insert and allowed to migrate for 19 hours (for H1299 cells) or 20 hours (HeLa cells) 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. Cell invasion assay was performed by following the same procedures as those for the cell migration assay, except that transwell inserts were precoated with Growth Factor-Reduced Matrigel (BD Biosciences).

vi. Immunofluorescence

HeLa cells were transfected with PSG5-HA-Empty vector, HA-ERK3, HA-ERK3 KD, HA-ERK3 S189A, HA-ERK3 L290A, HA-ERK3 L290P, or HA-ERK3 L290V mutant. Two days later, cells were analyzed by immunofluorescent staining using a mouse anti-HA antibody (Sigma) or myc antibody (Cell Signaling). Cell nucleus were 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).

vii. Transient lentiviral transduction

Pseudotype lentiviruses expressing ERK3 cDNAs were produced in 293T cells by cotransfecting lentiviral pCDH-Myc6-ERK3 expressing construct and pPACK Packaging Plasmid Mix (System Biosciences), following the manufacturer’s instructions. Pseudoviral particles were harvested 48 hours after transfection and concentrated using PEG-it Virus Precipitation Solution. A549 cells were transiently transduced with lentiviruses expressing either an empty vector pCDH or pCDH-Myc6-ERK3, pCDH-ERK3-L290A, pCDH-ERK3-L290P, pCDH- ERK3-L290V in the presence of 4 μg/ml polybrene for two days.

viii. In vitro ERK3 kinase assay

HA-tagged ERK3 and ERK3 mutant proteins were purified from mammalian cells as follows. PSG5-HA-ERK3, PSG5-HAERK3KD, PSG5-HAERK3-L290P or PSG5-
HAERK3-L290V were transiently overexpressed in 293T cells. Cells were lysed with lysis buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 0.5% NP-40, 10% glycerol, 0.4 mM EDTA, 1 mM Complete protease inhibitors [Roche Diagnostics], and 1 mM Phosphatase Inhibitor Cocktail III [Sigma-Aldrich]). The protein lysate supernatant was precleared using Protein A Affinity gel beads then incubated with anti-HA affinity agarose beads (Sigma-Aldrich) for 3 hours. The beads were then washed 3 times with wash buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 0.5% NP-40, 10% glycerol, 0.2 mM EDTA, 1 mM Complete protease inhibitors [Roche Diagnostics], 10 mM β-glycerophosphate, and 1 mM Phosphatase Inhibitor Cocktail III [Sigma-Aldrich]). Purified proteins were eluted off the beads using HA peptide. The elution was repeated twice to obtain eluate 1 and eluate 2. The proteins’ concentration was determined by bradford protein assay, and purity assessed by running 500 ng purified proteins on a 10 % gel and staining with coomassie blue solution.

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-glycerophosphate. Each reaction contained 100 ng of purified ERK3 or ERK3 mutant and 1 μg of the purified protein substrate GST-SRC3-CID, 5 μCi 32P-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.
ix. **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 mutants display decreased phosphorylation at S189 in HeLa cells

We found cancer-related mutations at R64 and L290 residues of ERK3 in COSMIC database (Table 1), both of which are located in the kinase domain. We were interested in elucidating the effects of these mutations on ERK3 kinase activity and cellular functions. First, we decided to study their effects on the phosphorylation of S189 in ERK3’s activation motif. Three different mutants were generated on each amino acid residue by changing R64 to alanine, cysteine or histidine, and L290 to alanine, proline or valine. These ERK3 point mutations were generated in pSG5-ERK3 plasmid backbone for expression in mammalian cells. Upon transfection of HeLa cells with the empty vector, or pSG5-ERK3 or ERK3 mutants, we observed that cancer-related ERK3 mutants had expression levels equivalent to that of wild type ERK3 (ERK3WT) (Figure 4). We then analyzed ERK3 phosphorylation at S189 (P-ERK3) by Western blotting using a phospho-S189 specific ERK3 antibody. The ratio of P-ERK3 signal over total ERK3 was calculated by performing densitometry analysis of the blots and was normalized the ratio of wild type ERK3 (WT) (Figure 4). As expected, wild type ERK3 demonstrated high level of phosphorylation at S189, whereas ERK3 kinase dead (KD) mutant that has mutations of the ATP-binding site and significant loss of its kinase activity had a remarkable reduction of S189 phosphorylation. Interestingly, all of the R64 and L290 mutants demonstrated a reduction in phosphorylation at S189, suggesting that these mutations may decrease ERK3 kinase activity in HeLa cells.
Figure 4: Expression levels and S189 phosphorylation of ERK3 and ERK3 mutants in HeLa cells. Western blot analysis of ERK3 and P-ERK3 (S189) in HeLa cells transfected with either a pSG5 empty vector, wild-type (WT) ERK3, or each ERK3 mutant as indicated. β-actin was used as a loading control. Densitometry of the bands was performed and the ratio of phosphorylated ERK3 to total ERK3 is displayed at the bottom of the figure. The ratio of P-ERK3/ERK3 for ERK3 WT was set as “1”.

<table>
<thead>
<tr>
<th>Empty vector</th>
<th>ERK3 WT</th>
<th>ERK3 KD</th>
<th>ERK3 R64A</th>
<th>ERK3 R64C</th>
<th>ERK3 R64H</th>
<th>ERK3 L290A</th>
<th>ERK3 L290P</th>
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</table>

P-ERK3 (S189)

ERK3

β-actin

P-ERK3/ERK3
ii. Effects of ERK3 mutations on ERK3’s ability in promoting migration of HeLa cells

A previous study showed that ERK3 plays an important role in lung cancer cell motility: overexpression of wild type ERK3 increased and knockdown of ERK3 decreased cell migration (Long et al). To determine the functional importance of the cancer-related ERK3 mutations, we measured the abilities of ERK3 mutants in cell migration by comparing with that of wild-type ERK3 (WT ERK3). As expected, overexpression of WT ERK3 increased HeLa cell migration by about four folds in comparison with that of the empty vector control (Figure 5). For mutations on R64, both R64H and R64A led to decreased cell migration, whereas R64C did not show significant effect. Interestingly, both L290P and L290V cancer-related mutations resulted in a significant increase of cell migration as compared to WT ERK3, whereas L290A artificial mutant control did not have clear effect (Figure 5), suggesting that both L290P and L290V have gain-of-functions and confer EKR3 increased ability in promoting cancer migration.
**Figure 5: The effect of ERK3 mutations on ERK3’s ability in promoting HeLa cell migration.** HeLa cells were transfected with the empty expressing vector, WT ERK3 or each of the ERK3 R64 or L290 mutants. Two days post-transfection, cell migration was analyzed using a two-chamber transwell system and migrated cells were stained with crystal violet, photographed and counted under a microscope at 50X magnifications. Representative images were shown on the top. Quantitative results were presented as number of migrated cells per filed. Values in bar graph represent mean ± S.D. *P<0.05 by Student's *t*-test (in comparison with ERK3 WT).
iii. Subcellular localization of cancer-related ERK3 mutants

ERK3 protein shuttles between the nucleus and the cytoplasm (Déléris et al., 2008) but is localized mainly in the nucleus (Julen et al., 2003; Seternes et al., 2004), suggesting ERK3’s activity and function may be regulated by subcellular localization. To investigate whether ERK3 mutations affects ERK3 protein’s subcellular localization, HA-tagged ERK3 or ERK3 mutants were exogenously expressed in HeLa cells by transient transfection. The subcellular localization of ERK3 proteins was visualized by immunofluorescence (Figure 6A). Cells were grouped into three categories based on the subcellular localization of ERK3 proteins: group 1) cells with relatively equal distribution of ERK3 in the cytoplasm and nucleus; group 2) cells having more ERK3 in the nucleus; group 3) cells having more ERK3 in the cytoplasm (Figure 6B). In line with previous report, WT ERK3 (shown in red) was localized mainly in the nucleus in the majority of cell population. Similarly, both ERK3 KD and ERK3 S189A mutants are mostly in the nucleus. Interestingly, compared to WT ERK3, all of the ERK3L290 mutants have increased cytoplasmic localization. In particular, ERK3L290P is primarily localized in the cytoplasm.
<table>
<thead>
<tr>
<th>Variant</th>
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<th>DAPI</th>
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</table>
Figure 6: Subcellular distribution of ERK3 and its mutants in HeLa cells

Subcellular localization of WT or mutant ERK3 was determined by immunofluorescent staining of HA-tagged ERK3 exogenously expressed in HeLa cells. HeLa cells were transfected with WT or mutant ERK3 plasmids and two days post-transfection, cells were immuno-labelled with an anti-HA antibody (red) against the HA tag on ERK3 proteins and DNA was stained with DAPI (blue) to show the nucleus. Pictures were taken under 63X magnification and representative images are shown on the top. For each transfection, at least 50 cells expressing ERK3 or each ERK3 mutant were analyzed and grouped as follow: cells showing predominant cytoplasmic ERK3 localization (C>N), cells showing relatively equal distribution of ERK3 in the nucleus and cytoplasm (N=C) and cells showing predominant nuclear localization of ERK3 (N>C). The bars represent the percentage of total transfected cells for each different group.
iv. Stable knockdown of ERK3 decreases HeLa cell migration

HeLa cells have endogenous expression of ERK3, which may attenuate the effect of exogenously expressed ERK3 (wild or mutants) on cellular activities. Hence, we generated HeLa stable cell line with stable knockdown of ERK3 (Figure 7A) and tested the effects of ERK3 mutations in this stable cell line. In line with previous findings, HeLa cells with stable knockdown of ERK3 (HeLa shERK3) showed a significant reduction in cell migration as compared to control cells (HeLa shGIPZ, Figure 7B).

v. ERK3L290P and EKR3L290V mutants have increased ability in promoting cell migration in HeLa shERK3 stable cells

We then used HeLa shERK3 cell line to investigate the effects of L290P (or V) mutations on ERK3’s role in promoting HeLa cell migration. First, we then examined the expression and phosphorylation levels of ERK3L290 mutants in comparison with wild type ERK3 in HeLa shERK3 cells by exogenously expressing either empty vector, WT ERK3 or each different ERK3 mutant. Two days post-transfection, cells were harvested and analyzed by Western blotting. Neither ERK3L290P nor L290V showed a clear change in protein expression level as compared to WT ERK3 (Figure 8). In contrast, both L290P and L290V mutants showed a remarkable reduction in phosphorylation at S189, so did ERK3L290A, suggesting that L290 affects ERK3 phosphorylation at S189. Reintroduction of WT ERK3 HeLa shERK3 increased cell migration about as two-fold compared to the empty vector (Figure 9). However, overexpression of ERK3-KD mutant did not significantly affect cell migration, suggesting EKR3 kinase activity is important for its function in promoting cell migration. Surprisingly, ERK3S189A was capable of promoting
HeLa cell migration to the same level as WT ERK3 did, indicating S189 phosphorylation is not required for ERK3 in promoting cell migration. In agreement with their effects in parental HeLa cells, both ERK3 L290P and ERK3 L290V have increased ability of promoting migration of HeLa shERK3 cells migration as compared to WT ERK3.
**Figure 7: Generation of HeLa cells with stable knockdown of endogenous ERK3.**

A. HeLa shERK3 stable cells were generated by lentiviral expression of a shRNA targeting the non-coding region of ERK3 mRNA. HeLa cells with stable expression of a non-targeting shRNA (HeLa shGIPZ) were generated to be used as a control. ERK3 knockdown was confirmed by Western blot analysis and β-actin was used as a loading control. B. Transwell migration assay of HeLa shGIPZ and shERK3 cells. Values in bar graph represent mean number of migrated cells per field ± S.D. *P<0.05 by Student's t-test.
Figure 8: Phosphorylation of ERK3 and ERK3 mutants at S189 in HeLa shERK3 cells. HeLa shERK3 cells were transfected with empty vector, WT ERK3 or ERK3 mutants. Cells were then harvested and protein lysates were analyzed by Western blotting for ERK3 expression and phosphorylation using an ERK3 Ab and a phospho-S189 specific ERK3 Ab, respectively. β-Actin was probed to serve as a loading control. Quantification of ERK3, P-ERK3 and β-actin protein levels by densitometry analysis of the immunoblots was done using Image J software and the ratio of P-ERK3 to ERK3 was calculated. The band density in “ERK3 WT” was set as 1.
Figure 9: The effects of ERK3 mutations on ERK3’s migration-promoting role in HeLa-shERK3 cells. HeLa shERK3 cells were transiently transfected with empty vector, ERK3 or each different ERK3 mutant as indicated. Two days post-transfection, cell migration was analyzed using a modified two-chamber transwell system and migrated cells were stained with crystal violet. Quantitative results were presented as number of migrated cells per field. Values in bar graph represent mean ± S.D. *P<0.05 by Student's t-test (in comparison with ERK3 WT).
vi. Knockdown of endogenous ERK3 decreases migration and invasion of H1299 cells

As ERK3L290P/V mutations are identified in lung tumors (COSMIC database), we wanted to investigate these ERK3 mutants in lung cancer cell lines. By using similar approaches, we first generated H1299 lung cancer cells with stable knockdown of endogenous ERK3 using the shRNA that targets the non-coding region of ERK3 mRNA. We examined the extent of ERK3 knockdown in these stable cells by Western blotting, and found that endogenous ERK3 was sufficiently depleted in H1299-shERK3 (Figure 10A). As expected, stable depletion of ERK3 dramatically reduced H1299 cell migration and invasion (shERK3 vs shGIPZ) (Figures 10B and C).

vii. ERK3 L290P and L290V mutants have increased ability in promoting migration and invasion of H1299-shERK3 cells

To measure the activation loop phosphorylation at S189 of ERK3 mutants in lung cancer cells, WT or each mutant ERK3 was expressed H1299 shERK3 cells. Both L290P and L290V mutants are expressed at equivalent levels to that of WT ERK3. ERK3 L290P had reduced phosphorylation at S189, whereas ERK3L290V had an increase of S189 phosphorylation as compared to WT ERK3 (Figure 11). As to their effects on migration and invasion of H1299 shERK3 cells, both ERK3 L290P and L290V mutants significantly increased cell migration (Figure 12) and invasion (Figure 13) in comparison with those of WT ERK3. These results are consistent with what we observed in HeLa cells, further suggesting that L290P (or V) mutations confer ERK3 increased ability in promoting cancer cell invasiveness.
Figure 10: Stable knockdown of EKR3 in H1299 cells decreased migration and invasion. ERK3 was stably knocked down in H1299 by shRNA targeting the non-coding region of ERK3 (shERK3). H1299 cells expressing a non-targeting control shRNA (shGIPZ) was used as a control. A. Western blot analysis of ERK3 in H1299 stable cell lines. β-actin was used as a loading control. B & C, Transwell migration and invasion assays of H1299 shGIPZ or shERK3 cells. Quantitative data under each condition is presented as number of migrated or invaded cells per field (lower panel). Values in bar
graph represent mean ± S.D. *P<0.05 by Student's t-test. On the top are representative images of migrated or invaded cells stained with crystal violet.
Figure 11: Phosphorylations of ERK3 and ERK3 Mutants at S189 in H1299 shERK3 cells. H1299 shERK3 stable cells were transfected with empty vector, ERK3, or each of ERK3 mutants as indicated. Cells were harvested and protein lysates were analyzed by Western blotting using P-ERK3 (S189) and ERK3 antibodies as indicated. β-actin was used as a loading control. The ratio of P-ERK3 to ERK3 is shown below the immunoblots.
Figure 12: Effects of ERK3 mutations on promoting H1299 shERK3 cell migration

H1299 shERK3 cells were transfected with WT ERK3 or each of ERK3 L290 mutants. Two days post-transfection, cell migration was analyzed using a modified two-chamber transwell system and migrated cells were stained with crystal violet, as shown in the upper panel. Quantitative migration values under each condition were presented as number of migrated cells per field. Values in bar graph represent mean ± S.D. *P<0.05 by Student's t-test.
Figure 13: Effects of ERK3 mutations on promoting H1299 shERK3 cell invasion

H1299 shERK3 cells were transfected with WT ERK3 or each of ERK3 L290 mutants. Two days post-transfection, cell invasion was analyzed using a modified two-chamber transwell system and invaded cells were stained with crystal violet, as shown in the representative images in the upper panel. Quantitative invasion values under each condition were presented as number of invaded cells per field. Values in bar graph represent mean ± S.D. *P<0.05 by Student's t-test.
viii. **ERK3L290P (or V) mutants also promote invasion of A549 cells**

To further demonstrate the increased ability of ERK3L290 mutants in promoting lung cancer cell invasion, WT ERK3 or ERK3L290 mutants with a Myc tag were overexpressed by lentiviral transduction in A549 cells (Figure 14A) and their effects on cell invasion were determined. As shown in Fig. 14B, both L290P and L290V mutants had significantly increased ability in promoting cell invasion as compared to ERK3 WT.

ix. **ERK3 L290P (or V) mutants localize primarily in the cytoplasm of A549 cells**

We also examined the subcellular localization of WT ERK3 and ERK3 L290 mutants in A549 lung cancer cells by immunofluorescent staining following lentiviral overexpression of ERK3 proteins. WT ERK3 is primarily localized in the nucleus in the majority (over 60%) of cells (Figure 15). In contrast, both L290P and L290V mutations caused remarkable increase in cytoplasmic localization. Over 70% of the cells overexpressed with either L290P or L290V had predominant cytoplasmic ERK3 localization (Figure 15).
A

Empty
vector
ERK3
ERK3
L290A
ERK3
L290P
ERK3
L290V

ERK3

β-actin

B

EV
ERK3
ERK3
L290P
ERK3
L290V
ERK3
L290A

Number of invaded cells/field

Empty
vector
ERK3
ERK3
L290A
ERK3
L290P
ERK3
L290V
ERK3
L290A

Number of invaded cells/field

EV
ERK3
L290P
L290V
L290A
Figure 14: Effects of ERK3 mutations on its ability in promoting A549 cell invasion

A: Western blot analysis of ERK3 expression in A549 cells that were transduced with lentivirus vector CDH, CDH-ERK3 WT, CDH-ERK3 L290A, CDH- ERK3 L290P, or CDH- ERK3 L290V. β-Actin was used as a loading control. B: A549 cells were transduced with lentivirus as shown in A. Two days post-transduction, cell invasion was analyzed using a modified two-chamber transwell system in which the inserts were precoated with growth factor-reduced Matrigel and invaded cells were stained with crystal violet. Quantitative values under each condition were presented as number of invaded cells per field. Values in bar graph represent mean ± S.D. *P<0.05 by Student's t-test (in comparison with ERK3 WT).
Subcellular location of ERK3 in transfected cells (% of total transfected cells)

- ERK3
- ERK3-L290P
- ERK3-L290V

- C>N
- N>C
- N>C

Myc-ERK3

DAPI
Figure 15: Subcellular localization of ERK3 and ERK3 mutants in A549 cells

The subcellular localization of WT or mutant ERK3 was determined by immunofluorescent staining of myc-tagged ERK3 which were exogenously expressed in A549 cells by lentiviral transduction. Cells were immuno-labelled with an anti-myc antibody (red) against the myc tag on ERK3 proteins and DNA was stained with DAPI (blue) to show the nucleus. Pictures were taken under 63X magnification. For each transfection, at least 50 cells expressing either WT ERK3 or each of ERK3L290 mutants were analyzed and grouped as follow: cells showing predominant cytoplasmic ERK3 localization (C>N), cells showing relatively equal distribution of ERK3 in the nucleus and cytoplasm (N=C) and cells showing predominant nuclear localization of ERK3 (N>C). The bars represent the percentage of total transfected cells for each different group.
x. **ERK3 L290 mutants do not affect ERK3 kinase activity in phosphorylating SRC-3**

ERK3 phosphorylates the CBP-interacting domain (CID) of SRC-3 (Long et al., 2012), which is at least partly important for ERK3’s role in promoting cancer cell migration and invasion. We thus tested the effects of L290 mutations on ERK3 kinase activity towards SRC-3 by performing in vitro kinase assay using the purified CID of SRC-3 as the substrate. As shown in Figure 16, WT ERK3, ERK3 L290P and ERK3L290V exhibited similar kinase activity of phosphorylating SRC-3CID (autoradiograph of Figure 16B).
Figure 16: In vitro kinase assay for ERK3 L290 mutants

A. ERK3L290 mutants with a HA tag were expressed in 293T cells and purified using HA Ab-conjugated agarose beads. Coomassie stained gel shows the purified ERK3 proteins.

B. In vitro kinase assay was conducted by incubating 1 μg of purified GST-SRC3-CID (as a substrate) with 100 ng of ERK3 WT, ERK3L290P, ERK3L290V or ERK3. GST-SRC3-CID proteins in each reaction was detected by coomassie staining (Left panel). ERK3 autophosphorylations and phosphorylations of GST-SRC3-CID are seen in the autoradiograph. Note that while ERK3 autophosphorylation was seen in autoradiograph, ERK3 protein (~100 Kd) was not shown by coomassie staining (Left Panel) as only 100 ng of ERK3 protein kinase was used in each reaction.
IV. Discussion

ERK3 is member of the atypical MAPK subfamily. ERK3 has a single Ser-Glu-Gly phospho-acceptor motif in its activation loop instead of the Thr-Xaa-Tyr motif found in the conventional MAPKs, such as ERK1/2 (coulmbe et al, 2007). While the classical MAPKs have been studied extensively, much less is known about the regulation, substrates and pathological functions of ERK3.

Recent studies have shown that ERK3 plays important roles in cancer. ERK3 is highly expressed in multiple cancers, such as lung cancer (Long et al., 2012), oral squamous cell carcinoma (Rai et al., 2004) and gastric cancer (Liang et al., 2005). Importantly, ERK3 was shown to increase cancer cell invasiveness (migration and invasion) by phosphorylating oncoprotein SRC-3, hence upregulating MMP gene expression (Long et al., 2012). Similarly, it was recently shown that ERK3 increases breast cancer cell migration speed by regulating breast cancer cell morphology and motility (Al-Mahdi et al., 2015). In addition, ERK3 increases the chemo resistance of lung cancer cells to topoisomerase-II inhibitors (Bian et al., 2016). However, little is known about ERK3 mutations in cancers. As ample evidence has proved that alteration of protein kinase activity and its signaling can lead to cancer growth and progression (Dhillon et al., 2007), it would be interesting to investigate the roles of ERK3 mutations on its kinase activity and cellular functions in cancer.

In this study, we found in COSMIC database that ERK3 gene mutations do occur in multiple cancers including lung cancer, skin cancer and colon cancers. Of particular
interests are L290 (P or V) mutations that are located in the kinase domain and are conserved in ERKs1-4, indicating these mutations may have important roles in cancer cells. Indeed, our study clearly shows that both L290P and L290V mutations confer ERK3 increased cytoplasmic localization and ability in promoting cancer cell migration and invasion. In contrast, ERK3R64H had reduced ability in promoting HeLa cell migration and R64C mutation had no significant effect. Thus, we thought ERK3 mutations on R64 may be random or passenger mutations and may not have significant clinic outcomes.

ERK3 is phosphorylated at S189 of the SEG activation motif, which is thought to be important for ERK3 kinase activation. S189 can be auto-phosphorylated by ERK3 or trans-phosphorylated by other kinases, such as PAKs (Déléris et al., 2011) (De la Mota-Peynado A et al., 2011). In addition, this residue was found to be important for the interaction of ERK3 with MK5, which results in MK5 activation and shuttling of both the proteins to the cytoplasm (M Cargnello. et al., 2011). Thus we tested the effects of ERK3 mutations on S189 phosphorylation. Interestingly, all these cancer-related mutations (R64C, R64H, L290P and L290V) cause a decrease of ERK3 phosphorylation at S189 in cancer cells. But this does not seem to be associated with the increased ability of ERK3L290P (or V) in promoting cancer cell migration and invasion, as mutation of S189 to alanine did not change ERK3’s role in promoting HeLa cell migration.

ERK3 is known to phosphorylate SRC-3 and upregulated SRC-3-mediated MMP gene expression, which is important for cancer migration and invasion (Long et al., 2012). To elucidate the underlying mechanism by which ERK3 L290 mutants increase cancer cell invasiveness, we then evaluated the kinase activity of WT and ERK3 mutants in vitro by assessing the phosphorylation of SRC-3. We found that ERK3 L290P and L290V mutants
phosphorylate SRC-3 at similar level as WT ERK3 does. Even though we were not able to assess the effects of L290 mutations on phosphorylating SRC-3 in cultured cancer cells due to lack of a phospho-specific Ab, these results imply that L290 mutants might promote cancer cell invasiveness through mechanisms other than SRC3-mediated MMP gene expression.

Interestingly, besides highly increased ability in promoting cancer migration and invasion, another phenotypic change with both L290P and L290V mutants is their remarkable increase of localization in the cytoplasm. Previous studies have shown that ERK3 interacts with PAK1 and PAK2, both of which are primarily localized in the cytoplasm and play critical roles in cancer cell morphology and motility. In addition, ERK3 was shown to be localized at the plasma membrane of migrating breast cancer cells, where it enhances cancer cell migration speed. Moreover, unpublished data in our lab indicates that ERK3 might positively regulate PAK1 phosphorylation and activity, thereby promoting cancer cell migration. Taken together, we propose a hypothetic mechanistic model for the enhanced ability of ERK3L290P (or V) mutants to promote cancer cell migration (Figure 17): as compared to wild type ERK3 proteins, which are primarily localized in the nucleus, ERK3L290P (or V) mutants are mainly localized in the cytoplasm or even in the plasma membrane region, where they upregulate PAKs signaling and promote cancer cell migration. As MK5 is known to interact with ERK3 and promotes its cytoplasmic translocation from the nucleus (Schumacher et al., 2004), we are interested in testing whether ERK3 L290P or L290V mutations lead to increased ERK3’s interaction with MK5.
In summary, our present study demonstrates that the cancer-related ERK3 L290P and L290V mutants increase the ability of ERK3 to promote cancer cell invasion and migration, possibly through the increased cytoplasmic localization. For future work, we plan to investigate the role of ERK3 L290P and L290V mutations in tumor invasiveness/metastasis using xenograft animal models and their clinical association with tumor grades, invasiveness and metastasis by examining tumor specimens of cancer patients.

**Figure 17:** A hypothetic model for the enhanced ability of ERK3L290P (or V) mutants in promoting cancer cell migration
V. References


