# A CRITICAL ROLE OF MIXED-LINEAGE KINASE 3 IN BREAST CANCER CELL MIGRATION, INVASION AND METASTASIS

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## ABSTRACT

A CRITICAL ROLE OF MIXED-LINEAGE KINASE 3 IN BREAST CANCER CELL MIGRATION, INVASION AND METASTASIS

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Despite improvements in early detection and treatment, breast cancer metastasis to distant sites remains a clinical problem and is a major cause of death. Thus, discovery of key molecular players that drive cancer cell local invasion and metastasis could reveal novel therapeutic targets for combating such diseases. Mitogen-activated protein kinases (MAPKs) mainly comprise c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, ERK5/BMK and control a variety of intercellular events. Mixed-lineage kinase 3 (MLK3), an MAP3K, can activate all the MAPK signaling pathways.

We find that MLK3 is highly expressed in breast cancer cells compared with mammary epithelial cells. Active MLK and signaling to JNK are required for migration of breast cancer cells. Conversely, induced overexpression of MLK3 promotes migration and invasion of mammary epithelial cells. Consistent with this, MLK3 activates AP-1 transcription factor through JNK, which induces the expression of invasion genes. In contrast, Inhibition of AP-1 blocks MLK3-induced invasion. In a 3D extracellular matrix culture model, MLK3 increases acinar size, perturbs apico-basal polarity and induces luminal repopulation through inhibition of pro-apoptotic protein BimEL. Taken together,

we demonstrate that MLK3 is critical for breast cancer cell migration, invasion and acquisition of a malignant phenotype of mammary epithelial cells.

In addition to induction of invasion gene expression, we also hypothesize MLK3 signaling can directly impact the cell migration machinery in breast cancer cells. We show that the prometastatic factors, CXCL12 and HGF, signal through MLK3-JNK to promote phosphorylation of focal adhesion scaffold protein, paxillin on Ser 178 (by JNK), which is required for migration of breast cancer cells. Furthermore, we show that MLK3 promotes phosphorylation of paxillin at Tyr 118 (by FAK/Src), possibly through facilitating the recruitment of FAK to S178-phosphorylated paxillin. Supporting this model, expression of nonphosphorylatable paxillin mutant S178A blocks Y118 phosphorylation of paxillin. Y118 phosphorylation of paxillin is a critical event for focal adhesion turnover by limiting activation of small GTPase Rho. We show silencing or inhibition of *Mlk3*, inhibition of JNK, or expression of paxillin S178A all increase Rho activity, suggesting that the MLK3-JNK-paxillin axis limits Rho activity in breast cancer cells. Consistently, MLK3 knockdown in breast cancer cells increases focal adhesion numbers, suggesting that loss of MLK3 causes a defect in focal adhesion turnover. Using mouse xenograft models, we provide evidence that MLK3 is necessary for metastases of human breast cancer cells to lung. Furthmore, paxillin phosphorylation on S178 is elevated in high metastatic cells. We conclude that Inhibition of MLK3-paxillin axis may be a novel therapeutic strategy to prevent breast cancer metastasis.

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| | |
|---|---|
| 2D | 2 dimensional |
| 3D | 3 dimensional |
| ABC | avidin-biotin complex |
| ADH | atypical ductal hyperplasia |
| AI | aromatase inhibitor |
| AP-1 | activating protein-1 |
| ARP2/3 | actin-related protein |
| ASK | apoptosis signal-regulating kinase |
| ATF2 | activating transcription factor 2 |
| ATP | adenosine triphosphate |
| Bcl2 | B-cell lymphoma 2 |
| BimEL | bim-extra long |
| BMK1 | big MAP kinase 1 |
| BRCA1 | breast cancer 1 |
| BSA | bovine serum albumin |
| CCL18 | Chemokine (C-C motif) ligand 18 |
| CD44 | cluster of differentiation 44 |
| Cdc42 | cell division cycle 42 |
| CRIB | Cdc42/Rac interactive binding |
| CSF | colony stimulating factor |
| CTSL1 | cathepsin L1 |
| CXCL12 | chemokine (C-X-C motif) ligand 12 |

| | |
|---|---|
| CXCR4 | C-X-C chemokine receptor type 4 |
| DAB | 3, 3'-diaminobenzidine |
| DAPI | 4'-6-diamidino-2-phenylindole |
| DCIS | ductal carcinoma in situ |
| DLK | dual leucine zipper bearing kinase |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EMT | epithelial-to-mesenchymal transition |
| ER | estrogen receptor |
| ERK | extracellular signal-regulated kinase |
| Ets | E-twenty six |
| FAK | focal adhesion kinase |
| FBS | fetal bovine serum |
| Fra-1 | Fos-related antigen 1 |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factors |
| GFP | green fluorescent protein |
| GM130 | Golgi matrix protein 130 |
| GPCR | G-protein coupled receptor |
| GSK | glycogen synthase kinase |

| | |
|---|---|
| GTP | guanosine-5'-triphosphate |
| GTPases | guanosine triphosphatases |
| HEK-293T | human embryonic kidney 293 contains SV40 large T-antigen |
| HER2 | human epidermal growth factor receptor 2 |
| HGF | hepatocyte growth factor |
| HGF/SF | hepatocyte growth factor/scatter factor |
| HIV-1 | human immunodeficiency virus-1 |
| HMEC | human mammary epithelial cell |
| HPV E7 | human papillomaviruses E7 |
| i.p | Intraperitoneal injection |
| IDC | invasive ductal carcinoma |
| IGF | insulin growth factor |
| IL-11 | Interleukin 11 |
| JIP | JNK-interacting protein |
| JNK | c-Jun N-terminal kinase |
| LPS | lipopolysaccharide |
| LZK | leucine zipper-bearing kinase |
| MAP3K11 | mitogen-activated protein kinase kinase kinase 11 |
| MAPK | mitogen-activated protein kinase |
| MAPKAPK2 | mitogen-activated protein kinase-activated protein kinase 2 |
| MAPKKK /MAP3K | mitogen-activated protein kinase kinase kinase |
| MEF2 | myocyte enhancer factor 2 |
| MKK | MAPK kinases |

| | |
|---|---|
| MLK | mixed lineage kinase |
| MLTK | MLK-like mitogen-activated protein triple kinase |
| MMP | matrix metalloproteinase |
| MPP+ | 1-methyl-4-phenylpyridinium |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MSI | microsatellite instability |
| MUK | MAPK-upstream protein kinase |
| NF-2 | neurofibromin 2 |
| NFAT | nuclear factor of activated T cells |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGF | nerve growth factor |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| PARP | poly (ADP-ribose) polymerase |
| PBS | phosphate buffer saline |
| PDGF | platelet-derived growth factor |
| PKB | protein kinase B |
| PKC | protein kinase C |
| POSH | plenty of SH3s |
| PR | progesterone receptor |
| PSD-95 | postsynaptic density protein 95 |
| PTEN | phosphatase and tensin homolog |
| Rac | Ras-related C3-botulinum toxin substrate |
| Rho | Ras homologous |

| | |
|---|---|
| RhoGEF | Rho guanine nucleotide exchange factor |
| RIPA | RadioImmunoPrecipitation Assay |
| RNAi | ribonucleic acid interference |
| RSK | ribosomal S6 kinase |
| RTKs | receptor tyrosine kinases |
| SATB1 | special AT-rich sequence binding 1 |
| SDF1-α | stromal cell-derived factor1-alpha |
| SERMS | selective estrogen receptor modulators |
| SH3 | Src homology 3 |
| shRNA | short hairpin ribonucleic acid |
| siRNA | small interfering ribonucleic acid |
| STR | short tandem repeat |
| TEY | Thr–Glu–Tyr |
| TGF-β | transforming growth factor beta |
| TNC | tenascin C |
| TNF-α | tumor necrosis factor-alpha |
| TRAF2 | TNF-α receptor-associated factor 2 |
| UV | ultraviolet |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VEGF | vascular endothelial growth factor |
| WASP | Wiskott-Aldrich syndrome protein |
| ZAK | zipper sterile-alpha-motif kinase |
| ZPK | zipper protein kinase |

# CHAPTER I

# LITERATURE REVIEW

## 1. Breast cancer and subtypes

In 2012, approximately 200,000 women will be diagnosed with invasive breast cancer in the US. Approximately 40,000 patients will die due to metastatic breast cancer (Siegel et al., 2012). Breast cancer remains the second leading cause of cancer death in women, despite the continuous progress achieved by physicians and scientists in early diagnosis and disease treatment.

Breast cancer is a heterogeneous disease with diverse subtypes which present with distinct histopathological, genetic or epigenetic variations (Carey et al., 2006). Recently, gene expression profiling has been used to classify breast cancer into four major subtypes, including luminal A, luminal B, basal, and HER2 (ErBB2) (Perou et al., 2000). Breast cancers of the luminal A subtype (42-59%), which typically express hormone receptor including estrogen receptor (ER), progesterone receptor (PR), and lack ErBB2 overexpression, are usually associated with a favorable outcome. A worse prognosis is predicted for luminal B subtype (6-19%) tumors which are hormone receptor positive (ER+, PR+), can be either ErBB2, -postive or negative, and have a high level of Ki-67, a proliferation marker (Sorlie et al., 2003). If diagnosed with luminal A or B breast cancers, patients are often treated with anti-estrogens or other endocrine therapies, in addition to the primary treatment strategies which include surgery, radiation and adjuvant chemotherapy. For example, tamoxifen and raloxifene are commonly used selective estrogen receptor modulators (SERMS) for premenopausal women. The mechanism of action for SERMS is blocking ER function to inhibit proliferation of cancer cells. In addition, Fulvestrant is a pure antiestrogen that induces

degradation of ER. For postmenopausal women, aromatase inhibitors (AIs) such as anastrozole, letrozole or exemestane which block nonovarian estrogen synthesis, effectively reduce recurrence and mortality (Cuzick et al., 2010; Howell et al., 2005).

The HER2 subtype (7-12%) is characterized by overexpression of the growth factor receptor ErBB2. ErBB2 overexpression is most often caused by gene amplification, but loss of repressors can also lead to increased transcription of ErbB2 (Zuo et al., 2007). ErBB2 belongs to epidermal growth factor receptor (EGFR) family of tyrosine kinases. ErBB2 lacks a ligand binding domain and is believed to exert its function through forming a heterodimeric complex with other EGFR family members, ErbB1, ErbB3 and ErbB4 (Hynes et al., 2005; Yarden et al., 2001). ErBB2 signaling can initiate mammary tumorigenesis by promoting proliferation and suppressing apoptosis of mammary epithelium (Muthuswamy et al., 2001). Transgenic mice expressing constitutive active ErBB2 in the mammary gland develop invasive ductal carcinoma (Muller et al., 1988; Ursini-Siegel et al., 2007). ErBB2 overexpression is associated with a high rate of relapse, poor prognosis and shortened overall survival (Berger et al., 1988; Slamon et al., 1987). Targeted therapies for HER2-overexpressing breast cancer include the monoclonal antibody Trastuzumab (Herceptin) that targets the ErBB2 extracellular domain; and the small molecule kinase inhibitor Lapatinib (Tykerb) that targets the kinase domain of both ErBB1 and ErBB2 (Bilancia et al., 2007; Piccart-Gebhart et al., 2005). Although acquired resistance to ErBB2-targeted therapies is not uncommon (Sergina et al., 2007), the combination of Trastuzumab or Lapatinib with chemotherapeutics is a widely used strategy to treat advanced and metastatic HER2-overexpressing breast cancers (Geyer et al., 2006; Romond et al., 2005).

Approximately 14-20% of human breast tumors fall into the basal subtype. Basal breast tumors, also typically called triple negative, lack ER, PR and ErBB2 and express basal marker cytokeratin 6 (Abd El-Rehim et al., 2004; Livasy et al., 2006; Rakha et al., 2008; van de Rijn et al., 2002). Basal breast tumors are aggressive, more malignant and metastatic. Accordingly patients with basal breast tumors have the worst outcome (Rakha et al., 2008). Although studies have identified several potential targets, such as EGFR, the growth factor receptor c-KIT, which are expressed in a high proportion of basal type breast cancers (Nielsen et al., 2004; Simon et al., 2004), the clinical evidence supporting their efficacy is lacking. Since basal type breast cancers share fundamental biologic similarities with BRCA1-associated tumors (Foulkes et al., 2003; Foulkes et al., 2010; Rakha et al., 2008), which are characterized by DNA-repair defects, especially defective homologous recombination, inhibitors for the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP) could be relevant in treating this type of breast cancer (Farmer et al., 2005; Kennedy et al., 2004; Turner et al., 2005). However, the efficacy of these therapeutic strategies remains to be determined. Thus, for basal breast cancer, currently available targeted therapies are very limited.

## 2. Breast cancer metastasis

The normal human mammary gland is composed of ducts and lobules. A duct contains a layer of epithelial cells surrounded by a layer of myoepithelial cells with a hollow lumen. Transformation of normal mammary epithelial cells could result from a series of genetic or epigenetic alterations in the cells and/or interaction with the

microenvironment. Abnormal cells are found in atypical ductal hyperplasia (ADH) that is proposed to be a precursor for ductal carcinoma in situ (DCIS). In DCIS, the hollow lumen is filled with abnormal cells while the cells have not breached the basement membrane. Once the cells invade through the basement membrane, they become invasive ductal carcinoma (IDC) and have the potential to travel to the lymph node or distant sites. Breast cancer invasion dramatically increases the risk of developing distant metastases. Breast cancer metastasis is an inefficient multi-step process. Cancer cells must invade the basement membrane, migrate through the surrounding stroma tissue, enter the vasculature (intravasate), survive in the circulation, transmigrate through the endothelium to the tissue (extravasate) and colonize foreign microenvironments. Although regional lymph node metastases are commonly used as a prognostic factor for breast cancer patients, distant metastases to vital organs cause serious problems. Breast cancer commonly metastasizes to bone, lung, liver and brain (Lee et al., 1983; Weigelt et al., 2005).

The dominant view during the past decades was that metastasis is a progressive, late-stage event deriving from evolution of the primary tumor (Cairns et al., 1975; Greco et al., 2009; Pantel et al., 2008). However, this model is challenged now because it is not consistent with two clinical observations. First, metastatic recurrence in breast cancer not uncommonly develops many years after removal of primary tumors from patients who had no detectable metastases at diagnosis. Second, a small portion of patients with metastatic cancers do not present with identifiable primary tumors. An alternative model proposes that metastasis is an inherent feature of the early primary tumor (Hellman et al., 1994; Hüsemann et al., 2008; Klein et al., 2009; Podsypanina et

al., 2008; van 't Veer et al., 2002). A recent view of breast cancer metastasis suggests that breast tumor cells disseminate early in the tumorigenesis to form micrometastases, which can later be converted to overt metastases, in response to triggers such as oncogene activation, immune escape, angiogenic factors, or other factors in the microenvironment.

Metastasis of breast cancer is site specific. In 1889, Stephen Paget proposed the 'seed-and-soil' hypothesis of metastasis, suggesting that metastasis arises from specific interactions between selected cancer cells (seeds) and the microenvironment of the specific target organs (soil) (Fidler et al., 2003; Nguyen et al. 2009). It is clinically observed that certain cancers are predisposed to disseminate to certain second organs. Chemokines, in the metastatic milieu, were found to play an important role in breast cancer site-specific metastasis to lung and bone (Müller et al., 2001; Nguyen et al., 2009). Chemokines exert their effects by binding to specific transmembrane receptors, which are members of a large family of G protein-coupled receptors (GPCRs). Over 20 chemokine receptors and more than 47 chemokines have been characterized in humans (Lazennec et al., 2010). Among them, CXCL12, also known as stromal-derived factor-alpha (SDF-α), is highly abundant in lung and bone marrow, two common metastatic sites of breast cancer. Metastatic tumor cells that express high levels of CXCR4, the CXCL12 receptor, have a propensity to colonize in the lung and bone marrow in response to the CXCL12 expressed in those organs. Mechanistically, CXCL12 can elicit profound cytoskeletal remodeling leading to migration and invasion of breast cancer cells. Blockade of CXCL12 or CXCR4 impairs cell migration (Chen et al., 2003; Müller et al., 2001; Scotton et al., 2001). Using gene expression analysis of

breast cancer cell lines that metastasize to different sites, a number of gene sets have been identified that are associated with site-specific metastasis (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005; Nguyen et al., 2009). Some critical gene products mediating the interaction between the tumor cells and the microenvironment of the metastatic site have been validated in both experimental and clinical samples. For example, the brain metastasis gene signature includes the sialyltransferase ST6GALNAC5, which mediates the adhesion of breast cancer cells to endothelial cells to facilitate the crossing of the brain-blood barrier, which is required for brain metastasis (Bos et al., 2009). In contrast, in the gene signature for lung metastasis, the cytokine, angiopoietin-like 4, made by cancer cells, disrupts lung endothelial cell junctions, enhances permeability of lung capillaries, and promotes colonization of the lung (Padua et al., 2008). In addition, lung metastatic breast cancer cells highly express cyclooxygenase COX2, and matrix metalloproteinases MMP1/2 which are involved in remodeling the lung vascular structures to enable metastatic seeding in the lung. Combined inhibition of these gene products dramatically blocks colonization of lung metastatic breast cancer cells (Gupta et al., 2007).

## 3. Hallmarks of breast cancer metastasis

### 3.1 Migration and invasion

Tumor cell motility and invasion are essential features of metastasis. Local invasion of the primary tumor, intravasation and extravasation are required steps of

metastasis. An early event in the process of metastasis is dysregulation of cellular adhesions, which allows tumor cells to detach from the primary tumor mass and acquire an invasive phenotype (Hanahan et al., 2011). Loss of E-cadherin is a hallmark of cancer invasion (Berx et al., 1995; Onder et al., 2008). E-cadherin is a key molecule required for maintaining the epithelial cell sheet by forming adherens junctions between the adjacent cells (van Roy et al., 2008). E-cadherin level is negatively associated with invasion and metastasis in a variety of human cancers including breast cancer (Berx et al., 2001; Oka et al., 1993). Besides loss of, or downregulation of E-cadherin, changes in the cell shape or adhesions also involve up-regulation of the mesenchymal cadherins, such as, N-cadherin, which potently promotes an invasive phenotype and breast cancer metastasis (Hazan et al., 2000; Hulit et al., 2007; Suyama et al., 2002). The process, referred to as epithelial-to-mesenchymal transition (EMT), is an embryonic development program hijacked by carcinoma cells to overcome the constraints of epithelial cells and to gain mesenchymal features (Lee et al., 2006). This process typically involves downregulation of E-cadherin, upregulation of N-cadherin and the intermediate filament protein vimentin, and secretion of matrix metalloproteinases (Hanahan et al., 2011; Thiery et al., 2009). By undergoing EMT, cancer cells acquire motile and invasive properties.

Multiple extracellular cues including the inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) (Li et al., 2012), the growth factors transforming growth factor beta (TGF-β) (Shi et al., 2003), platelet-derived growth factor (PDGF) (Jechlinger et al., 2006), insulin growth factor (IGF) (Kim et al., 2007), hepatocyte growth factor/scatter factor (HGF/SF) (Elliott et al., 2002) and EGF, can induce EMT (Hardy et al, 2010).

Binding of these factors to their receptors triggers intracellular signaling pathways that eventually alter expression of a group of transcription factors such as Snail, Slug, Twist, and Zeb1/2. For example, transcription of E-cadherin is downregulated by Snail, Slug, Zeb1, which repress E-cadherin promoter activity in breast cancer cells (Hajra et al., 2002; Sánchez-Tilló et al, 2010). Twist is also an E-cadherin repressor and plays a critical role in breast cancer cell invasion, intravasation to the blood stream and lung metastasis (Smit et al., 2009; Yang et al., 2004). Clinical evidence for EMT is limited, perhaps because EMT may be transient or may occur only in the invasive front of the tumor (Ledford et al., 2011; Mani et al., 2008). Nonetheless, EMT-associated features are significantly linked to high histological grade and metastasis in basal breast cancer (Jeong et al., 2012). EMT could be an important event in the malignant progression that drives invasion and metastasis.

Mesenchymal migration is a form of directed cell migration. During mesenchymal migration, in response to gradients of chemoattractants, such as chemokines or growth factors, polarization of an elongated cancer cell establishes a leading edge, which is followed by contraction of the cell body and rear retraction (Ridley et al., 2003; Roussos et al., 2011) to move the cell forward. In addition to the adoption of a fibroblast-like morphology, mesenchymal migration and invasion also involves remodeling of the extracellular matrix (ECM) (Friedl et al., 2011). Degradation of basement membranes and clearance of a migratory path in the ECM requires the activity of proteases (Joyce et al., 2009). Matrix metalloproteinases (MMPs) have been proposed to play major roles in these processes. MMPs are a large family of zinc-dependent endopeptidases with over 20 members. Most MMPs are secreted within the extracellular matrix, whereas

MMPs 14-17 are found in plasma membranes by virtue of a membrane translocation domain, and therefore are referred to as MT-MMPs (Egeblad et al., 2002; Stamenkovic et al., 2000). Many experiments indicate that MMPs degrade ECM components to promote breast cancer invasion. For example, invasion promoted through oncogenic signaling from ErBB2 requires activity of MMPs (Bosc et al., 2001; Ke et al., 2006). Elevated MT1-MMP (MMP14) expression correlates with metastasis in biopsies from human triple-negative breast cancers and has been shown to promote breast cancer cell migration and invasion *in vitro*, as well as blood vessel infiltration and lung metastasis in mouse xenograft models (Perentes et al., 2011).

Numerous extracellular factors, including cytokines and growth factors, can enhance expression of MMPs through transcriptional activation. AP-1 transcription factors are comprised homodimers of the Jun family (c-Jun, JunB, and JunD), or more commonly Jun heterodimers with Fos family members (cFos, Fra-1, Fra-2, and FosB) (Karin et al., 1997; Shaulian et al., 2002). AP-1 elements are identified in the promoter of MMP genes. Experiments using transfected cDNAs or siRNA-mediated gene silencing support a critical role of AP-1 in expression of MMPs (Benbow et al., 1997; Westermarck et al., 1999). In addition to regulation at the transcription level, activation of MMPs also involves proteolytic activation of inactive MMP precursor forms by other families of proteases (Westermarck et al., 1999). For example, the cathepsin family of cysteine proteases, translocate from lysosomes to the cell surface or extracellular milleu, where they elicit a proteolytic cascade to activate MMPs. Cysteine cathepsins, upstream of MMP activation, have been recently shown to be up-regulated by ErBB2 oncogenic signaling and to mediate ErBB2-induced breast cancer invasion (Rafn et al.,

2012). Furthermore, cysteine cathepsins are found frequently up-regulated in many human cancers, suggesting that cathepsin inhibitors could be useful therapeutics for limiting cancer invasion (Gocheva et al., 2007).

Expression of invasion genes is driven by key transcription factors. In addition to the classical EMT inducers, other transcription factors also contribute to activation of the invasion gene program during metastasis. For instance, SATB1 (special AT-rich sequence binding 1) has been implicated in potent activation of the breast cancer invasion and metastasis gene signatures (Han et al., 2008). AP-1 activity has also been associated with more aggressive forms of breast cancers (Belguise et al., 2005). Basal activity of AP-1 is significantly higher in more metastatic breast cancer sublines of MDA-MB-231 sublines than in poorly metastatic MDA-MB-231 sublines. AP-1 enhances transcription of many invasion-associated genes, including interleukin 11 (IL-11), which is critical for formation of breast cancer bone metastases (Kang et al., 2005). Furthermore, EpCAM, a cell-surface glycoprotein that is overexpressed in breast cancer and is associated with breast cancer cell dissemination, promotes breast cancer invasion through activation of AP-1 and AP-1 target invasion genes (Sankpal et al., 2011).

Acquired invasiveness involves not only genetic or epigenetic changes within cancer cells, but changes in neoplastic stroma cells have also been proposed to be critical (Friedl et al., 2011; Qian et al., 2010). Tumor-infiltrating fibroblasts can secrete CXCL12 or HGF, which have been shown to promote invasion (Jedeszko et al., 2009; Kalluri et al., 2006). Tumor-associated macrophages that are activated by breast cancer cell-derived colony stimulating factor (CSF) secrete EGF to facilitate intravasation and

metastasis in a mouse model of metastatic breast cancer (Joyce et al., 2009). Furthermore, the chemokine CCL18, supplied by tumor-associated macrophages, correlates with metastasis and shorter survival of breast cancer patients. Mechanistically, CCL18 through binding to its receptor, PITPNM3, activates integrin-focal adhesion kinase (FAK) signaling pathways to promote local invasion of breast cancer cells into the tumor stroma as well as distant metastasis to lung and liver (Chen et al., 2011). These studies suggest that targeting invasion-promoting chemokines could be a novel therapeutic strategy for metastatic breast cancer.

In summary, during chemotaxis driven by chemokines and growth factors in the tumor microenvironment, mesenchymal breast cancer cells utilize the epithelial-to-mesenchymal program to change to an elongated, fibroblast-like morphology for the ability to migrate and secrete matrix-degrading MMPs. Additionally, two other migratory modes have been implicated in breast cancer invasion (Hanahan et al., 2011). Collective cell migration, is characterized by a cluster of cancer cells which maintain cell-cell junctions and migrate as a multicellular strand into stroma, and is basically movement of a cell sheet (Friedl et al., 2004; Rørth et al., 2009). Gradients of chemokines and growth factors stimulate formation of a polarized leader cell and rear cells in the strand of the cells. In collective migration, the leader subsets of cells also polarize, undergo EMT and remodel the ECM with proteases (Rørth et al., 2009). Finally, amoeboid cell motility is a migration mode originally characterized in lymphocytes and neutrophils. However, a variety of epithelial cancers including breast cancer can adapt to this type of migration, suggesting plasticity of cancer cells in migration. For instance, inhibition of MMPs causes mesenchymal breast cancer cells to switch to amoeboid

migration (Wolf et al., 2003). Amoeboid cancer cells are able to squeeze through existing gaps in the ECM. Amoeboid migration is characterized by rounded cell morphology, poor adhesion to ECM, low activity of cell migration machinery and lack of MMP activity (Friedl et al., 2003).

### 3.2 Survival in the circulation

While large numbers of motile tumors cells are capable of entering the circulation, only few eventually form metastases (Fidler et al., 1970). The circulation system is a hostile environment where the circulating cancer cells are stressed by shear forces, natural killer cell-mediated immune surveillance, nutrient depletion and detachment-induced cell death (anoikis) (Hanahan et al., 2011; Mehlen et al., 2006). Only those circulating tumor cells that have acquired resistance to anoikis can survive in the circulation and disseminate. Anoikis involves release of pro-apoptotic proteins Bcl2 family members such as BCL2-interacting mediator of cell death (Bim) to trigger mitochondrial-mediated apoptosis (Frisch et al., 2001). Thus, overexpression of the anti-apoptotic Bcl2 can promote survival of circulating cancer cells and metastasis (Pinkas et al., 2004). In addition, induction of certain oncogenes, such as ErBB2 or Src, or loss of the tumor suppressor PTEN can lead to anoikis resistance in mammary epithelial cells by activation of survival signaling pathways (Haenssen et al., 2010; Mehlen et al., 2006; Schafer et al., 2009; Vitolo et al., 2009). Moreover, loss of E-cadherin and acquired EMT properties result in insensitivity to anoikis and promote metastatic dissemination in breast cancer (Derksen et al., 2006).

### 3.3 Proliferation at secondary site

Upon extravasation to a secondary site, most breast cancer cells die in the foreign environment. Survival after extravasation is essential for colonization and formation of frank metastases. The extravasated tumor cells may be kept in a dormant stage for long periods before forming an overt metastasis. In this case, survival in the inhospitable environment must be very relevant for disseminating tumor cells (Pantel et al., 2008). A recent study demonstrates that breast cancer cells express high levels of the membrane protein vascular cell adhesion molecule-1 (VCAM-1), through which cancer cells interact with metastasis-associated lung macrophages. Following extravasation this interaction promotes cancer cell survival. The colonized breast cancer cells activate a survival signaling pathway involving VCAM-1-Ezrin-PI3K/Akt to inhibit apoptosis (Chen et al., 2011). In addition to VCAM-1-mediated cell-cell adhesion, disseminating breast cancer cells express the ECM protein, tenascin C (TNC) in the metastatic niche, to promote survival and outgrowth of lung micrometastasis. TNC is a key component of ECM in the lung microenvironment, eliciting Wnt and Notch signaling for survival and outgrowth of infiltrated breast cancer cells (Oskarsson et al., 2011). Fibronectin has also been implicated in promoting transition from growth arrest dormant stage to overt growth in breast cancer cells using *in vitro* 3D culture and *in vivo* mouse model (Barkan et al., 2008). Furthermore, ECM-mediated adhesion signaling involving FAK and β1 integrin has been shown to promote cell cycle progression of breast cancer cells in lung parenchyma *in vivo* (Shibue et al., 2009). In a transgenic mouse model expressing oncogene polyoma middle T antigen (PyMT), breast cancer cells depleted of

β1 integrin fail to interact with fibronectin and undergo growth arrest (Barkan et al., 2010). These studies highlight the importance of ECM in survival and outgrowth of breast cancer cells in the metastatic niche. It is also proposed that an angiogenic switch, characterized by transition from an anti-angiogenic to neoplastic vascularization, contributes to escape from dormancy in micrometastatic cells (Aguirre-Ghiso et al., 2007). A better understanding of interaction between breast cancer cells and secondary sites will reveal potential therapeutic targets that prevent development of overt metastases from dormant cancer cells.

## 4. Small Rho family GTPases in cell migration

Cell migration is essential for development of metastasis. However, the molecular basis of cell migration is still poorly understood. Generally, cell migration is a dynamic process that involves cycles of cellular polarization, protrusion, adhesion to ECM, contraction and retraction. These steps are highly coordinated in a temporal and spatial manner and driven by rearrangements of the actin cytoskeleton (Ridley et al., 2003). Small Rho family GTPases, Cdc42, Rac and Rho, play critical roles in the regulation of actin cytoskeleton in cell migration.

Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form which results in a conformational change. Only the GTP-bound active form can bind downstream targets to modulate their activities and localization. GTPase effector proteins regulate cell migration, vesicular trafficking and gene expression. Guanine nucleotide exchange factors (GEF) activate Rho GTPases by promoting GDP release to

facilitate binding of abundant cytosolic GTP. Binding of various chemokines or growth factors to GPCRs or RTKs, respectively, or engagement of integrins with the ECM, elicits translocation and activation of GEFs which, in turn, activate their cognate Rho GTPases. Conversely, Rho family GTPases can be inactivated by specific GTPase activating proteins (GAPs) that catalyze hydrolysis of GTP (Heasman et al., 2008; Vega et al., 2008).

How does Rho GTPase regulate actin cytoskeleton in each step of the cell migration process? Studies showed Rho GTPases mediate actin cytoskeleton through their effectors. Briefly, in response to a chemoattractant, migrating cells first polarize to establish a leading edge. Establishment of polarity and directionality of movement is initiated by differential localization of phosphatidylinositol 4,5-bisphosphate at the leading edge, which results in localized activation of Cdc42. Either inhibition of Cdc42 activity or global activation of Cdc42 impairs polarity, suggesting activity of Cdc42 is tightly controlled for cell polarity. Active Cdc42 binds to its effector, WASP, whose gene is mutated in Wiskott-Aldrich syndrome, leading to WASP association with Arp2/3 (Actin-related protein) to initiate actin nucleation in the leading edge to establish cell polarity. Secondly, Rac and its effector WAVE then induce actin polymerization to form cell protrusion. For cell body contraction in the following step, the contraction force is generated by the interaction of myosin II and actin filaments and regulated through modulation of myosin II activity. Rho activates its effector Rho kinase (ROCK), which enhances phosphorylation of myosin light-chain (MLC) to regulate myosin activity and contraction. Activation of Rho also promotes maturation of focal adhesions. Focal adhesions are the intracellular multi-protein structures by which the cell links the ECM to

actin cytoskeleton. Focal adhesion contains numerous signaling molecules and scaffold proteins that undergo dynamic changes during cell migration. At the last step of cell migration, the focal adhesions at the cell rear must be disassembled to allow retraction of the cell body to move forward. The molecular mechanisms for focal adhesion turnover remain largely unknown. However, experimental data show cells depleted of FAK or Src have more and larger focal adhesions, suggesting FAK/Src tyrosine kinases are required for focal adhesion disassembly (Cain and Ridley, 2009; Raftopoulou and Hall, 2004; Ridley et al., 2003; Wozniak et al., 2004).

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Chen J, Rattanasinchai C and Gallo KA. MLK3. *Encyclopedia of Signaling Molecules*. Springer. 2013.

5. Mixed-lineage kinase 3 (MLK3)

The mixed-lineage kinases are so-named for their sequence similarity to both tyrosine kinases and serine/threonine kinases. However, based on biochemical assays, only serine/threonine kinase activity has been demonstrated for the MLKs. The three subfamilies of MLKs reside within the “tyrosine kinase-like” branch of the human kinome. Members of the MLK subfamily, comprised of MLK1-4, share a conserved domain arrangement and 75% identity within their catalytic domains (Figure 1.1). The dual leucine zipper bearing kinase (DLK) subgroup of MLKs, which includes DLK/ZPK/MUK and LZK, is characterized by a kinase catalytic domain followed by two leucine zipper motifs. A third subgroup of MLKs represented by ZAK/MLTK contains both a leucine zipper motif and a sterile-alpha motif. MLK3 has emerged as the paradigm for the MLK subfamily.

There is no ortholog for MLK3 in yeast but in *Drosophila*, the MLK1-4 ortholog, Slipper, is critical for the cell sheet movement during dorsal closure in the fly embryo, which involves activation of the JNK pathway. MLK3-deficient mice are fully viable but have a reduced thickness of the dorsal epidermal tissue, which parallels the effects of disruption of Slipper in *Drosophila* (reviewed in Gallo and Johnson. 2002; Schachter et al., 2006).

Figure 1.1

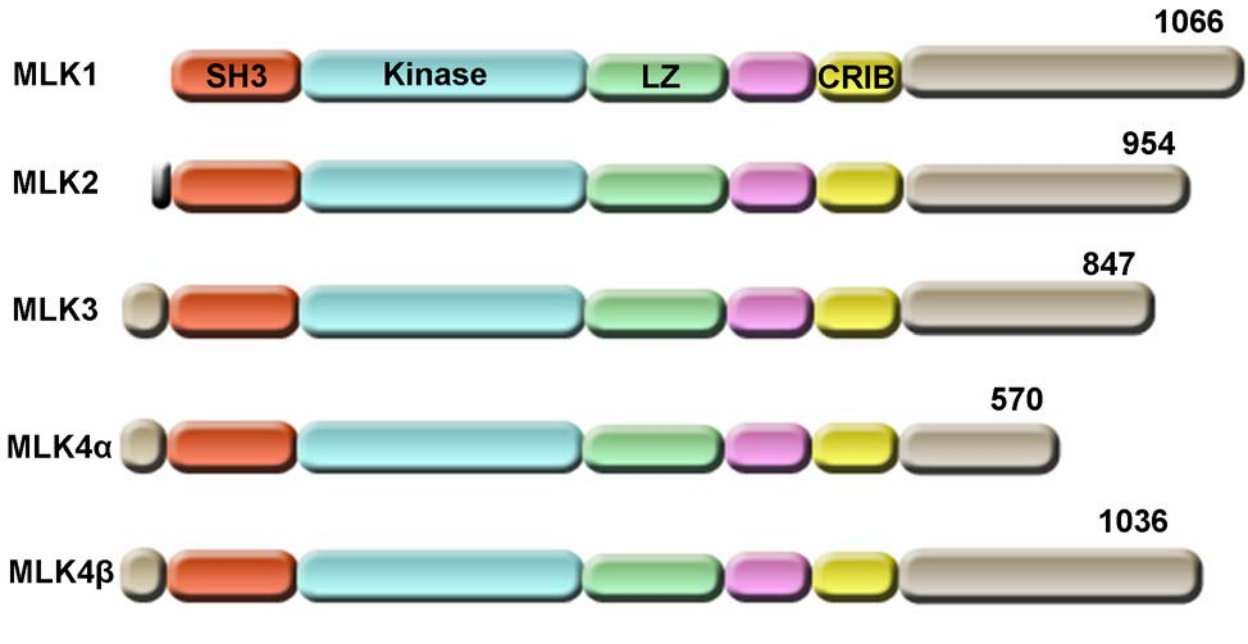


Figure 1.1. Conserved domains in the MLK subfamily. The domain arrangement within the MLK subfamily, depicting the relative positions of the Src-homology-3 (SH3), kinase, leucine-zipper (LZ) and Cdc42/Rac1 interactive binding (CRIB) motifs. The number of amino acids in each kinase is shown. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

## 5.1 Regulation of MLK3 activity

MLK3 contains several protein interaction domains that are important in regulating its activity. A short glycine-rich region is followed sequentially by a Src homology 3 (SH3) domain, a kinase catalytic domain, a leucine zipper region and a Cdc42/Rac Interactive Binding (CRIB) motif (Figure 1.1). The carboxyl terminal region of MLK3 is rich in proline, serine, and threonine residues (Gallo and Johnson 2002). Like many protein kinases, MLK3 activation involves phosphorylation within the activation loop of the kinase domain. Based on site-directed mutagenesis studies, Thr 277 and Ser 281 in the activation loop act as positive regulatory phosphorylation sites; and phospho-specific antibodies directed against these sites are widely used to monitor MLK3 activity. Notably these potential phosphorylation sites are conserved within the activation loops of MLK1-4, suggesting that they may serve a similar function. Leucine zippers form coiled coil dimers that are stabilized by the interaction of leucine or other nonaromatic aliphatic residues at the interface of the helices. Deletion of the entire zipper or introduction of a helix disrupting Pro residue for one of the conserved Leu residues prevents dimerization and activation loop phosphorylation of MLK3. Thus, leucine zipper-mediated dimerization can lead to MLK3 activation. These findings are consistent with the high MLK3 activity observed upon overexpression in many cultured cell lines. Presumably when MLK3 is expressed at high levels, a portion of MLK3 is dimerized in the absence of a physiologically appropriate stimulus, leading to high basal MLK3 activity (Gallo and Johnson 2002).

MLK3 contains a CRIB motif, a short conserved sequence required for binding the Rho family GTPases, Cdc42 and Rac. Subsequent work demonstrated that the small GTPases, Cdc42 and Rac, are indeed capable of activating MLK3. Like other GTPase effector proteins, MLK3 interacts with the active, GTP-bound GTPase, but not the inactive, GDP-bound form. Binding of activated Cdc42 (or Rac) promotes MLK3 dimerization, activation loop phosphorylation, increases MLK3 catalytic activity, and translocates MLK3 to the cell periphery (Du et al., 2005). Posttranslational COOH-terminal prenylation (geranylgeranylation) of Cdc42 and Rac allows for membrane targeting. A prenylation-defective site-directed mutant of activated Cdc42 retains the ability to bind MLK3 and promote activation loop phosphorylation of MLK3, but fails to translocate MLK3 to membranes. Thus, the physical interaction between the activated GTPase and MLK3 is the key mechanism by which it activates MLK3. However, membrane targeting by activated, prenylated Cdc42, is accompanied by additional phosphorylation events on MLK3 and further enhances MLK3 *in vitro* kinase and cellular signaling activities.

SH3 domains are modular domains of about 60 amino acids that typically bind proline-rich sequences to interact with intracellular signaling partners. The N-terminal SH3 domain of MLK3 functions as an autoinhibitory domain. A single Pro residue located between the leucine zipper and CRIB motif is required for the interaction with its SH3 domain. Though not formally shown, this autoinhibitory interaction is presumed to be intramolecular. Since this Pro residue is conserved in MLK1-4, it is likely that these MLKs are also regulated by SH3-mediated autoinhibition.

An integrated model for MLK3 activation by Cdc42 and Rac consistent with available data is shown in Figure 1.2. Binding of the active, GTP-bound GTPase to MLK3 in a region containing the CRIB motif disrupts the SH3 autoinhibitory interaction, promoting leucine zipper-mediated dimerization and trans autophosphorylation within the activation loop, yielding the active kinase at cell membranes (reviewed in Gallo and Johnson. 2002; Schachter et al., 2006).

In addition to phosphorylation within the activation loop, numerous MLK3 phosphorylation sites have been identified through mass spectrometry, the majority of which are Ser or Thr residues followed immediately by Pro residues, conforming to the consensus sequence for proline-directed kinases. The downstream MAPK, JNK, has been shown to phosphorylate MLK3 at multiple proline-directed kinase sites within the COOH terminal region in a positive feedback loop that stabilizes MLK3 and/or redistributes MLK3 into Triton-soluble cellular fractions. Ser 674 has been identified as an Akt (PKB)-mediated phosphorylation site on MLK3 that inhibits MLK3-mediated apoptosis (reviewed in Handley et al., 2007; Schachter et al., 2006). In contrast, Ser 789 and Ser 793 have been identified as sites for GSK-3beta-mediated phosphorylation on MLK3 that promote neuronal cell death (Mishra et al., 2007).

5.2 MLK3 signaling triggered by cell-surface receptors

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that are crucial for responding to extracellular stimuli and regulate various cellular activities including proliferation, differentiation, migration survival and apoptosis. MAPKs

Figure 1.2

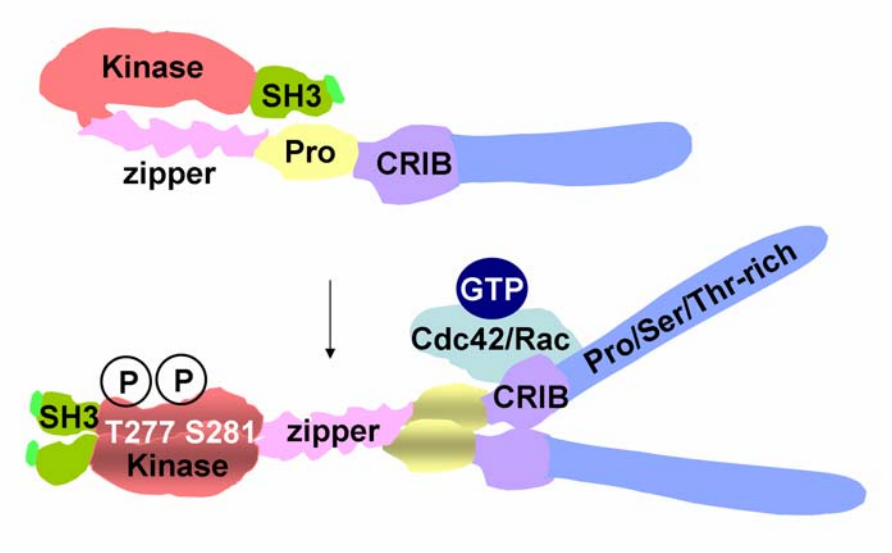


Figure1.2 Model for MLK3 activation by small GTPases, Cdc42 and Rac. Autoinhibition of MLK3 is maintained by an interaction between SH3 domain of MLK3 and a proline-containing sequence located between the LZ region and CRIB motif. The binding of GTP-bound GTPase(s) through the CRIB motif disrupts the autoinhibition, promotes dimerization and trans-autophosphorylation at Thr 277 and Ser 281 ensues.

are tightly controlled through a phosphorylation cascade in which a MAPK kinase kinase (MAPKKK) activates a MAPKK, which in turn activates a MAPK, leading to diverse cellular responses. In eukaryotic cells, the three best characterized MAPK subfamilies are extracellular signal regulated kinases (ERK), c-Jun NH2-terminal kinase (JNK), and p38.

The Raf/MEK/ERK pathway is the first identified and most widely studied MAP kinase cascade. In response to a variety of stimuli including growth factors and cytokines, MEK1/2 is phosphorylated and activated by MAPKKK including Raf and Mos. MEK1/2 then phosphorylates threonine and tyrosine residues in the Thr–Glu–Tyr (TEY) sequence in the activation loop of the catalytic domain of ERK1/2, resulting in the activation of ERK1/2. Activated ERK1/2 translocates to the nucleus and phosphorylates many substrates including transcription factors, such as Elk1, c-Myc and NF-κB, and other protein kinases, such as ribosomal S6 kinase (RSK) (Lewis et al., 1998). ERK also phosphorylates cytosolic substrates. The Raf/MEK/ERK pathway is involved in the regulation of many cellular processes, such as proliferation and differentiation (Marshall, 1995). ERK5, also known as big MAP kinase 1 (BMK1), is another member of the ERK family and shares the TEY activation motif found in ERK1/2. ERK5 is activated by MEKK2 and MEKK3, other MAPKKK members that signal to MEK5 (Chao et al., 1999; Sun et al., 2001). Subsequently, activated ERK5 phosphorylates substrates including myocyte enhancer factor 2 (MEF2) (Kato et al., 1997).

The JNK pathway represents one major group of MAP kinases that is activated primarily by inflammatory cytokines, environmental stress, such as heat shock, ultraviolet (UV), ionizing radiation, DNA damage as well as growth factors (Davis, 2000; Weston and Davis, 2002). In response to multiple stimuli, MAP3Ks including TAK, MLKs and ASK are activated and, in turn, phosphorylate and activate the MAP2K isoforms MKK4 and MKK7. MKK4 and MKK7, in turn, phosphorylate and activate JNKs. In addition, the JNK activation is also mediated by scaffold proteins, such as JIPs (Morrison and Davis, 2003). JNKs phosphorylate transcription factors c-Jun, ATF-2, p53, Elk-1, and nuclear factor of activated T cells (NFAT) (Chen et al., 2001). JNK can also phosphorylate cytoplasmic substrates including paxillin and beta-catenin (Lee et al., 2009). JNK isoforms are encoded by three genes. Jnk-1 and Jnk-2 are ubiquitously expressed, while the expression of Jnk-3 is restricted to certain tissues including the brain, heart and testis (Davis, 2000; Tournier et al., 2000).

The p38 MAPK pathway is activated in response to physical and chemical stresses, inflammatory cytokines and growth factors (Widmann, 1998). In response to cellular stress and cytokines, p38 MAPKs are activated through phosphorylation primarily by MAPK kinase 3 (MKK3) and MKK6. Substrates of p38 MAPK include protein kinases like MAPKAPKs and transcription factors such as ATF2 (Cuadrado and Nebreda, 2010). The four p38 MAPK isoforms are p38α, p38β, p38γ and p38δ and p38α is the best characterized isoform and regulates various cellular activities.

MLK3 signals through multiple MAPK pathways including JNK, p38, and ERK.

MLK3, also known as MAP3K11, activates the JNK pathway by phosphorylation and activation of MKK4/7, whereas it activates the p38 MAPK pathway through phosphorylation of activation of MKK3/6. Catalytic activity of MLK3 is required for activation of the JNK and p38 MAPK pathways, whereas MLK3 acts as an indispensable scaffold required for B-Raf mediated ERK activation (reviewed in Kyriakis 2007). Scaffold proteins, like the JIPs and POSH, are able to bind MLK3 as well as downstream MKKs and MAPKs, and may serve to localize, organize or assemble MLK3 pathway complexes at specific subcellular locations (reviewed in Dhanasekaran et al., 2007). Substantial evidence indicates that MLK3 can signal through multiple receptor types, including receptor tyrosine kinases, cytokine receptors, and G-protein coupled receptors (GPCRs) (Figure 1.3), but the details of these signaling pathways are largely unknown. TNF-α is the best-described stimulus for activating MLK3 signaling to JNK. The TNF-α receptor-associated factor 2 (TRAF2) complexes with both the receptor and MLK3 and is critical for TNF-α-induced JNK activation (Sondarva et al., 2010). It should be noted that other MAP3Ks, such as ASK, have also been implicated in TNF-α-induced JNK activation. Receptor tyrosine kinase signaling also involves MLK3, since silencing of MLK3 prevents EGF-induced activation of JNK, p38 and ERK (reviewed in Kyriakis 2007). MLK3 can be activated by stimulation of cells with carbachol, a chemical ligand for G protein-coupled acetylcholine receptors (Swenson-Fields et al., 2008). In this context, MLK3 complexes with the Rho GEF, p63RhoGEF to inhibit RhoA activation. Finally free fatty acids can activate MLK3 and signaling to JNK, implicating MLK3 in insulin resistance (Jaeschke et al., 2007).

Figure 1.3

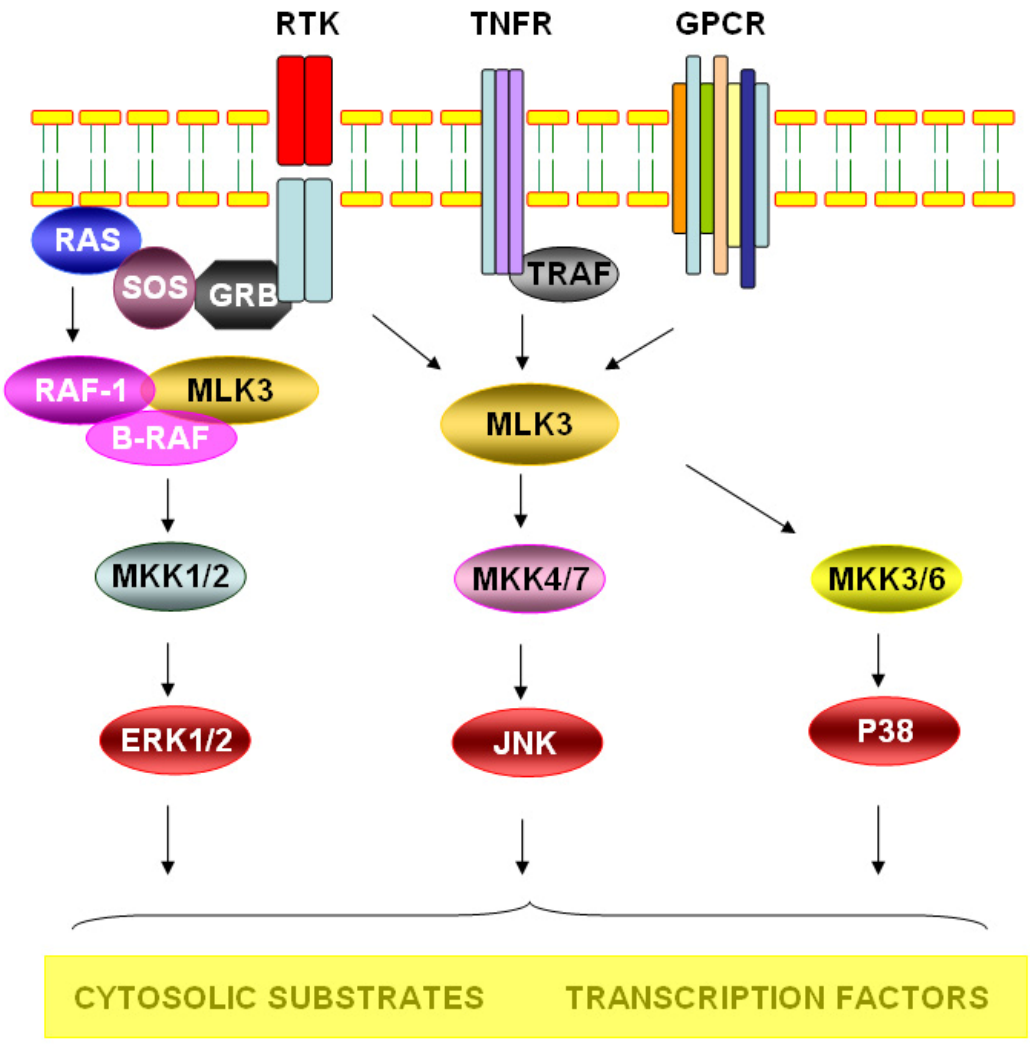


Figure 1.3 Role of MLK3 in signaling through cell surface receptors to MAPKs. MLK3-mediated signaling pathways downstream of various receptors are shown. EGFR, epidermal growth factor receptor; TNFR, tumor necrosis factor receptor; GPCR, G-protein coupled receptor. Activated MAPKs can phosphorylate cytosolic substrates or enter the nucleus to phosphorylate nuclear substrates, including transcription factors that regulate gene expression.

5.3 MLK3 is implicated in neurodegenerative diseases

Numerous neurotoxic insults can induce JNK activation and mitochondria-mediated apoptosis. MLK3 has been implicated as an upstream mediator of JNK in neuronal cell death in several experimental systems, primarily based on the use of dominant negative forms of MLK3 and the pan-MLK inhibitors, CEP-1347 and CEP-11004. For example, deprivation of nerve growth factor (NGF) induces JNK activation and apoptosis in PC12 cells and in cultured superior cervical rat ganglia, which can be attenuated by blocking MLK activity (reviewed in Wang et al., 2004). Studies in rodents support a role for MLK3 in apoptosis in response to cerebral ischemia-reperfusion, a model for stroke (Zhang et al, 2009). MLK3 has also been linked to JNK activation in kainate-induced neurotoxicity, with the scaffolding protein PSD-95 interacting with both the GluR6 receptor and MLK3 (reviewed in Gallo and Johnson, 2002). Finally substantial evidence has accumulated for MLK3 in JNK activation and neuronal death in cell-based and in vivo models of Parkinson's disease that use the dopaminergic selective neurotoxin MPTP or its derivative MPP+, respectively (reviewed in Wang et al., 2004). In response to treatment with MPTP/MPP+, the MLK inhibitor CEP-1347 was able to suppress JNK activation and increase survival of dopaminergic neurons. These studies suggested that CEP-1347 might be a promising therapeutic for treating patients with Parkinson's disease. CEP-1347 ultimately progressed to Stage II/III clinical trials, but failed to delay progression of patients with early stage Parkinson's disease (Parkinson Study Group PRECEPT Investigators, 2007).

## 5.4 MLKs in inflammation

MLK3 signaling is important in the production of pro-inflammatory proteins. In the context of interferon-gamma activated macrophages, MLK signals to p38 MAPK increasing the mRNA levels of TNF-α and interferon inducible protein 10, presumably through increased mRNA stability. In microglia activated by the bacterial endotoxin, lipopolysaccharide (LPS), an MLK or JNK inhibitor reduces AP-1 mediated transcription of TNF-α (reviewed in Handley et al., 2007). In primary cortical astrocytes, activated by a mixture of pro-inflammatory cytokines, MLK signaling to both p38 and JNK has been implicated in the induction of inflammation-responsive genes (Falsig et al., 2004).

Thus, there is considerable interest in MLK inhibitors as anti-inflammatory drugs, particularly in attenuating neuroinflammation associated with HIV infection. Recently, the MLK inhibitor, CEP-1347, was shown to prevent the production of cytokines and chemokines in HIV-infected human macrophages and to elicit antiinflammatory and neuroprotective effects in mouse models of HIV-1 encephalitis (Eggert et al., 2010).

## 5.5 MLK3 in cancer

Deregulation of signal transduction pathways drives development of human malignancies. MLK3 has been shown to be overexpressed in breast cancer cell lines compared with non-tumorigenic mammary epithelial cells, suggesting that MLK3 might contribute to acquisition of malignant phenotypes in breast cancer (Chen et al., 2010). Ectopic expression of wildtype MLK3 causes cellular transformation of immortalized fibroblasts (reviewed in Kyriakis, 2007) and promotes a malignant phenotype of

mammary epithelial spheroids in 3D culture (Chen et al., 2010). MLK3 silencing or inhibition can inhibit proliferation in some, but not all, tumor cell lines, perhaps depending upon the oncogenic signaling signature in those cells (reviewed in Schachter et al., 2006; Kyriakis, 2007).

Several lines of evidence demonstrate a critical role of MLK3 in migration and invasion of cancer cells of epithelial origin. Induced expression of MLK3 promotes migration of poorly invasive breast cancer cells and invasion of mammary epithelial cells (Chen et al., 2010). Small interfering RNA-mediated silencing of MLK3 blocks migration of highly invasive breast, lung, and gastric carcinoma cells, indicating an essential function for MLK3 in migration and/or invasion of a broad array of epithelial-derived tumor cells (Chen et al., 2010; Mishra et al., 2010; Swenson-Fields et al., 2008). A major mechanism by which MLK3 controls migration and invasion is through activation of JNK and its downstream transcription factor AP-1, leading to expression of genes that promote invasion or epithelial-to-mesenchymal transition, such as MMP-7, Fra-1, vimentin and N-cadherin (Chen et al., 2010; Mishra et al., 2010; Shintani et al., 2008). An AP-1-independent role in which MLK3 blocks activation of RhoA has been identified in lung carcinoma cells (Swenson-Fields et al., 2008). The inactivation of the Neurofibromatosis-2 tumor suppressor gene is associated with the formation of benign brain tumors. NF-2/merlin can interact with MLK3 to inhibit its activity, preventing proliferation and invasion of schwann cells (Zhan et al., 2011).

Missense mutations in *Mlk3* have been identified in human gastrointestinal cancers and are significantly associated with microsatellite instability (MSI) phenotype in mismatch repair-deficient gastrointestinal carcinomas. The identified mutations are

found in different functional domains of MLK3 including the SH3 domain (Y99C), the kinase domain (A165S; P252H) and the COOH-terminal proline-rich region (R799C; P840L). When ectopically expressed in fibroblasts, these mutant forms of MLK3 are more transforming and tumorigenic than wildtype MLK3 (Velho et al. 2010). How these specific mutations affect MLK3 signaling activities has yet to been determined.

The objective of the thesis is to study the role of MLK3 in breast cancer cell proliferation, migration, invasion and metastasis. The goal of this study is to elucidate how MLK3 and its signaling pathways contribute to the malignant phenotypes of breast cancer. The impact of MLK3 signaling on cell migration machinery is further investigated. Finally, the role of MLK3 signaling in breast cancer metastasis is studied using xenograft mouse model.

# REFERENCES

## REFERENCES


Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF, et al. Expression of luminal and basal cytokeratins in human breast carcinoma. *J. Pathol.* 2004; 203(2):661-71.

Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007; 7(11):834-46.

Barkan D, Green JE and Chambers AF. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. *Eur J Cancer.* 2010; 46(7):1181-8.

Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, et al. Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Res.* 2008; 68(15):6241-50.

Belguise K, Kersual N, Galtier F and Chalbos D. FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells. *Oncogene*. 2005; 24(8):1434-44.

Benbow, U and Brinckerhoff, C. The AP-1 site and MMP gene regulation: What is all the Fuss about? *Matrix Biol.* 1997; 15(8-9):519-26.

Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B, et al. Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res*. 1988; 48(5):1238–1243.

Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, et al. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J.* 1995; 14(24):6107-15.

Berx G and van Roy F. The E-cadherin/catenin complex: An important gatekeeper in breast cancer tumorigenesis and malignant progression. *Breast Cancer Res*. 2001; 3(5):289–293.

Bilancia D, Rosati G, Dinota A, Germano D, Romano R and Manzione L. Lapatinib in breast cancer. *Ann Oncol*. 2007; 18 Suppl 6:vi26-30.

Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. *Nature.* 2009; 459(7249):1005-9.

Bosc DG, Goueli BS and Janknecht R. HER2/Neu-mediated activation of the ETS transcription factor ER81 and its target gene MMP-1. *Oncogene.* 2001; 20(43):6215-24.

Cain RJ and Ridley AJ. Phosphoinositide 3-kinases in cell migration. *Biol Cell*. 2009;101(1):13-29.

Cairns J. Mutation selection and the natural history of cancer. *Nature.* 1975; 255(5505):197–200.
Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA*. 2006; 295(21):2492-502.

Chao TH, Hayashi M, Tapping RI, Kato Y and Lee JD. MEKK3 directly regulates MEK5 activity as part of the big mitogen-activated protein kinase 1 (BMK1) signaling pathway. *J Biol Chem*. 1999; 274(51): 36035–36038.

Chen J, Miller EM and Gallo KA. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. *Oncogene*. 2010; 29(31):4399-411.

Chen J, Yao Y, Gong C, Yu F, Su S, Chen J, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer Cell*. 2011; 19(4):541-55.

Chen Q, Zhang XH and Massagué J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invadethe lungs. *Cancer Cell*. 2011; 20(4):538-49.

Chen YC, Stamatoyannopoulos G and Song CZ. Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. *Cancer Res*. 2003; 63(16): 4801–4804.

Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, et al. MAP kinases. *Chem Rev*. 2001; 101(8): 2449–2476.

Cuadrado A and Nebreda AR. Mechanisms and functions of p38 MAPK signaling. *Biochem. J*. 2010; 429(3):403–417.

Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncol*. 2010; 11(12):1135-41.

Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell.* 2000; 103(2):239-252.

Derksen PW, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, et al. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell*. 2006; 10(5):437-49.

Dhanasekaran DN, Kashef K, Lee CM, Xu H and Reddy EP. Scaffold proteins of MAP-kinase modules. *Oncogene.* 2007; 26(22):3185-202.

Du Y, Böck BC, Schachter KA, Chao M and Gallo KA. Cdc42 induces activation loop phosphorylation and membrane targeting of mixed lineage kinase 3. *J Biol Chem.* 2005; 280(52):42984-93.

Egeblad M and Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer.* 2002; 2(3):161-74.

Eggert D, Dash PK, Gorantla S, Dou H, Schifitto G, Maggirwar SB, et al. Neuroprotective activities of CEP-1347 in models of neuroAIDS. *J. Immunology*. 2010; 184(2):746-56.

Elliott BE, Hung WL, Boag AH and Tuck AB. The role of hepatocyte growth factor (scatter factor) in epithelial-mesenchymal transition and breast cancer. *Can J Physiol Pharmacol.* 2002; 80(2): 91–102.

Falsig J, Pörzgen P, Lotharius J and Leist M. Specific modulation of astrocyte inflammation by inhibition of mixed lineage kinases with CEP-1347. *J. Immunology*. 2004; 173(4): 2762-70.

Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature.* 2005; 434(7035):917-21.

Fidler IJ. Metastasis: quantitative analysis of distribution and fate of tumor embolilabeled with 125 I-5-iodo-2'-deoxyuridine. *J Natl Cancer Inst.* 1970; 45(4):773-82.

Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer*. 2003; 3(6):453-8.

Foulkes WD, Smith IE and Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med.* 2010; 363(20):1938-48.

Foulkes WD, Stefansson IM, Chappuis PO, Bégin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J. Natl Cancer Inst*. 2003; 95(19):1482-5.

Friedl P and Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer*. 2003; 3(5):362-74.

Friedl P and Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell.* 2011; 147(5):992-1009.

Friedl P, Hegerfeldt Y and Tusch M. Collective cell migration in morphogenesis and cancer. *Int J Dev Biol.* 2004; 48(5-6):441-9.

Frisch SM and Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol*. 2001; 13(5):555-62.

Gallo KA and Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol*. 2002; 3(9):663-72.

Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med*. 2006; 355(26):2733–2743.

Gocheva V and Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle*. 2007; 6(1):60-4.

Greco FA and Hainsworth JD. Introduction: unknown primary cancer. *Semin. Oncol*. 2009; 36(1): 6–7.

Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, et al. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature*. 2007; 446(7137):765-70.

Haenssen KK, Caldwell SA, Shahriari KS, Jackson SR, Whelan KA, Klein-Szanto AJ, et al. ErbB2 requires integrin alpha5 for anoikis resistance via Src regulation of receptor activity in human mammary epithelial cells. *J Cell Sci.* 2010; 123(Pt 8):1373-82.

Hajra KM, Chen DY and Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res*. 2002; 62(6):1613-8.

Han HJ, Russo J, Kohwi Y and Kohwi-Shigematsu T. SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. *Nature*. 2008; 452(7184):187-93.

Hanahan D and Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011; 144(5):646-74.

Handley ME, Rasaiyaah J, Chain BM and Katz DR. Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity? *Int J Exp Pathol*. 2007; 88(2):111-26.

Hardy KM, Booth BW, Hendrix MJ, Salomon DS and Strizzi L. ErbB/EGF signaling and EMT in mammary development and breast cancer. *J Mammary Gland Biol Neoplasia*. 2010; 15(2):191-9.

Hazan RB, Phillips GR, Qiao RF, Norton L and Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol*. 2000; 148(4):779-90.

Heasman SJ and Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol*. 2008; 9(9):690-701.

Hellman S. Karnofsky Memorial Lecture. Natural history of small breast cancers. *J Clin Oncol*. 1994; 12(10):2229-34.

Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet*. 2005; 365(9453):60-2.

Hulit J, Suyama K, Chung S, Keren R, Agiostratidou G, Shan W, et al. N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation. *Cancer Res*. 2007; 67(7):3106-16.

Hüsemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. 2008; 13(1):58-68.

Hynes NE and Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*. 2005; 5(5):341-54.

Jaeschke A and Davis RJ. Metabolic stress signaling mediated by mixed-lineage kinases. *Mol Cell*. 2007; 27(3):498-508.

Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. *J Clin Invest*. 2006; 116(6):1561-70.

Jedeszko C, Victor BC, Podgorski I and Sloane BF. Fibroblast hepatocyte growth factor promotes invasion of human mammary ductal carcinoma in situ. *Cancer Res*. 2009; 69(23):9148-55.

Jeong H, Ryu YJ, An J, Lee Y and Kim A. Epithelial-mesenchymal transition in breast cancer correlates with high histological grade and triple-negative phenotype. *Histopathology*. 2012; 60(6B):E87-E95.

Joyce JA and Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009; 9(4):239-52.

Kalluri R and Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006; 6(5):392-401.

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell.* 2003; 3(6):537-49.

Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci U S A*. 2005; 102(39):13909-14.

Karin M, Liu Z and Zandi E. AP-1 function and regulation. *Curr Opin Cell Biol*. 1997; 9(2):240-6.

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ and Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* 1997; 16(23):7054–7066.

Ke Z, Lin H, Fan Z, Cai TQ, Kaplan RA, Ma C, et al. MMP-2 mediates ethanol-induced invasion of mammary epithelial cells over-expressing ErbB2. *Int J Cancer*. 2006; 119(1):8-16.

Kennedy RD, Quinn JE, Mullan PB, Johnston PG and Harkin DP. The role of BRCA1 in the cellular response to chemotherapy. *J Natl Cancer Inst*. 2004; 96(22):1659-1668.

Kim HJ, Litzenburger BC, Cui X, Delgado DA, Grabiner BC, Lin X, et al. Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-kappaB and snail. *Mol Cell Biol*. 2007; 27(8):3165-75.

Klein CA. Parallel progression of primary tumours and metastases. *Nat. Rev. Cancer*. 2009; 9(4):302–312.

Kyriakis JM. The integration of signaling by multiprotein complexes containing Raf kinases. *Biochim Biophys Acta*. 2007; 1773(8):1238-47.

Lazennec G and Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med*. 2010; 16(3):133-44.

Ledford H. Cancer theory faces doubts. *Nature*. 2011; 472(7343):273.

Lee JM, Dedhar S, Kalluri R and Thompson EW. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol*. 2006; 172(7):973-81.

Lee MH, Koria P, Qu J and Andreadis ST. JNK phosphorylates beta-catenin and regulates adherens junctions. *FASEB J*. 2009; 23(11):3874-83.

Lee YT. Breast carcinoma: pattern of metastasis at autopsy. *J Surg Oncol*. 1983; 23(3):175-80.

Lewis TS, Shapiro PS and Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res.* 1998; 74: 49–139.

Li CW, Xia W, Huo L, Lim SO, Wu Y, Hsu JL, et al. Epithelial-mesenchymal transition induced by TNF-α requires NF-κB-mediated transcriptional upregulation of Twist1. *Cancer Res*. 2012; 72(5):1290-300.

Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT et al. Phenotypic evaluation of the basallike subtype of invasive breast carcinoma. *Mod Pathol*. 2006; 19(2):264-71.

Marshall, CJ. Specificity of receptor tyrosine kinase signal ing: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 1995; 80(2):179–185.

Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer*. 2006; 6(6):449-58.

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005; 436(7050):518-24.

Mishra P, Senthivinayagam S, Rangasamy V, Sondarva G and Rana B. Mixed lineage kinase-3/JNK1 axis promotes migration of human gastric cancer cells following gastrin stimulation. *Mol Endocrinol*. 2010; 24(3):598-607.

Mishra R, Barthwal MK, Sondarva G, Rana B, Wong L, Chatterjee M, et al. Glycogen synthase kinase-3beta induces neuronal cell death via direct phosphorylation of mixed lineage kinase 3. *J Biol Chem*. 2007; 282(42):30393-405.

Morrison DK and Davis RJ: Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu Rev Cell Dev Biol*. 2003; 19:91-118.

Wozniak MA, Modzelewska K, Kwong L and Keely PJ. Focal adhesion regulation of cell behavior. *Biochim Biophys Acta*. 2004; 1692(2-3):103-19.

Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001; 410(6824):50-6.

Muller WJ, Sinn E, Pattengale PK, Wallace R and Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell.* 1988; 54(1):105-15.

Muthuswamy SK, Li D, Lelievre S, Bissell MJ and Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol*. 2001; 3(9):785-92.

Nguyen DX, Bos PD and Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer*. 2009; 9(4):274-84.

Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res*. 2004; 10(16):5367-74.

Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayashi T, et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.* 1993; 53(7):1696-701.

Onder TT, Gupta PB, Mani SA, Yang J, Lander ES and Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res*. 2008; 68(10):3645-54.

Oskarsson T, Acharyya S, Zhang XH, Vanharanta S, Tavazoie SF, Morris PG, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. 2011; 17(7):867-74.

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, et al. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell*. 2008; 133(1):66-77.

Pantel K, Brakenhoff RH, and Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat. Rev. Cancer.* 2008; 8(5):329–340.

Parkinson Study Group PRECEPT Investigators. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. *Neurology*. 2007; 69(15):1480-90.

Perentes JY, Kirkpatrick ND, Nagano S, Smith EY, Shaver CM, Sgroi D, et al. Cancer cell-associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors. *Cancer Res*. 2011; 71(13):4527-38.

Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature*. 2000; 406(6797): 747–752.

Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-postive breast cancer. *N. Engl. J. Med*. 2005; 353 (16):1659–1672.

Pinkas J, Martin SS and Leder P. Bcl-2-mediated cell survival promotes metastasis of EpH4 βMEKDD mammary epithelial cells. *Mol Cancer Res.* 2004; 2(10):551-6.

Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, and Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Scienc*e. 2008; 321(5897):1841–1844.

Qian BZ and Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010; 141(1):39-51.

Rafn B, Nielsen CF, Andersen SH, Szyniarowski P, Corcelle-Termeau E, Valo E, et al. ErbB2-Driven Breast Cancer Cell Invasion Depends on a Complex Signaling Network Activating Myeloid Zinc Finger-1-Dependent Cathepsin B Expression. *Mol Cell*. 2012; 45(6):764-76.

Raftopoulou M and Hall A. Cell migration**:** Rho GTPases lead the way. *Dev Biol.* 2004; 265(1):23-32.

Rakha EA, Reis-Filho JS and Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol*. 2008; 26(15):2568-81.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. *Science*. 2003; 302(5651):1704-9.

Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N. Engl. J. Med*. 2005; 353(16):1673–1684.

Rørth P. Collective cell migration. *Annu Rev Cell Dev Biol*. 2009; 25:407-29.

Roussos ET, Condeelis JS and Patsialou A. Chemotaxis in cancer. *Nat Rev Cancer*. 2011; 11(8):573-87.

Sánchez-Tilló E, Lázaro A, Torrent R, Cuatrecasas M, Vaquero EC, Castells A, et al. ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene*. 2010; 29(24):3490-500.

Sankpal NV, Mayfield JD, Willman MW, Fleming TP and Gillanders WE. Activator protein 1 (AP-1) contributes to EpCAM-dependent breast cancer invasion. *Breast Cancer Res*. 2011; 13(6):R124.

Schachter KA, Liou GY, Du Y and Gallo KA. Mlk3. *UCSD Nature Molecule Pages*. 2006.

Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature*. 2009; 461(7260):109-13.

Scotton CJ, Wilson JL, Milliken D, Stamp G and Balkwill FR. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res*. 2001; 61(13):4961–4965.

Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature*. 2007; 445(7126):437-41.

Shaulian E and Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 2002; 4(5):E131-6.

Shi Y and Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*. 2003; 113(6):685-700.

Shibue T and Weinberg RA. Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci U S A*. 2009; 106(25):10290-5.

Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ and Johnson KR. Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. *J Cell Biol*. 2008; 180(6):1277-89.

Siegel R, Naishadham D and Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012; 62(1):10-29.

Simon R, Panussis S, Maurer R, Spichtin H, Glatz K, Tapia C, et al. KIT (CD117)-positive breast cancers are infrequent and lack KIT gene mutations. *Clin Cancer Res.* 2004; 10(1Pt1):178-183.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER2/neu oncogene. *Science*. 1987; 235(4785):177-82.

Smit MA, Geiger TR, Song JY, Gitelman I and Peeper DS. A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis. *Mol Cell Biol*. 2009; 29(13):3722-37.

Sondarva G, Kundu CN, Mehrotra S, Mishra R, Rangasamy V, Sathyanarayana P, et al. TRAF2-MLK3 interaction is essential for TNF-alpha-induced MLK3 activation. *Cell Res*. 2010; 20(1):89-98.

Sørlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl Acad. Sci. USA.* 2003; 100(14):8418-23.

Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. *Semin Cancer Biol*. 2000; 10(6):415-33.

Sun W, Kesavan K, Schaefer BC, Garrington TP, Ware M, Johnson NL, et al. MEKK2 associates with the adapter protein Lad/RIBP and regulates the MEK5–BMK1/ERK5 pathway. *J Biol Chem.* 2001; 276(7): 5093–5100.

Suyama K, Shapiro I, Guttman M and Hazan RB. A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell*. 2002; 2(4):301-14.

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K, et al. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. *Mol Cell*. 2008; 32(1):43-56.

Thiery JP, Acloque H, Huang RY and Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009; 139(5):871-90.

Tournier C, Hess P, Yang D, Xu J, Turner TK, Nimnual A, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science.* 2000; 288(5467): 870-874.

Turner N, Tutt A and Ashworth A: Targeting the DNA repair defect of BRCA tumours. *Curr Opin Pharmacol*. 2005; 5(4):388-393.

Ursini-Siegel J, Schade B, Cardiff RD and Muller WJ. Insights from transgenic mouse models of ERBB2-induced breast cancer. *Nat. Rev. Cancer*. 2007; 7(5):389-97.

van de Rijn M, Perou CM, Tibshirani R, Haas P, Kallioniemi O, Kononen J, et al. Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. *Am. J. Pathol*. 2002; 161(6):1991-6.

van Roy F and Berx G. The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci*. 2008; 65(23):3756-88.

van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002; 415(6871):530-6.

Vega FM and Ridley AJ. Rho GTPases in cancer cell biology. *FEBS Lett*. 2008; 582(14):2093-101.

Velho S, Oliveira C, Paredes J, Sousa S, Leite M, Matos P, et al. Mixed lineage kinase 3 gene mutations in mismatch repair deficient gastrointestinal tumours. *Hum Mol Genet*. 2010; 19(4):697-706.

Vitolo MI, Weiss MB, Szmacinski M, Tahir K, Waldman T, Park BH, et al. Deletion of PTEN promotes tumorigenic signaling, resistance to anoikis, and altered response to chemotherapeutic agents in human mammary epithelial cells. *Cancer Res*. 2009; 69(21):8275-83.

Wang LH, Besirli CG and Johnson EM Jr. Mixed-lineage kinases: a target for the prevention of neurodegeneration. *Annu Rev Pharmacol Toxicol*. 2004; 44:451-74.

Weigelt B, Peterse JL and van 't Veer LJ. Breast cancer metastasis: markers and models. *Nat Rev Cancer*. 2005; 5(8):591-602.

Westermarck J and Kähäri VM. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J*. 1999; 13(8):781-92.

Weston CR and Davis RJ: The JNK signal transduction pathway. *Curr Opin Genet Dev.* 2002; 12(1):14-21.

Widmann C, Gibson S and Johnson GL. Caspase-dependent cleavage of signaling proteins during apoptosis. *J. Biol. Chem*. 1998; 273(12):7141–7147.

Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, et al. Compensation mechanism in tumor cell migration: mesenchymal–amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol*. 2003; 160(2):267-77.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004; 117(7):927-39.

Yarden Y and Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Bio*l. 2001; 2(2):127-37.

Zhan Y, Modi N, Stewart AM, Hieronimus RI, Liu J, Gutmann DH, et al. Regulation of mixed lineage kinase 3 is required for Neurofibromatosis-2-mediated growth suppression in human cancer. *Oncogene*. 2011; 30(7):781-9.

Zhang Q-G, Wang R, Hana D, Dong Y and Branna DW. Role of Rac1 GTPase in JNK signaling and delayed neuronal cell death following global cerebral ischemia. *Brain Research*. 2009; 1265:138-147.

Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, et al. FOXP3 Is an X-Linked Breast Cancer Suppressor Gene and an Important Repressor of the HER-2/ErbB2 Oncogene. *Cell*. 2007; 129(7):1275-86.

# CHAPTER II

# MLK3 IS CRITICAL FOR BREAST CANCER CELL MIGRATION AND PROMOTES A MALIGNANT PHENOTYPE IN MAMMARY EPITHELIAL CELLS

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## Abstract

The malignant phenotype in breast cancer is driven by aberrant signal transduction pathways. Mixed-lineage kinase 3 (MLK3) is a mammalian mitogen-activated protein kinase kinase kinase (MAP3K) that activates multiple MAPK pathways. Depending upon the cellular context, MLK3 has been implicated in apoptosis, proliferation, migration, and differentiation. Here we investigated the impact of MLK3 and its signaling to MAPKs in the acquisition of malignancy in breast cancer. We show that MLK3 is highly expressed in breast cancer cells. We provide evidence that MLK3 catalytic activity and signaling to c-Jun N-terminal kinase (JNK) is required for migration of highly invasive breast cancer cells and for MLK3-induced migration of mammary epithelial cells. Expression of active MLK3 is sufficient to induce invasion of mammary epithelial cells, which requires AP-1 activity and is accompanied by the expression of several proteins corresponding to AP-1-regulated invasion genes. To assess MLK3's contribution to the breast cancer malignant phenotype in a more physiological setting, we implemented a strategy to inducibly express active MLK3 in preformed acini of MCF10A cells grown in 3D Matrigel. Induction of MLK3 expression dramatically increases acinar size and modestly perturbs apico-basal polarity. Remarkably, MLK3 expression induces luminal repopulation and suppresses expression of the pro-apoptotic protein BimEL, as has been observed in Her2/Neu-expressing acini. Taken together, our data demonstrate that MLK3-JNK-AP1 signaling is critical for breast cancer cell migration and invasion. Our current study reveals both a proliferative role and a novel antiapoptotic role for MLK3 in acquisition of a malignant phenotype in

mammary epithelial cells. Thus, MLK3 may be an important therapeutic target for the treatment of invasive breast cancer.

## Introduction

Invasive ductal carcinoma accounts for 70% of invasive breast cancer. Cancer cell migration is fundamentally required for breast tumor invasion and metastasis. The migratory phenotype afforded to breast cancer cells is thought to be accompanied by a transient epithelial-to-mesenchymal transition (EMT) in which genes associated with migration and invasion are upregulated. Malignant breast cancer cells must also bypass normal growth arrest, evade apoptosis, and gain limitless replicative potential (Hanahan and Weinberg, 2000). These alterations are not always cancer cell autonomous. Interactions of breast cancer cells with extracellular matrix components and other cell types clearly influence phenotypic outcome.

Aberrant signal transduction pathways within tumor cells govern malignant processes. Delineating the signaling pathways that contribute to the acquisition of the malignant phenotype in breast cancer is critical for developing interventional strategies. Of the numerous proteins that have been implicated in breast cancer, kinases have emerged as excellent therapeutic targets (Zhang *et al.*, 2009), as evidenced by the use of the monoclonal antibody, Trastuzumab, and the small molecule, ATP-mimetic, HER2/EGFR dual receptor tyrosine kinase inhibitor, lapatinib, in treating many patients with HER2-positive breast cancer (Nielsen *et al.*, 2009).

MAPK pathways respond to diverse extracellular stimuli to regulate cellular processes including proliferation, differentiation, migration, survival and apoptosis

(Johnson and Lapadat, 2002). The three best-characterized mammalian MAPKs are extracellular signal-regulated kinase (ERK), JNK, and p38. MAPKs have numerous cellular substrates including transcription factors. For instance, JNK activates the heterodimeric transcription factor, AP-1, by phosphorylation of one of its components, c-jun. While the number of MAPK isoforms is rather limited, there are many more MAP3Ks, suggesting that MAP3Ks may function as key nodes for integrating diverse extracellular stimuli with MAPK signaling. MLK3 is a widely-expressed MAP3K that contributes to the activation of multiple MAPK pathways. MLK3 activates JNK and, in some experimental settings, also the p38 pathway, by phosphorylating the activation segment of the cognate MAP2K (Gallo and Johnson, 2002). MLK3 apparently acts as a scaffold for the MAP3K, B-Raf, that is required for B-Raf-mediated ERK activation (Chadee and Kyriakis, 2004).

MLK3, with a predicted molecular weight of 92 kDa, contains an N-terminal *src*-homology 3 (SH3) domain, followed by a kinase catalytic domain with similarity to both serine/threonine and tyrosine kinases, leucine zipper regions, a Cdc42/Rac interactive binding (CRIB) motif, and a large C-terminal tail rich in Ser, Thr and Pro residues (Gallo *et al*., 1994; Gallo and Johnson, 2002). We previously discovered that MLK3 is autoinhibited through its SH3 domain (Zhang and Gallo, 2001). Activated Cdc42/Rac increases MLK3 catalytic activity (Teramoto *et al*., 1996) through binding the CRIB motif (Bock *et al.*, 2000) and promoting activation loop (auto) phosphorylation (Du *et al.*, 2005). Based on work from our lab and from others (Leung and Lassam, 1998; Vacratsis and Gallo, 2000; Nihalani *et al*., 2001), we propose that binding of the small GTPase, Cdc42 or Rac, disrupts SH3-mediated autoinhibition, promoting leucine

zipper-mediated dimerization and subsequent transphosphorylation within the catalytic domain to yield active MLK3.

Given its potential for diverse signaling, we undertook an investigation of the role of MLK3 in human breast cancer. Using gene silencing, as well as an MLK inhibitor, we demonstrate a critical requirement for MLK3 in migration of invasive breast cancer cells. In complementary experiments, induced expression of active MLK3 is shown to promote migration of poorly invasive breast cancer and non-tumorigenic mammary epithelial cells. Our data indicate that MLK3 signaling to JNK is critical for breast cancer cell migration. In addition, MLK3 induces the expression of c-jun, Fra-1, and vimentin, all of which have been associated with EMT and invasive breast cancer cells. We further demonstrate that MLK3 promotes invasion in an AP-1 dependent manner. To mimic how aberrant MLK3 signaling might impact normal mammary ductal epithelium, active MLK3 was induced in preformed MCF10A mammary acini grown in a 3D Matrigel culture system. MLK3 induction causes the mammary acini to bypass growth arrest, increase in size, and reinitiate luminal filling. Thus, in the physiological context of 3D Matrigel culture, MLK3 promotes acquisition of key aspects of the malignant phenotype in mammary acini that, in some respects, mimic the pathological findings in DCIS and invasive breast cancer.

## Experimental procedures

*Chemicals, Antibodies, siRNAs, and DNA constructs*- Antibodies against phospho-ERK (T202/Y204), phospho-JNK (T183/Y185), phospho-p38 (T180/Y182), phospho-MLK3 (T277/S281), and phospho-MAPKAPK2 (Thr334) were from Cell Signaling

Biotechnology, Inc. (Danvers, MA, USA); phospho-c-Jun (S63), c-Jun, ERK1/2, JNK1/2, p38 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Ki-67 was from Zymed (San Francisco, CA, USA); GM130 was from BD Biosciences (San Jose, CA, USA); Bim antibody was from Assay Designs/Stressgen (Ann Arbor, MI, USA); and vimentin was from Abcam (Cambridge, UK). The Flag M2 and actin monoclonal antibodies were from Sigma-Aldrich (St Louis, MO, USA). Additional antibodies were the MLK3 antibody (homemade), horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA), fluorescent secondary antibodies: IRDye 800CW goat anti-mouse and IRDye 680 goat anti-rabbit IgG (Li-COR Biosciences, Lincoln, NE, USA). Inhibitors SP600125, U0126 and SB203580 were purchased from Calbiochem (San Diego, CA, USA); and CEP-11004 was generously provided by Cephalon (West Chester, PA, USA). Mlk3 siRNA was from Invitrogen (Carlsbad, CA, USA); control siRNA was from Dharmacon (Lafayette, CO, USA); and c-jun siRNA (5'-CUGCUCAUCUGUCACGU-UCTT-3') was from Qiagen (Valencia, CA, USA). AP21967 was generously provided by Ariad Pharmaceuticals (Cambridge, MA, USA). Oligonucleotides for short hairpin RNAs targeting human MLK3 5'-GATCCGGGCAGTGACGTCTCCAGTTTTTCAAGAGAAAACTCCAG-ACGTCACTGCTTTTTA-3' and 5'-AGCTTAAAAAGCAGTGACGTCTGGAGTTT-TCTCTTGAAAAACTCCAGACGTCACTGCGGG-3' (derived from the siRNA sequence as previously described (Chadee and Kyriakis, 2004), were designed using OligoEngine 2.0, annealed and subcloned into pSuper-retro vector (OligoEngine, Inc., Seattle, WA, USA). Construction of the inducible vector encoding Flag-tagged MLK3, pLH-Z12I-PL2-MLK3, was previously described (Zhang et al., 2004).

*Cell Lines, Cell Culture, Transfection and Lysis*- Human mammary epithelial and breast cancer cell lines were from ATCC (Manassas, VA, USA). MCF-7 cells engineered to inducibly express MLK3 (MCF-7-MLK3) were previously described (Zhang et al., 2004). MCF10A cells were maintained as previously described (Debnath et al., 2003). A population of MCF10A cells was engineered to inducibly express MLK3 (MCF10A-MLK3) or an empty vector control. MCF10A cells were infected with retrovirus/VSV-G pseudotypes produced in the 293GPG packaging cell line (a gift from R. Mulligan, Harvard Medical School, Children's Hospital, Boston, MA, USA; (Ory et al., 1996)) containing the pL2N2-RHS3H-ZF3 transcriptional regulation vector (Ariad Pharmaceuticals, Cambridge, MA, USA). Cells were selected in 300 μg/ml G418 and clones were isolated, infected with pLH-Z12I-PL2 -MLK3-containing retrovirus and selected in 50 μg/ml hygromycin. Hygromycin-selected MCF10A-MLK3 populations were induced with vehicle (ethanol) or 50 nM AP21967 (Ariad) and screened by immunoblotting for robust inducible expression and minimal background. The corresponding empty vector control (pLH-Z12I-PL2) was generated for the selected population. Breast cancer cell lines were cultured in DMEM (Gibco BRL, Paisley, PA, USA) with 10% FBS. Transfection of siRNA (Mlk3-specific siRNA (10 nM), c-jun-specific siRNA (100 nM) or a universal control siRNA) was performed using InterferIN (Polyplus-transfection, New York, NY) according to the manufacturer's instructions. Cells were lysed as previously described (Bock et al., 2000). Protein concentrations of the lysates were determined by the Bradford method (Bio-Rad). For detection of MLK3 protein levels in human mammary epithelial cells and breast cancer cells, cells were harvested and lysed in RIPA Buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-

40, 0.1% SDS) containing protease inhibitors. Protein concentrations of the lysates were determined by the BCA protein assay (Pierce, Rockford, IL, USA). For detection of proteins in MCF10A cells grown in 3D culture, cells were lysed by combining the Matrigel culture with an equal volume of RIPA lysis buffer containing protease inhibitors and passing through a 25-gauge needle; lysates were clarified by centrifugation at 14,000 rpm for 15 min prior to immunoblotting (Reginato et al., 2005).

*Gel electrophoresis and Western blot Analysis*- Cellular lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes and immunoblotted using appropriate antibodies followed by horseradish peroxidase-conjugated or IRDye-conjugated secondary antibody and developed by chemiluminescence method or visualized by fluorescence (Li-COR Biosciences), respectively.

*Transwell migration and invasion assays*- Chemotactic migration or invasion was quantified using a Boyden chamber transwell assay (8 μm pore size, Corning Costar, Cambridge, MA, USA) with uncoated or Matrigel-coated filters, respectively. Cells were deprived of serum overnight, trypsinized and introduced into the upper chamber ($5x10^4$ for MDA-MB-231, MDA-MB-435, Hs578T, 6h; $10^5$ for MCF-7-MLK3 and MCF10A-MLK3, 24h). For 24 h assays, Mitomycin C was included. The chemoattractant in the lower chamber was medium supplemented with 5% FBS (MDA-MB-231, MDA-MB-435, and Hs578T), 10% FBS (MCF-7-MLK3) or 2% horse serum (MCF10A-MLK3). Cells were fixed and stained as described (Goicoechea et al., 2009). Migrated cells in five randomly chosen fields were counted. Experiments were performed in triplicate wells and each experiment was performed at least 2 or 3 times as indicated.

*Wounding healing assay*- MDA-MB-231 cells and derivatives were grown to confluence. Growth medium was replaced with fresh medium supplemented with Mitomycin C (1 µg/ml) and the monolayer of cells was scratched using a 200 µl pipette tip. Wound width was monitored over time by microscopy. Percentage wound recovery was expressed as a [1-(width of the wound at a given time/width of the wound at t=0)]x100%.

*Three-dimensional morphogenesis assay*- A single cell suspension of 5000 cells was seeded per well on solidified Matrigel (BD Biosciences) in overlay media ((Debnath et al., 2003; Lee et al., 2007) (DMEM/F12 supplemented with 2% horse serum; 0.1 ng/ml or 5 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA); 10 µg/ml insulin; 100 µg/ml hydrocortisone; 1 ng/ml cholera toxin; 50 U/ml streptomycin/penicillin and 3% Matrigel (BD Biosciences, San Jose, CA, USA). After formation of mature acini, at day 10, MLK3 expression was induced with 50 nM AP21967 and cultures were assessed on day 20. Cultures were replenished with fresh medium every four days (Debnath et al., 2003; Lee et al., 2007). Phase contrast images were acquired with QCapturePro. All immunofluorescence procedures were done as previously described (Debnath et al., 2003) for antibodies against Ki-67, GM130 and vimentin. Nuclei were stained with 5 µg/ml DAPI (4', 6-diamidino-2-phenylindole) and cells were mounted with anti-fade reagent Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Fluorescence microscopy was performed on a Nikon Eclipse TE 2000 (for Ki-67 and vimentin) and on an Olympus Fluoview laser scanning microscope (for GM130). Acinar structures at day 20 were analyzed in Metamorph for size distribution, by digitally tracing the circumference of acini and expressing the cross sectional area as pixels squared. For proliferation, structures from each condition were counted; percent Ki67-positivity was

based on an acinus having one or more Ki-67-positive cells. Bar graphs were created in MS Excel and box plots were created using the "R" statistics package, version 2.8.1.

*Proliferation assay*- For MDA-MB-231 cells, 5000 cells per well were plated in 96-well microplate on Day 0. On Day 1 and 6, CCK-8 reagent was added to cells and absorbance at 450 nm was measured in a plate reader after 2 h of incubation, following the manufacturer's instructions (Dojindo Molecular Technologies, Rockville, MD, USA). For MCF10A-MLK3 cells, 15000 cells per well were seeded in 24-well plate.

## Results

**MLK3 is required for migration of highly invasive breast cancer cells.** Total cellular lysates were generated from a panel of human mammary epithelial and breast cancer cell lines and analyzed by western blotting with an MLK3-specific antibody. As shown in Figure 2.1, higher levels of MLK3 protein were observed in the breast cancer cells lines, MCF-7, T47D, SK-BR-3 and MDA-MB-231, compared to the nontumorigenic mammary epithelial cell lines, 184B5 and MCF10A. No apparent correlation was found between MLK3 protein levels and steroid hormone receptor status or metastatic potential.

To probe the function(s) of MLK3 in breast cancer, a gene silencing approach was taken. An Mlk3-shRNA based on a previously validated siRNA (Chadee and Kyriakis, 2004) was constructed in the pSuper vector and introduced into MDA-MB-231 cells using retrovirus. Stable populations of MDA-MB-231 cells containing pSuper-Mlk3 shRNA or control vector pSuper were selected and subjected to proliferation and transwell migration assays. MLK3 knockdown was highly efficient as judged by western

Figure 2.1

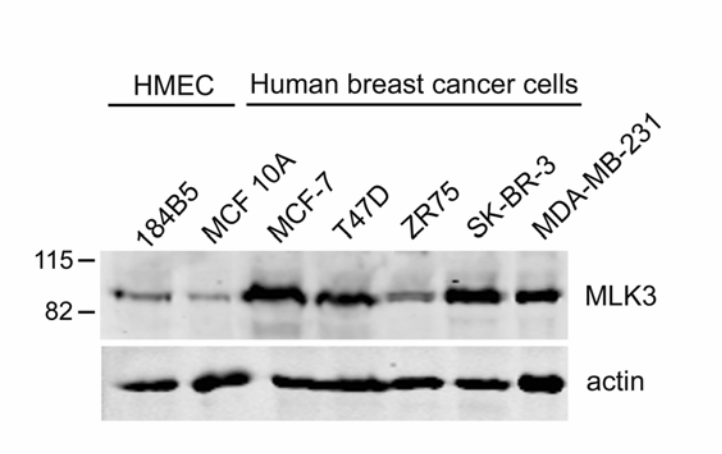



Figure 2.1 MLK3 levels in human nontumorigenic mammary epithelial cells and breast cancer cells. Cellular lysates containing 30 μg of total protein from each of the indicated cell lines were subjected to western-blot analysis using an MLK3 antibody. Western blot of actin serves as a loading control. Numbers to the left of the MLK3 western blot indicate mobility of molecular weight markers in kDa.

blotting (Figure 2.2a). MLK3 knockdown had no effect on proliferation of MDA-MB-231 cells in 2D culture on plastic (Supplementary Figure 2.1a). However, depletion of MLK3 dramatically reduced migration of MDA-MB-231 cells by over 4-fold in a 6 h transwell assay (Figure 2.2a). Comparable results were observed upon transient silencing of MLK3 (data not shown). As an alternative approach to study migration, the wound healing assay was employed. As shown in Figure 2.2b, MDA-MB-231 cells depleted of MLK3 are significantly impaired in wound recovery measured at 16 h. By 24 h, control MDA-MB-231 cells had completely closed the wound whereas an obvious wound remained in MLK3-depleted MDA-MB-231 cells. Due to the length of this assay, Mitomycin C was added to rule out any possibility of cell proliferation contributing to the observed phenotype.

To determine whether MLK3 plays a more general role in migration of other highly invasive breast cancer cell lines, a stable Mlk3-silenced population of MDA-MB-435 cells (Chambers, 2009), generated as described above, and Hs578T cells transiently transfected with Mlk3 siRNA were subjected to transwell migration assays. In agreement with our findings in MDA-MB-231 cells, Mlk3 silencing decreased migration of MDA-MB-435 and Hs578T cells by 2-fold (Figure 2.2c and 2.2d).

To assess whether catalytic activity of MLK3 is required for breast cancer cell migration, a small ATP analogue that selectively inhibits MLKs, CEP-11004 (Murakata et al., 2002; Shacka et al., 2006), was added to MDA-MB-231 cells in a transwell migration assay. As shown in Figure 2.3a, CEP-11004 inhibits the migration of MDA-MB-231 cells by 2-fold, supporting the notion that breast cancer cell migration depends upon MLK activity. Consistent with previously described catalytic roles of MLK3 in JNK

Figure 2.2

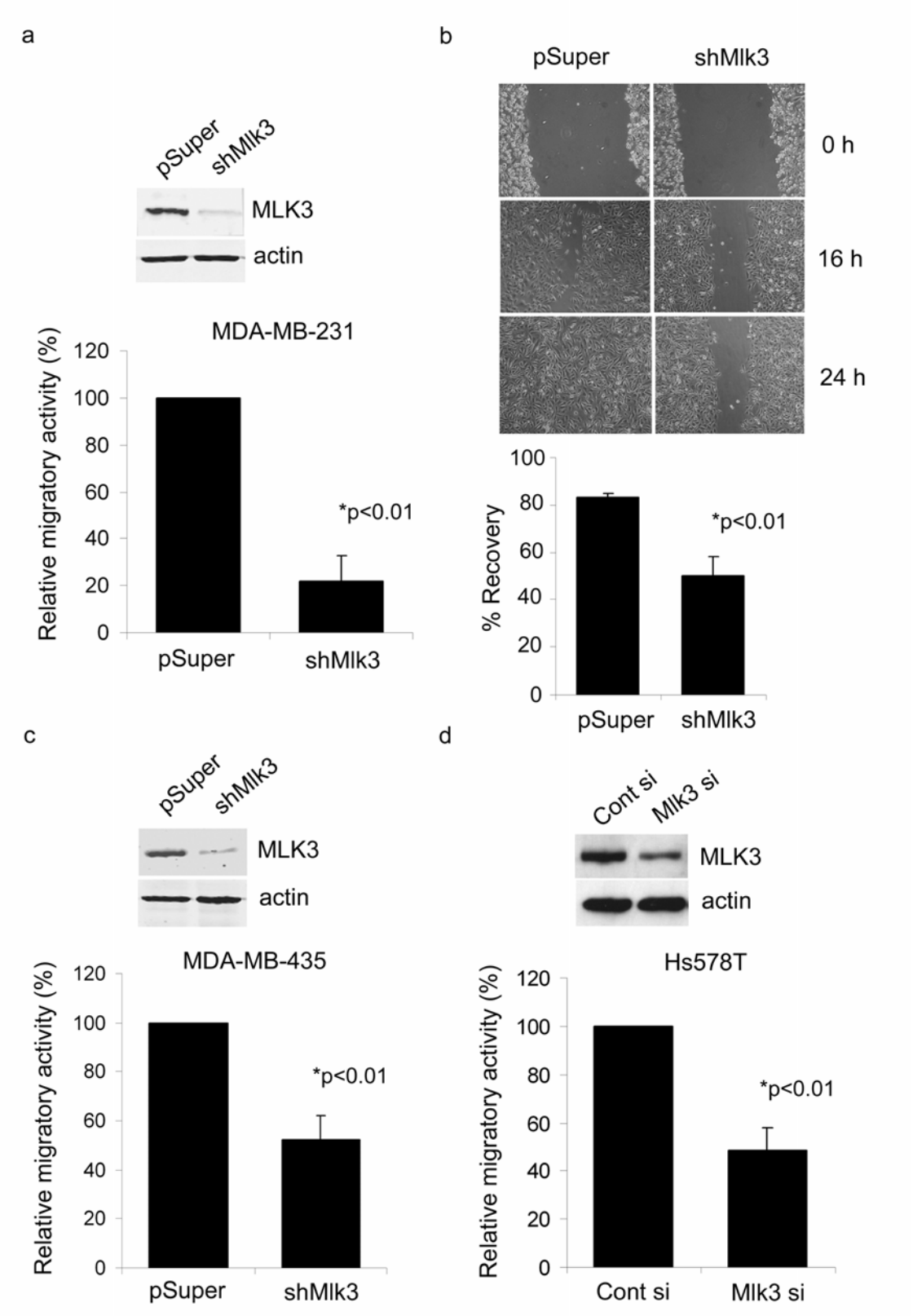

a
pSuper
shMlk3
MLK3
actin
MDA-MB-231
Relative migratory activity (%)
120
100
80
60
40
20
0
*p<0.01
pSuper
shMlk3
b
pSuper
shMlk3
0 h
16 h
24 h
% Recovery
100
80
60
40
20
0
*p<0.01
pSuper
shMlk3
c
pSuper
shMlk3
MLK3
actin
MDA-MB-435
Relative migratory activity (%)
120
100
80
60
40
20
0
*p<0.01
pSuper
shMlk3
d
Cont si
Mlk3 si
MLK3
actin
Hs578T
Relative migratory activity (%)
120
100
80
60
40
20
0
*p<0.01
Cont si
Mlk3 si

Figure 2.2 Silencing of MLK3 markedly reduces migration of highly invasive breast cancer cells. (a) MLK3 levels in a population of MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 were assessed by western-blot analysis (upper panel). Western blots of actin are shown as loading controls. MDA-MB-231 control cells or cells depleted of MLK3 were subjected to a transwell migration assay as described in Materials and methods. Cells were allowed to migrate towards 5% serum for 6 h. Triplicate wells were used for each of 3 independent experiments. The results are expressed relative to migration of control pSuper cells (=100%). Migration was significantly inhibited in MDA-MB-231 cells stably expressing shMlk3 compared to control pSuper-expressing cells. Column, mean of three experiments. Bar, SE. P-value was determined by Student's t-test *P<0.01. (b) MDA-MB-231 control cells or MDA-MB-231 cells depleted of MLK3 were subjected to a wound healing assay as described in Materials and methods. Representative photographs at the indicated time points from 3 independent experiments, each done in triplicate wells, are shown. Magnification 10x. Extent of wound recovery was determined as described in Materials and methods. Column, mean of three experiments. Bar, SE. *P<0.01. (c) MLK3 levels in a population of MDA-MB-435 cells stably expressing pSuper or pSuper-shMlk3 were assessed as in (a) (upper panel). Impact of silencing of Mlk3 on migration of MDA-MB-435 cells was determined as in (a). Column, mean of three experiments. Bar, SE. P-value was determined by Student's t-test. *P<0.01. (d) MLK3 levels in Hs578T cells upon transient transfection of control or Mlk3 siRNA for 48 h as described in Materials and methods and in (a) (upper panel). Impact of Mlk3 silencing on migration of Hs578T cells was determined by Student's t-test. *P<0.01.

Figure 2.3

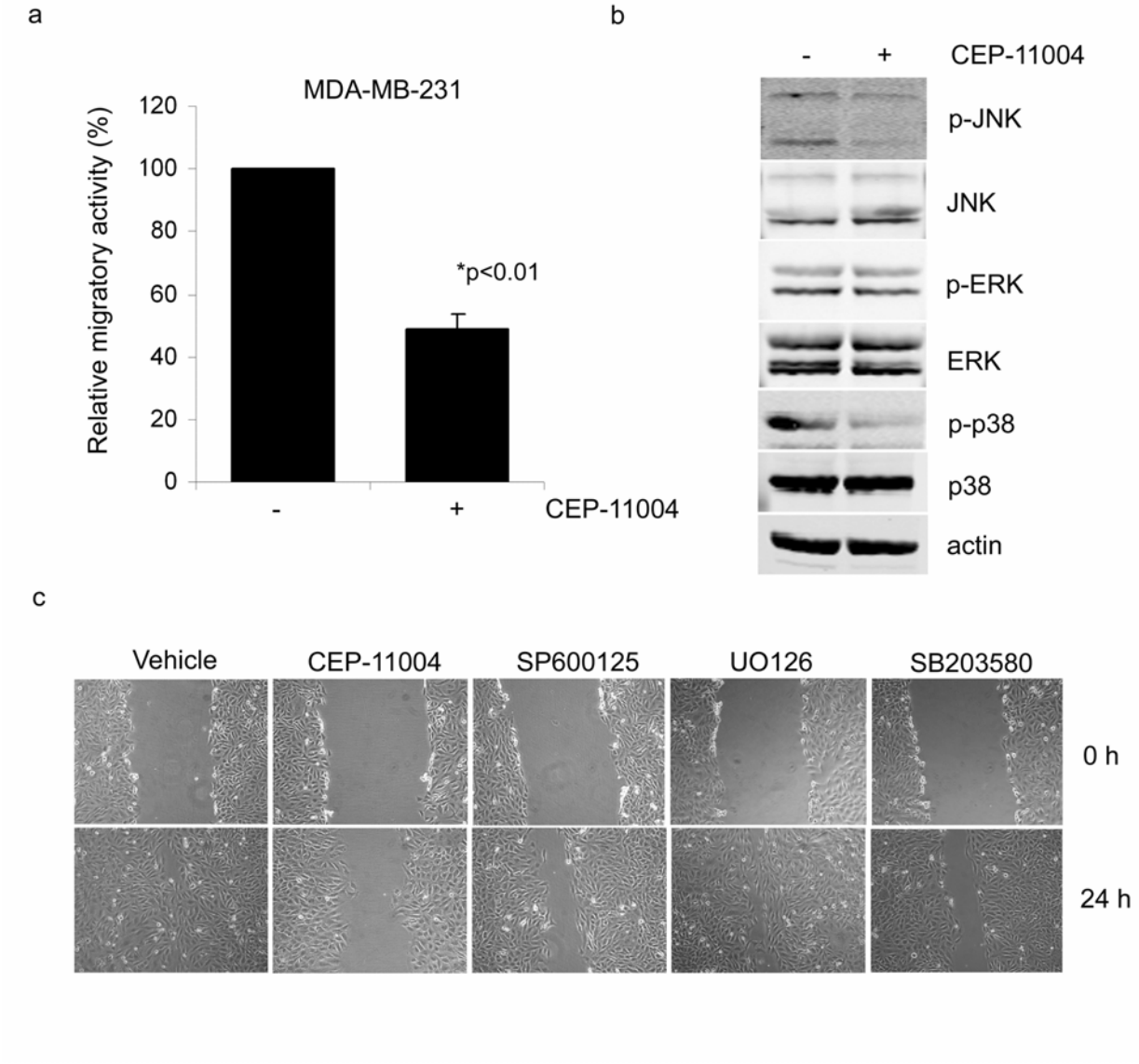

a
MDA-MB-231
Relative migratory activity (%)
120
100
80
60
40
20
0
*p<0.01
-
+
CEP-11004
b
-
+
CEP-11004
p-JNK
JNK
p-ERK
ERK
p-p38
p38
actin
c
Vehicle
CEP-11004
SP600125
UO126
SB203580
0 h
24 h

Figure 2.3 An MLK-selective inhibitor significantly decreases migration of MDA-MB-231 cells. (a) MDA-MB-231 cells were treated 400 nM CEP-11004 or vehicle for 30 min and subjected to a transwell migration assay as described in Materials and methods. Cells were allowed to migrate towards 5% serum for 6 h. Triplicate wells were used for each of 3 independent experiments. The results are expressed relative to migration of vehicle control (=100%). Migration was significantly inhibited in MDA-MB-231 cells treated with CEP-11004 compared to cells treated with vehicle. Column, mean of three experiments. Bar, SE. *P*-value was determined by Student's t-test. **P*<0.01. (b) Impact of CEP-11004 on MAPK activation in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or 400 nM CEP-11004 for 24 h. Total cellular lysates containing 60 µg of total protein were analyzed by Western blotting using the indicated antibodies. (c) Effects of MLK and MAPK pathway inhibitors on wound healing in MDA-MB-231 cells were determined as described in Materials and methods and Figure 2b. Immediately after wounding, media was replaced fresh growth medium and vehicle or the indicated inhibitor (400 nM CEP-11004, 15 µM SP600125, 10 µM U0126 or10 µM SB203580). Representative photographs from three independent experiments, each done in triplicate wells, at t=0 and at t=24 h are shown. Magnification 100x.

and p38 signaling, CEP-11004 blocked activation of JNK and p38, but not ERK, as judged by western blotting with phospho-specific antibodies (Figure 2.3b). CEP-11004 also dramatically inhibited closure in a wound healing assay of MDA-MB-231 cells (Figure 2.3c). A JNK inhibitor, SP600125, decreased wound closure. As has been previously reported, the p38 inhibitor, SB203580, decreased migration of MDA-MB-231 cells (Timoshenko et al., 2007). These data suggest that both p38 and JNK contribute to migration of MDA-MB-231 cells, which may explain why the MLK inhibitor, which decreases both p38 and JNK activity, is more efficacious than either the JNK or p38 inhibitor in blocking migration of these cells.

**Induced expression of MLK3 promotes migration of the poorly invasive MCF-7 breast cancer cells and of MCF10A mammary epithelial cells.** In complementary experiments, we asked whether induced expression of active MLK3 could promote migration of poorly invasive MCF-7 breast cancer cells (Schachter et al., 2006; Zhang et al., 2004) and MCF10A mammary epithelial cells. When a stable population of MCF-7-MLK3 cells was treated with the transcriptional inducer, AP21967 and subjected to a transwell migration assay, a 2.5-fold increase in migration of MCF-7 cells (Figure 2.4a) was observed. Stable populations of MCF10A that inducibly express MLK3 were also generated and showed a 2-fold increase in transwell migration upon transcriptional induction of MLK3 (Figure 2.5a). Induction of MLK3 potently induces JNK activation, but has no effect on ERK activation in either MCF-7 or MCF10A cells. Induced expression of MLK3 results in a 2-fold increase in p38 activity in both MCF-7 and MCF10A cells (Figure 2.4b, Figure 2.5b). In addition, MLK3 induction had no effect on ERK5 activation in MCF10A cells (Supplementary Figure 2.2).

Figure 2.4

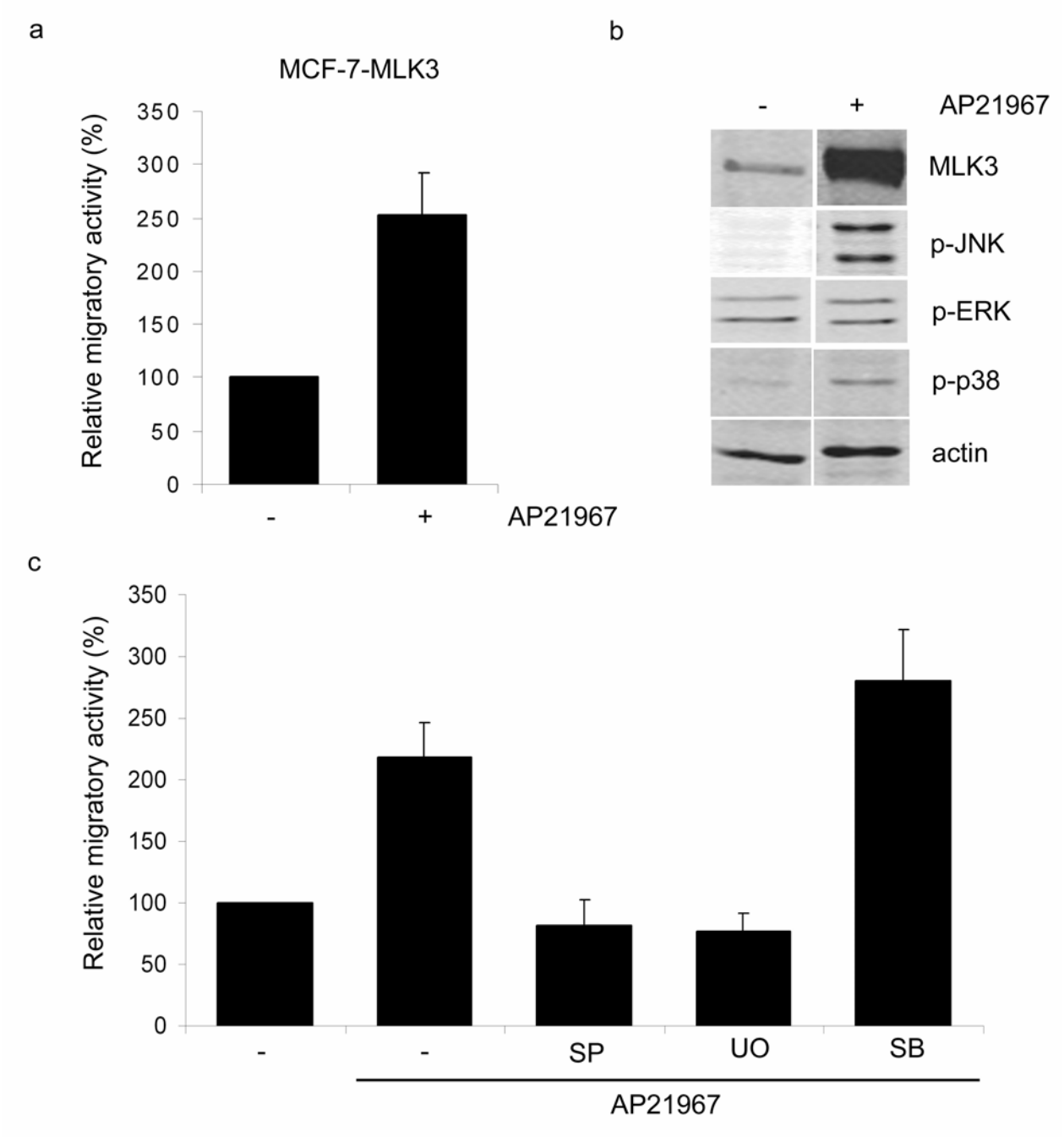

a
MCF-7-MLK3
Relative migratory activity (%)
350
300
250
200
150
100
50
0
-
+
AP21967
b
-
+
AP21967
MLK3
p-JNK
p-ERK
p-p38
actin
c
Relative migratory activity (%)
350
300
250
200
150
100
50
0
-
-
SP
UO
SB
AP21967

Figure 2.4 Induced expression of MLK3 promotes migration of MCF-7 cells. (a) MCF-7-MLK3 cells were seeded in the Boyden chamber with vehicle (-) or 25 nM AP21967 and subjected to a transwell migration assay as described in Materials and methods. The cells were allowed to migrate towards 10% FBS for 24 h. Triplicate wells were used for each of 3 independent experiments. The results are expressed relative to migration of uninduced MCF-7- MLK3 cells (=100%). Column, mean of three experiments. Bar, SE. P-value was determined by Student's t-test *P<0.01 (b) MCF-7-MLK3 cells were treated with vehicle or 25 nM AP21967 for 20 h. Total cellular lysates containing 60 μg of total protein were analyzed by Western blotting using the indicated antibodies. (c) MCF-7-MLK3 cells were seeded in the Boyden chamber in medium containing vehicle or 25 nM AP21967 and the indicated inhibitors (SP = 15 μM SP600125, U0 = 10 μM U0126 or SB = 10 μM SB203580) and subjected to a transwell migration assay as described in Materials and methods. Cells were allowed to migrate toward 10% FBS for 24 h. Triplicate wells were used for each of 2 independent experiments. The results are expressed relative to migration of uninduced MCF-7-MLK3 cells (=100%). Column, mean of two independent experiments. Bar, S.E.

Figure 2.5

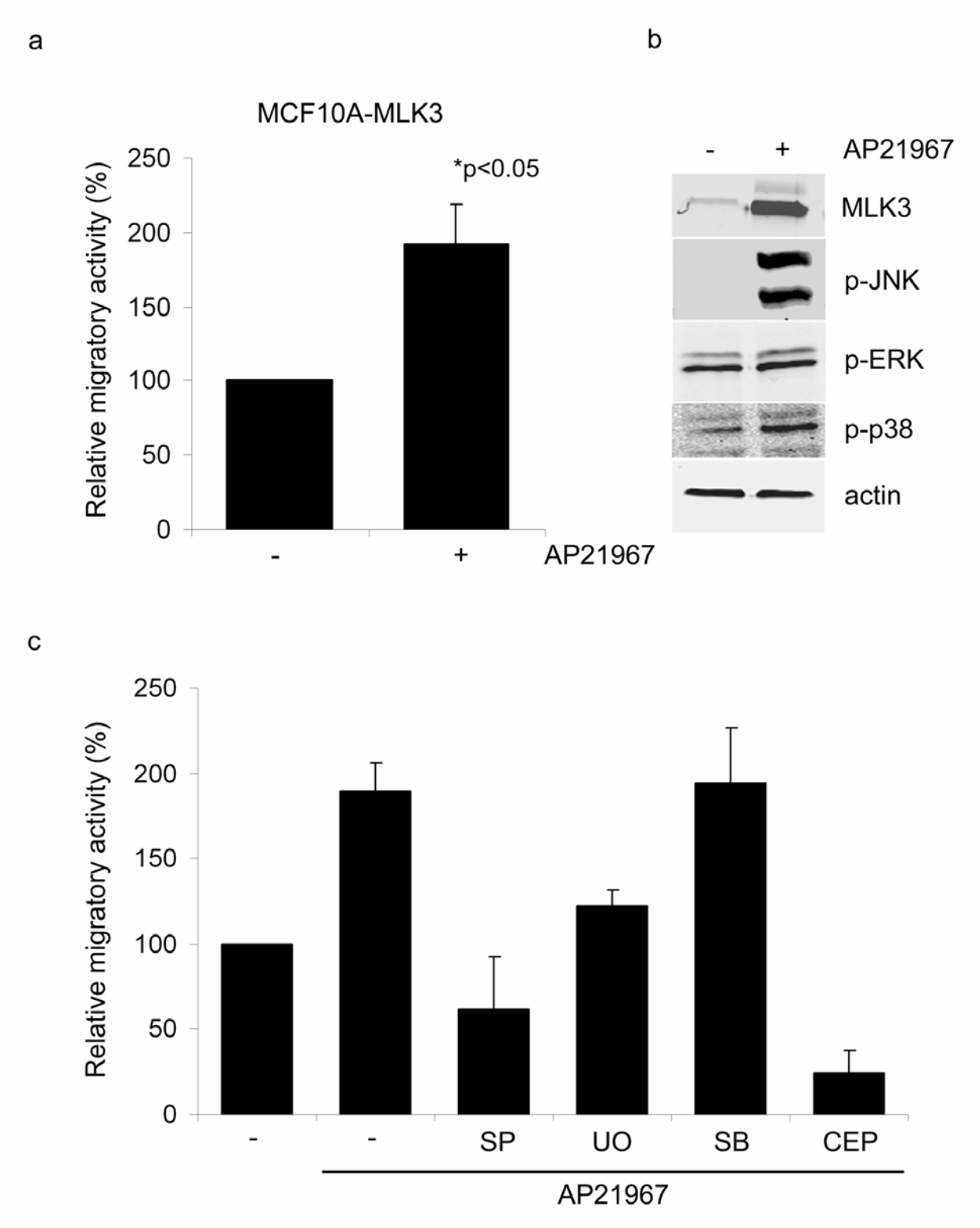
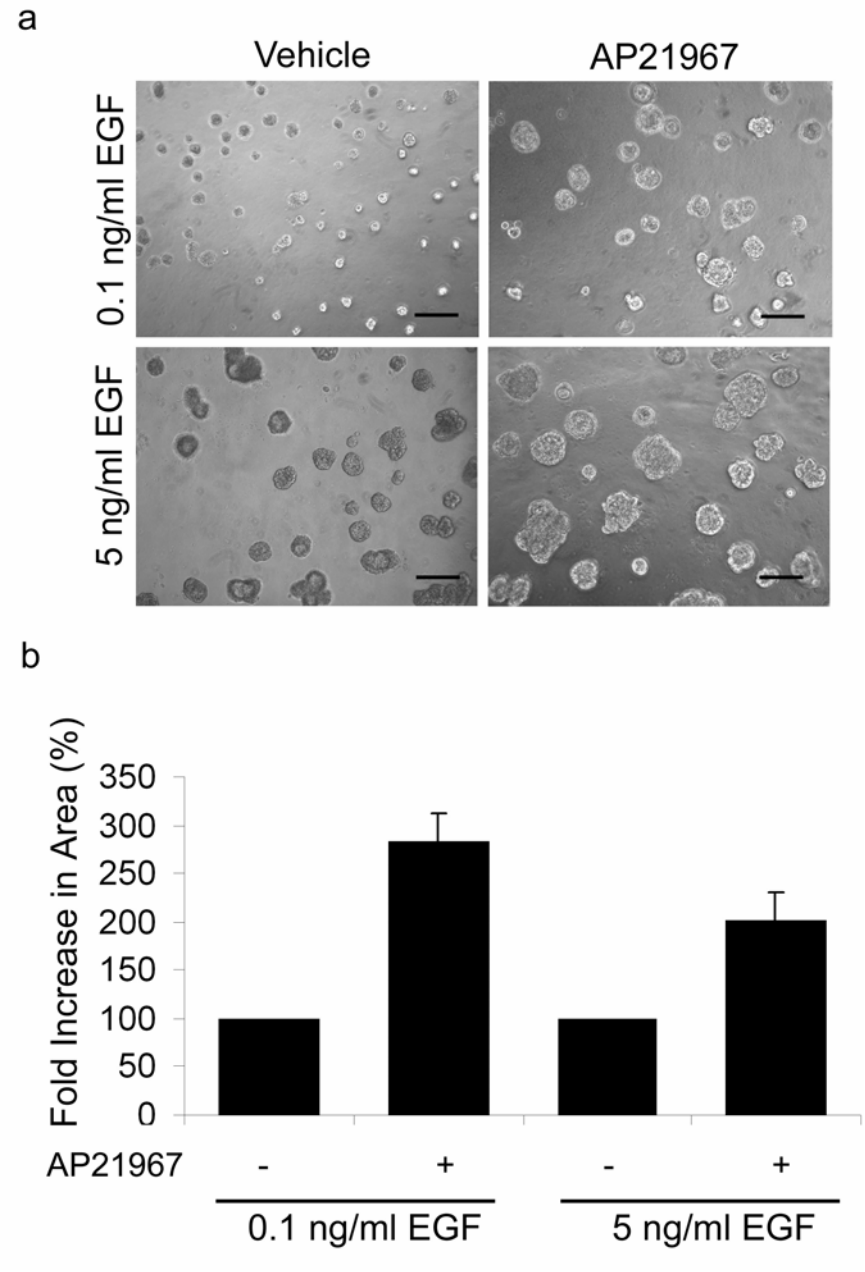

a
MCF10A-MLK3
*p<0.05
Relative migratory activity (%)
250
200
150
100
50
0
-
+
AP21967
b
-
+
AP21967
MLK3
p-JNK
p-ERK
p-p38
actin
c
Relative migratory activity (%)
250
200
150
100
50
0
-
-
SP
UO
SB
CEP
AP21967

Figure 2.5 Induced expression of MLK3 promotes migration of MCF10A cells. (a) MLK3-inducible MCF10A (MCF10A-MLK3) cells were treated with vehicle (-) or 25 nM AP21967 for 20 h. Cellular lysates containing 30 μg of total protein from the indicated cell lines were subjected to western blotting using an anti-MLK3 antibody with actin western blotting used as a loading control (upper panel). MCF10A-MLK3 cells were seeded in the Boyden chamber with vehicle or 25 nM AP21967 and subjected to a transwell migration assay as described in Materials and methods. Cells were allowed to migrate towards 2% horse serum for 24 h. Triplicate wells were used for each of 3 independent experiments. The results are expressed relative to migration of uninduced MCF10A-MLK3 cells (=100%) (lower panel). Column, mean of three experiments. Bar, SE. P-value was determined by Student's t-test. *P<0.05 (b) MCF10A-MLK3 cells were treated with vehicle or 25 nM AP21967 in media containing 2% horse serum and 20 ng/ml EGF for 20 h. Cellular lysates containing 60 μg of total protein were analyzed by Western blotting using the indicated antibodies. (c) MCF10A-MLK3 cells were seeded in the Boyden chamber in medium containing vehicle or 25 nM AP21967, and the indicated inhibitors (SP = 15 μM SP600125, U0 = 10 μM U0126, SB = 10 μM SB203580 or CEP = 400 nM CEP-11004) and subjected to a transwell migration assay as described in Materials and methods. Cells were allowed to migrate toward 2% horse serum for 24 h. Triplicate wells were used for each of 2 independent experiments. The results are expressed relative to migration of uninduced MCF10A-MLK3 cells (=100%). Column, mean of two independent experiments. Bar, S.E.

To assess the contributions of MAPK pathways to MLK3-induced MCF-7 and MCF10A cell migration, transwell migration assays were performed in the presence of MAPK pathway inhibitors. The JNK inhibitor SP600125 was effective in blocking MLK3-induced migration of both MCF-7 and MCF10A cells (Figure 2.4c, Figure 2.5c). The ERK pathway inhibitor also largely inhibited migration, consistent with previous reports using these cell lines (Wilsbacher et al., 2006; Lester et al., 2005). Our findings in MCF-7 and MCF10A cells that U0126 blocks MLK3-induced migration, even though MLK3 does not enhance ERK activation, is consistent with the idea that basal ERK activity is required for migration of these cells. Notably basal ERK activation is observed in the absence of MLK3 induction in both MCF-7 and MCF10A cells. The p38 inhibitor did not inhibit MLK3-induced migration in these cell lines, suggesting that MLK3-induced p38 activity is not sufficient to drive MLK3-induced migration of MCF-7 or MCF10A cells. Western blotting of cellular lysates, using phosphospecific antibodies against active JNK, ERK, and p38, as well as MAPKAPK2, a downstream effector of p38 (Young et al., 1997), confirms that each inhibitor appropriately blocked its cognate pathway (Supplementary Figure 2.3). The increased levels of active JNK and active ERK observed (Supplementary Figure 2.3), upon treatment with the p38 inhibitor, likely reflect the previously reported antagonistic crosstalk between the p38 pathway and other MAPKs (Hall and Davis, 2002; Schachter et al., 2006), as this phenomenon was also observed in MDA-MB-231 cells (Supplementary Figure 2.4). Collectively, these data indicate that MLK3 expression is sufficient to activate JNK signaling, which is critical for MLK3-mediated migration of MCF-7 and MCF10A cells.

**Induced expression of MLK3 increases mammary acinar size in an MLK3-JNK dependent pathway.** Three-dimensional cell culture systems provide a physiologically appropriate environment for assessing the impact of oncogenes on mammary epithelium (Debnath and Brugge, 2005; Lee et al., 2007). To determine whether MLK3 disrupts normal mammary morphogenesis, the inducible MLK3 expression system was utilized to express active MLK3 in preformed acinar structures composed of growth arrested, polarized MCF10A cells. A population of stable, inducible MCF10A-MLK3 cells was seeded in Matrigel cultures in low EGF (0.1 ng/ml) and standard EGF (5 ng/ml) concentrations. Polarized, hollow acini were established by day 10, as judged by confocal sectioning of acini after DAPI and GM130 staining (data not shown). After 10 days, cultures were treated with AP21967 to induce expression of MLK3. As is readily apparent at day 20 (Figure 2.6a), compared with uninduced MCF10A-MLK3 acini, induced expression of MLK3 dramatically increases acinar size at both EGF concentrations. Induction of MLK3 expression resulted in a 2.8- and a 2.0-fold increase in acinar cross-sectional area at low and standard EGF concentrations, respectively (Figure 2.6b). Data from a morphometric analysis of acinar cross-sectional area performed at day 20 are presented in a box-and-whiskers plot (Figure 2.6c). Using the corresponding control vector-expressing MCF10A cells, endogenous MLK3 levels (Supplementary Figure 2.5a) and acinar structures (Supplementary Figure 2.5b) were unaffected by AP21967. Highlighting the importance of the 3D environment for the MLK3-mediated effects on growth and morphogenesis, induced expression of MLK3 in MCF10A-MLK3 cells cultured on 2D plastic does not induce proliferation (Supplementary Figure 2.1b).

Figure 2.6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Vehicle
AP21967
0.1 ng/ml EGF
5 ng/ml EGF
b
Fold Increase in Area (%)
350
300
250
200
150
100
50
0
AP21967
-
+
-
+
0.1 ng/ml EGF
5 ng/ml EGF

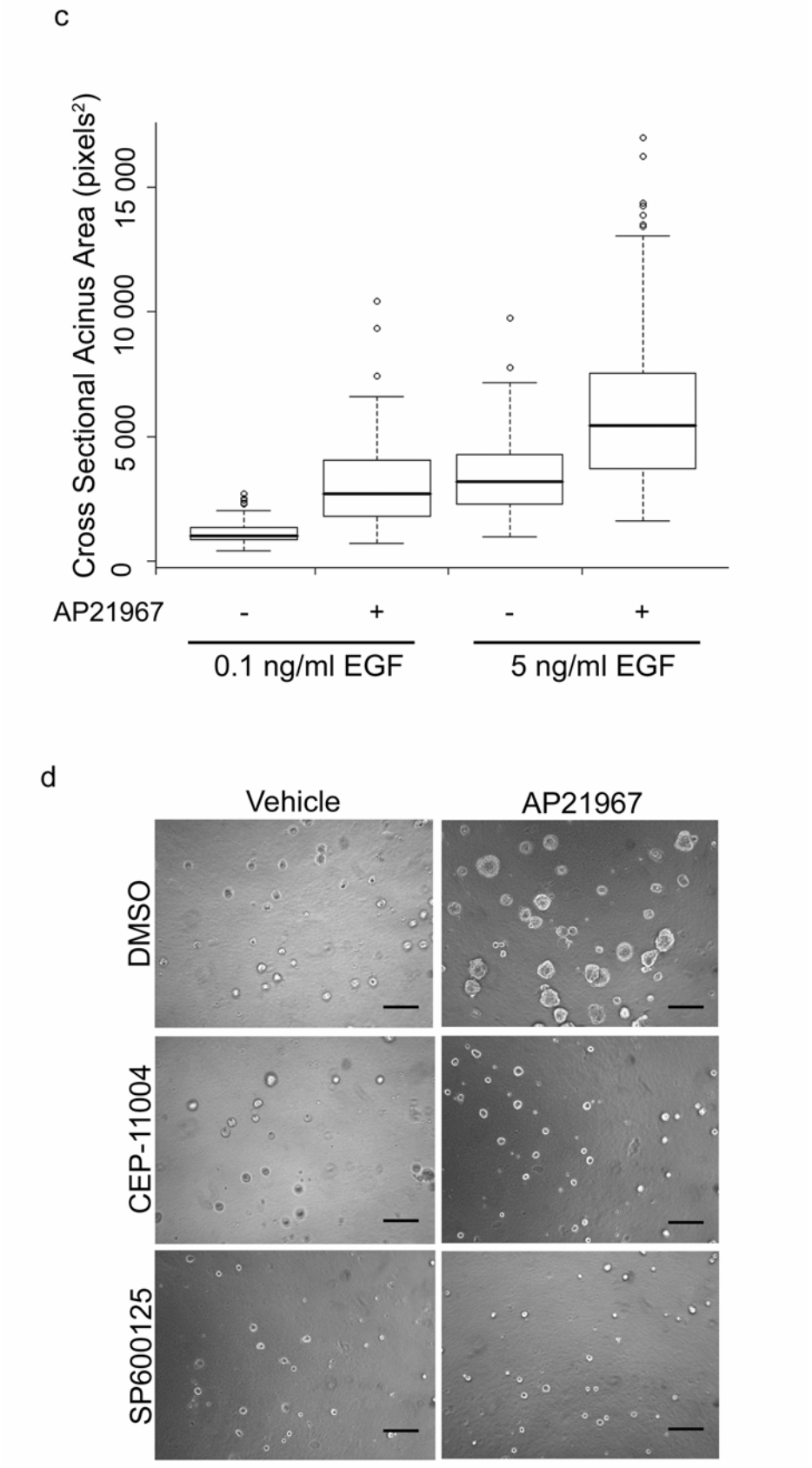
c
Cross Sectional Acinus Area (pixels²)
0
5 000
10 000
15 000
AP21967
-
+
-
+
0.1 ng/ml EGF
5 ng/ml EGF
d
Vehicle
AP21967
DMSO
CEP-11004
SP600125

Figure 2.6 Induced expression of MLK3 increases size of pre-established mammary acini. MCF10A-MLK3 cells were seeded in overlay medium containing 0.1 ng/ml or 5 ng/ml EGF on Matrigel as described in Materials and methods. (a) Fresh medium containing vehicle or 50 nM AP21967 was added to cultures on day 10 and images were acquired on day 20 of culture. Scale bar, 50 μm. (b) Cross-sectional area of MLK3-induced acini relative to uninduced acini (=100%). Each column represents at least 300 acini per condition combined from three independent experiments. Error bar, S.D. (c) Cross-sectional pixel areas of individual acini were determined as described in Materials and methods and plotted as a box plot. Black line, median value; spread, interquartile range; circles, outliers. Data are from a representative set of three independent experiments where ~150 acini per condition were measured. (d) MCF10A-MLK3 cells were seeded in overlay medium containing 0.1 ng/ml EGF on Matrigel as described in Materials and methods. Fresh medium containing vehicle or 50 nM AP21967 and the indicated inhibitor (400 nM CEP-11004 or 15 μM SP600125) was added on day 10 and images were acquired on day 18. Scale bar, 50 μm. Representative photographs from one of three independent experiments, each done in duplicate wells.

Addition of CEP-11004 at the time of MLK3 induction prevents the increased size of MCF10A-MLK3 acini, indicating a requirement for MLK3 activity (Figure 2.6d). The JNK inhibitor also prevented the MLK3-induced phenotype. The inhibitors had no observable effects on uninduced mammary acini. These data indicate that active MLK3 signaling to JNK is required for the effects of induced MLK3 in 3D culture.

**Induced MLK3 bypasses growth arrest, initiates luminal repopulation of preformed mammary acini, and suppresses Bim expression.** By day 20 in Matrigel culture, acini derived from MCF10A cells should be growth arrested (Debnath and Brugge, 2005). To determine whether induction of active MLK3 is sufficient to bypass growth arrest, acini were examined for expression of the proliferating-cell antigen, Ki-67, at day 20. As shown in Figure 2.7a, fewer than 10% of uninduced MCF10A-MLK3 acini contained Ki-67-positive cells in cultures containing both low and standard EGF concentrations, whereas greater than 70% and 90% of MLK3-induced acini contained at least one Ki-67 positive cell in cultures with low and standard EGF concentrations, respectively. It should be noted that although the majority of MLK3-induced acini contained multiple proliferating cells, Ki-67-positive cells were still in the minority. AP21967 does not affect Ki-67 positivity in vector control cells (Supplementary Figure 2.5c). The ability of induced MLK3 to substantially increase mammary acinar size and proliferation under minimal EGF concentrations suggests that MLK3 signaling partially overrides the growth factor dependence of the mammary acini.

To determine whether increased acinar size caused by MLK3 impacts polarity and luminal filling, structures were stained with nuclear and polarity markers, and optical sections through acini were examined. As shown in Figure 2.7b (left panel), at day 20,

Figure 2.7

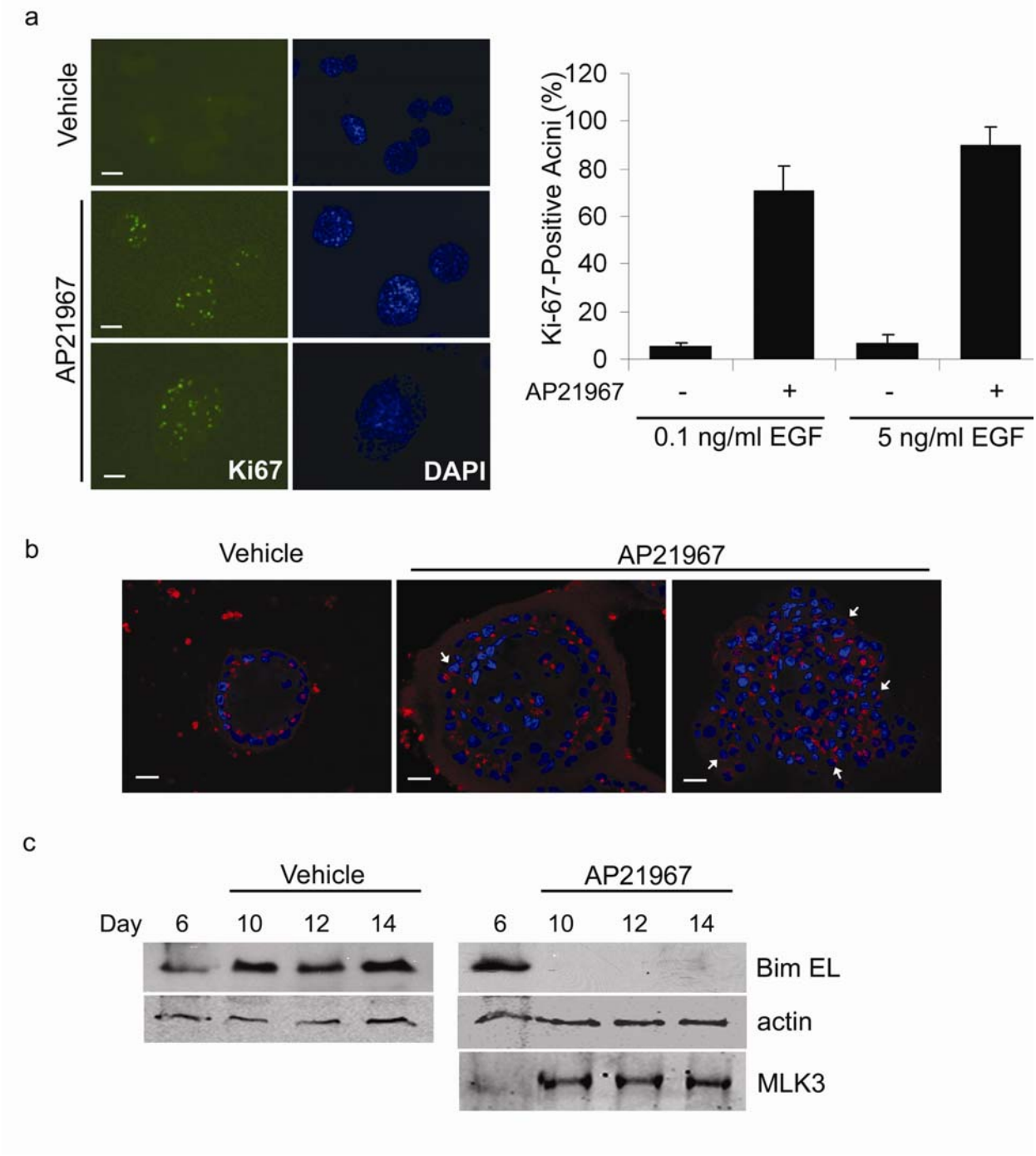
a
Vehicle
AP21967
Ki67
DAPI
Ki-67-Positive Acini (%)
120
100
80
60
40
20
0
AP21967
-
+
-
+
0.1 ng/ml EGF
5 ng/ml EGF
b
Vehicle
AP21967
c
Vehicle
AP21967
Day
6
10
12
14
6
10
12
14
Bim EL
actin
MLK3

Figure 2.7 Induced expression of MLK3 in pre-established mammary acini promotes proliferation, luminal filling and disruption of polarity in growth-arrested structures. (a) MCF10A-MLK3 cells were seeded in overlay medium containing 0.1 ng/ml or 5 ng/ml EGF on Matrigel as described in Materials and methods. Fresh medium containing vehicle or 50 nM AP21967 was added to cultures on day 10. On day 20, cultures were fixed and stained with anti-Ki-67 (green) and DAPI (blue). Representative images from cultures containing 5 ng/ml EGF. Scale bar, 50 μm. (left panel). Cultures were scored for the number of acini containing one or more Ki-67-positive cells under the indicated conditions. Column, percent Ki-67-positive acini based on at least 250 acini per condition combined from three independent experiments. Error bar, S.D. (right panel) (b) MCF10A-MLK3 cultures were established as above. On day 20, cultures were fixed, stained with anti-GM130 (red) and DAPI (blue), and analyzed by confocal microscopy. Optical sections through the largest cross-sectional area of representative acini are shown. White arrows indicate loss of polarity. Scale bar, 20 μm. (c) MCF10A-MLK3 cells were seeded in overlay medium containing 5 ng/ml EGF on Matrigel as described in Materials and methods. Fresh medium containing vehicle or 50 nM AP21967 was added to cultures on day 7. On the indicated days in culture, cellular lysates were prepared as described in Materials and methods and subjected to western blot analysis using the indicated antibodies. Actin was used as a loading control. Data are representative of two independent experiments.

in the absence of AP21967, the MCF10A-MLK3 cells form uniform spherical structures with hollow lumens, whereas after culturing in the presence of AP21967 from day 10 to day 20, the lumens are largely filled, indicating that MLK3 can initiate luminal repopulation (Figure 2.7b, center and right panels). Similar luminal filling was observed upon MLK3 induction in cultures containing 0.1 ng/ml EGF (data not shown). Whereas induction of the oncogene ErbB2 promotes the formation of multiacinar structures (Muthuswamy et al., 2001), the majority of the MLK3-expressing acini are still spherical (Figure 2.7b, central panel) with cell polarity largely maintained as determined by staining with the apical Golgi marker, GM130. However, some poorly defined mass-like structures with apparent disruption of cell polarity are also observed (Figure 2.7b, right panel). Taken together, these results demonstrate that expression of active MLK3 in mature acini reinitiates proliferation in growth-arrested structures, repopulates the lumen, and can perturb cell polarity.

BimEL is a BH3-only proapoptotic protein that has been shown to be critical in luminal apoptosis and lumen formation (Reginato et al., 2005). Oncogenes, like v-Src and ErbB2, which promote luminal filling of mammary acini have been shown to suppress the expression of BimEL (Reginato et al., 2005). Consistent with these findings, upon MLK3 induction, we observe a dramatic decrease in BimEL levels based on western blotting of lysates derived from 3D cultures of MCF10A-MLK3 cells (Figure 2.7c). These data demonstrate that, in the physiologically appropriate 3D context of mammary acini, MLK3 has both proliferative and antiapoptotic activities.

**MLK3 induces expression of proteins associated with invasive breast cancer.** Our data point to a central role for JNK in the MLK3-mediated malignant phenotypes.

MLK3 can act as a MAP3K to activate JNK which, in turn, can phosphorylate and activate c-jun, a component of the AP-1 transcription factor (Gallo and Johnson, 2002). We hypothesized that MLK3 signaling to JNK might activate c-jun to drive the expression of AP-1-regulated invasion genes in MCF10A cells. Notable AP-1-regulated invasion genes include c-jun (Karin, 1995; Minet et al., 2001; Zajchowski et al., 2001), fos-related antigen (Fra-1) (Bergers et al., 1995; Zajchowski et al., 2001) and vimentin (Rittling et al., 1989; Sommers et al., 1994b; Rizki et al., 2007; Zajchowski et al., 2001) Induced expression of MLK3 in MCF10A cells increases the level of c-jun (Figure 2.8a), Fra-1 (Figure 2.8b), and vimentin (Figure 2.8b) supporting the notion that MLK3 promotes a malignant phenotype, at least in part, by controlling AP-1 activity. Notably, vimentin expression is also induced upon induction of MLK3 in preformed MCF10A mammary acini (Figure 2.8c).

**MLK3 promotes invasion through signaling to AP-1.** To investigate whether MLK3 regulates AP-1 activity to promote a malignant phenotype, the impact of MLK3 on invasion of MCF10A cells was first examined. Induced expression of MLK3 in MCF10A-MLK3 cells increased invasion through Matrigel by 3-fold in transwell invasion assays (Figure 2.9a). To test the requirement of AP-1 in MLK3-induced invasion, we made use of the dominant negative mutant, Tam67 (Brown et al., 1993) which lacks the N-terminal transactivating domain, as an inhibitor of AP-1 activity. Expression of pBabe-Tam67 (Johung et al., 2007), but not pBabe control vector, effectively blocks MLK3-induced invasion (Figure 2.9a), indicating that MLK3-induced AP-1 activity is critical for the invasive phenotype. As shown in Figure 2.9b, Tam67 is efficiently expressed in

Figure 2. 8

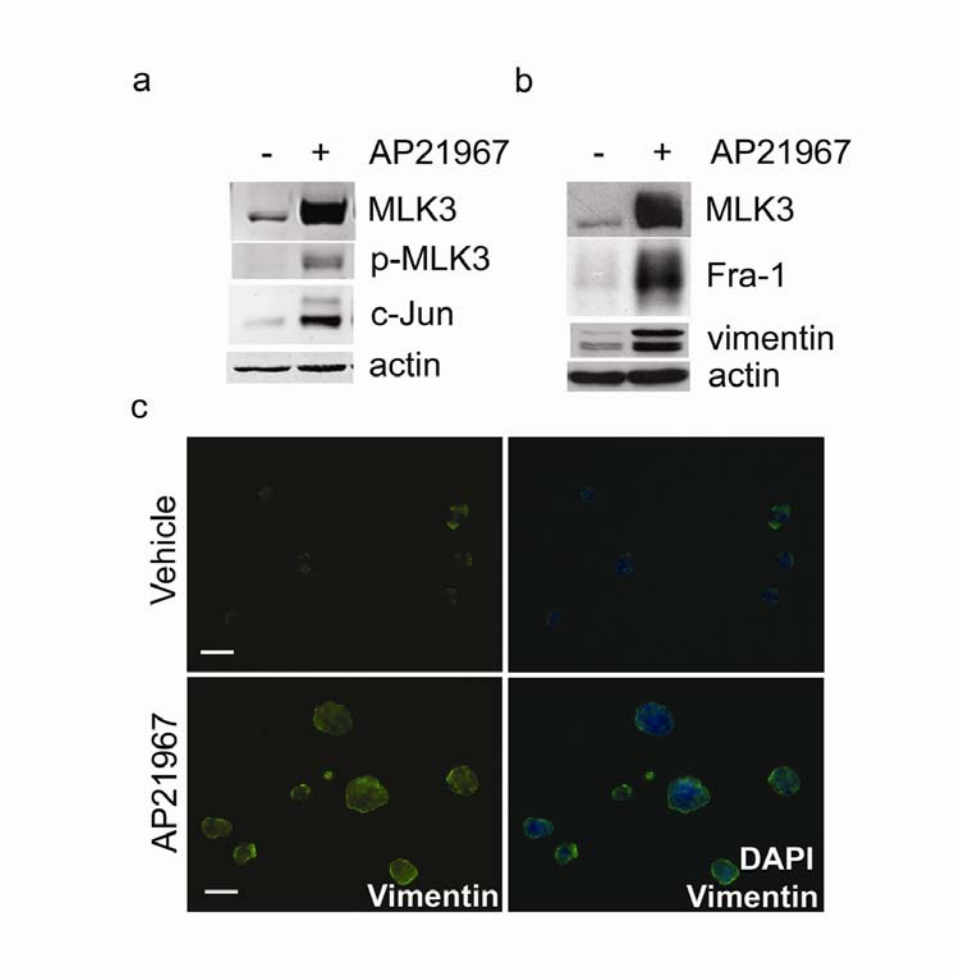


Figure 2.8 Induction of active MLK3 promotes expression of invasion-associated proteins: c-jun, Fra-1 and vimentin. (a, b) MCF10A-MLK3 cells were seeded in growth media and induced with 25 nM AP21967 for 20 h. Cellular lysates containing 25 µg of total protein were subjected to western blotting using the indicated antibodies. Data are representative of three independent experiments. (c) MCF10A-MLK3 cells were seeded as described in Materials and methods in 0.1 ng/ml EGF. Expression of MLK3 was induced with 50 nM AP21967 on day 10, and on day 20, cultures were immunostained with anti-vimentin (green) and stained with DAPI (blue). Data are representative of three independent experiments. Scale bar, 50 µm.

Figure 2.9

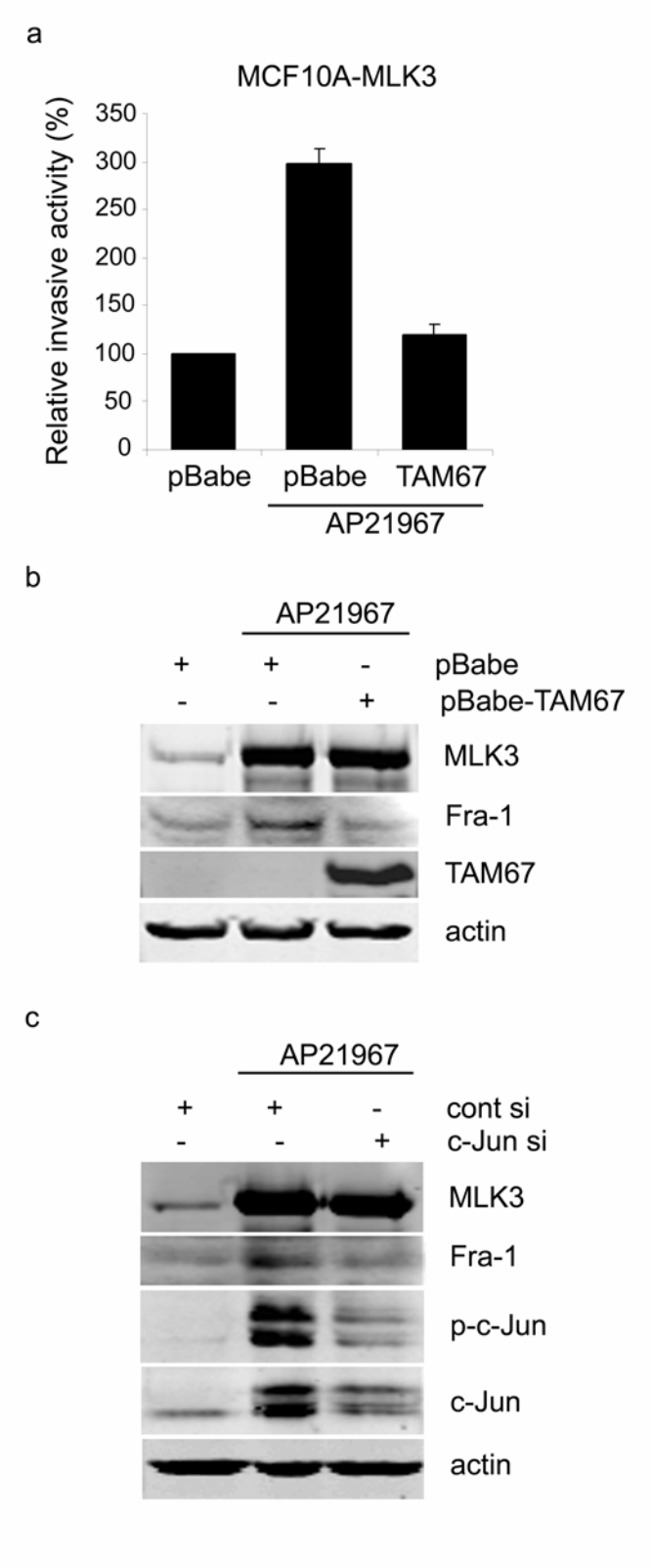
a
MCF10A-MLK3
Relative invasive activity (%)
350
300
250
200
150
100
50
0
pBabe
pBabe
TAM67
AP21967
b
AP21967
+ + - pBabe
- - + pBabe-TAM67
MLK3
Fra-1
TAM67
actin
c
AP21967
+ + - cont si
- - + c-Jun si
MLK3
Fra-1
p-c-Jun
c-Jun
actin

Figure 2.9 Induced expression of MLK3 promotes invasion of MCF10A cells and MLK3-induced invasion requires AP-1 activity. (a) MCF10A-MLK3 cells carrying pBabe or pBabe-Tam67 retroviral vectors were seeded in the Matrigel-coated Boyden chamber with vehicle or 25 nM AP21967 and subjected to a transwell invasion assay as described in Materials and methods. Cells were allowed to migrate towards 2% horse serum for 24 h. Triplicate wells were used for each of two independent experiments. The results are expressed relative to invasion of uninduced MCF10A-MLK3 cells (=100%). Column, mean of two experiments. Bar, SE. (b) MCF10A-MLK3 cells carrying pBabe or pBabe-Tam67 retroviral vectors were induced with 25 nM AP21967 for 20 h. Cellular lysates containing 25 μg of total protein were subjected to western blotting using the indicated antibodies. Data are representative of three independent experiments. (c) MCF10A-MLK3 cells were treated with control or c-Jun siRNA for 24 h followed by addition of 25 nM AP21967 for 20 h. Cellular lysates containing 25 μg of total protein were subjected to western blotting using the indicated antibodies. Data are representative of three independent experiments.

MCF10A-MLK3 cells, and prevents the MLK3-induction of the AP-1 regulated invasion gene, Fra-1. In a complementary experiment, silencing of c-jun, an AP-1 component and a direct substrate of JNK, also blocks the MLK3-induced expression of Fra-1 (Figure 2.9c). These data, taken together, demonstrate that MLK3-JNK signaling increases AP-1 activity to promote invasion of mammary epithelial cells.

## Discussion

Identifying signaling pathways that contribute to breast cancer progression is key to developing effective targeted therapies. MLK3 controls multiple MAPK signaling pathways and, depending upon cellular context, regulates proliferation, apoptosis, differentiation and migration. Several human breast cancer cell lines show higher levels of MLK3 compared with nontumorigenic mammary epithelial cell lines (Figure 2.1). Using the Oncomine database (www.oncomine.com) (Rhodes et al., 2007), we found that MLK3 was among the top 10% overexpressed transcripts in DCIS compared to normal breast tissue (Radvanyi et al., 2005), but it is also possible that MLK3 protein levels may be regulated at the translational and/or posttranslational levels.

Using Mlk3 silencing (Figure 2.2) as well as an MLK-selective inhibitor (Figure 2.3, Figure 2.5) we provide evidence that MLK3 catalytic activity is critical for migration of mammary cancer and epithelial cells. Studies with MAPK pathway inhibitors support the idea that MLK3-JNK signaling drives migration. Indeed, Slpr, the Mlk homolog in *Drosophila*, signals to JNK to promote epithelial sheet movement during dorsal closure of the fly embryo (Stronach and Perrimon, 2002), in a genetically mapped pathway that leads to induction of JNK and AP-1 (Jacinto et al., 2002). Interestingly, using an

MCF10A wound healing assay, a large scale RNAi screen targeting over 1000 genes encoding all protein kinases and phosphatases as well as a battery of proteins predicted to influence cell migration, identified over 60 genes as modulators of migration, one of which was Mlk3. (Simpson et al., 2008). A scaffolding function for MLK3 has been proposed for migration of A549 lung carcinoma cells (Swenson-Fields et al., 2008). However, our findings that an MLK inhibitor can block migration of invasive breast cancer cells and that JNK inhibition blocks MLK3-induced migration in MCF-7 and MCF10A cells supports the idea that, at least in the context of breast cancer, MLK3-JNK signaling is critical for cell migration. Indeed, a significant body of literature indicates a requirement for JNK in cancer progression (Ching et al., 2007; Khatlani et al., 2007; Vivanco et al., 2007; Cui et al., 2006; Dhillon et al., 2007; Su et al., 2009; Wagner and Nebreda, 2009).

It is interesting to note that both MDA-MB-231 and MCF-7 cells contain similar levels of MLK3, yet MDA-MB-231 cells are more migratory than MCF-7 cells. Since MDA-MB-231 cells are highly malignant, it is likely that multiple facets of the migratory machinery and signaling are aberrantly activated, contributing to their highly invasive phenotype. In MCF-7 cells, these signaling pathways are likely less active and thus high, induced MLK3-JNK signaling is needed to increase migration.

In DCIS, partial to complete filling of the luminal space is commonly observed, and a poorer prognosis is associated with disruption of apicobasal polarity (Debnath and Brugge, 2005). Three-dimensional mammary epithelial cell cultures provide a structurally appropriate milieu for investigating breast cancer phenotypes. MCF10A cells grown in 3D Matrigel form growth-arrested, hollow spheroids composed of

polarized cells that recapitulate many aspects of normal mammary glandular architecture (Debnath and Brugge, 2005). Upon expression of certain oncogenes such as Cyclin D1 and HPV E7 (Debnath and Brugge, 2005; Debnath et al., 2002; Shaw et al., 2004), MCF10A acini grown in Matrigel increase in size but maintain apicobasal polarity and hollow lumens. A few oncogenes, such as ErbB2, Notch and the Ets family of transcription factors, are able to confer DCIS and invasive breast cancer traits on MCF10A cells, forming filled lumens and disorganized structures when grown in Matrigel cultures (Muthuswamy et al., 2001, Shaw et al., 2004). In this DCIS model, we induced expression of active MLK3 in preformed, hollow MCF10A mammary acini and observed multiple features of DCIS, including bypass of growth arrest and reinitiation of luminal filling. The increase in the proliferative marker Ki67 and suppression of the proapoptotic BimEL (Figure 2.7) upon MLK3 induction reveals both proliferative and novel antiapoptotic roles for MLK3 in the context of breast cancer.

The ability of MLK3 to regulate cell proliferation is highly dependent upon 3D culture in extracellular matrix. Growth of MDA-MB-231 cells in 3D Matrigel culture is largely stunted by silencing of MLK3 (data not shown) whereas proliferation on 2D plastic is not enhanced by either induction of MLK3 in MCF10A cells or by silencing of Mlk3 in MDA-MB-231 cells (Supplementary Figure 2.1). These data point to the importance of physiologically relevant 3D culture systems in assessing oncogenic phenotypes. One of the major MLK3-JNK signaling outcomes is phosphorylation of c-jun and activation of AP-1. In mammary epithelial cells, MLK3 induces expression of c-jun, Fra-1, and vimentin whose genes are regulated by AP-1 and have been shown to be expressed at higher levels in invasive human breast cancer cell lines, compared with noninvasive

breast cancer cell lines (Sommers et al., 1994a; Zajchowski et al., 2001). Vimentin is a classical marker for EMT, a transient cellular differentiation that allows for cancer cell migration and invasion (Blick et al., 2008; Polyak and Weinberg, 2009). A subset of MLK3-expressing MCF10A acini also showed disruption of apicobasal polarity, a feature of invasive breast cancer. Furthermore, MLK3 promotes invasion of MCF10A cells (Figure 2.9). Our finding that the dominant negative AP-1 inhibitor, Tam 67, blocks MLK3-induced invasion indicates a critical role for MLK3-JNK-AP1 in invasion. Notably, decreased phospho-JNK1/2 levels are reported to correlate with improved overall survival in infiltrating ductal carcinoma (Yeh et al., 2006).

In summary, our investigation supports a critical role for MLK3-JNK-AP1 signaling in breast cancer cell migration and acquisition of a malignant phenotype. Although JNK has, in the past, largely been associated with stress signaling and neuronal apoptosis, a body of evidence linking JNK to cell migration, invasion, and proliferation is accumulating. Our data are consistent with the idea that JNK signaling to AP-1 activates genes associated with EMT and a breast cancer invasive phenotype. Global gene expression analyses are underway to determine the repertoire of genes, including AP-1 regulated genes, impacted by MLK3 in breast cancer. However, given the array of cellular substrates for JNK and other MLK3 regulated pathways, it will also be important to determine whether MLK3 signaling impacts the cytoskeleton in the context of breast cancer. Finally, our finding that an MLK inhibitor is able to inhibit breast cancer cell migration and suppress MLK3-induced phenotype of MCF10A cells in 3D culture indicates that MLK inhibitors may be useful in treating invasive breast cancer.

## Acknowledgements

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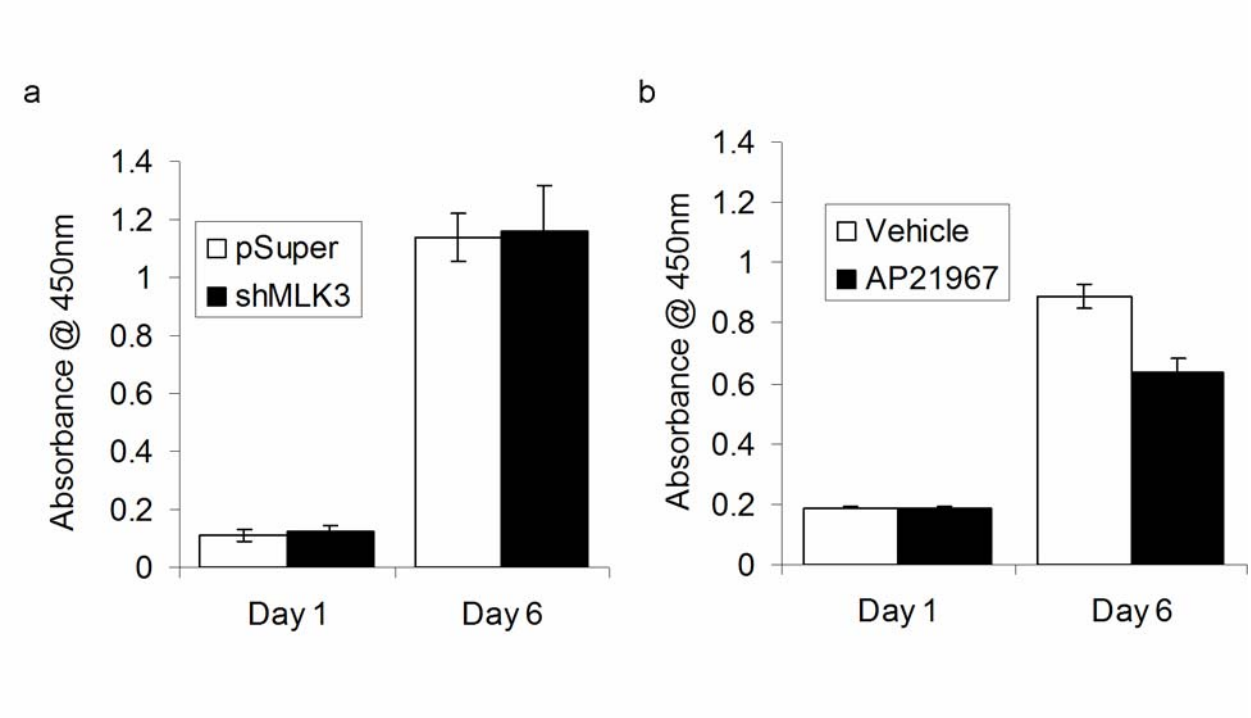
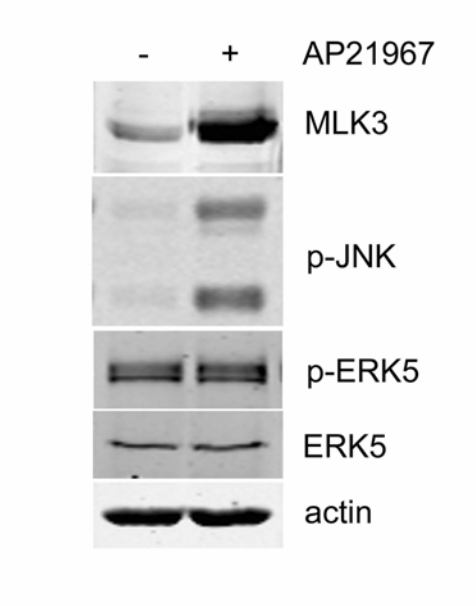
Supplementary figure 2.1



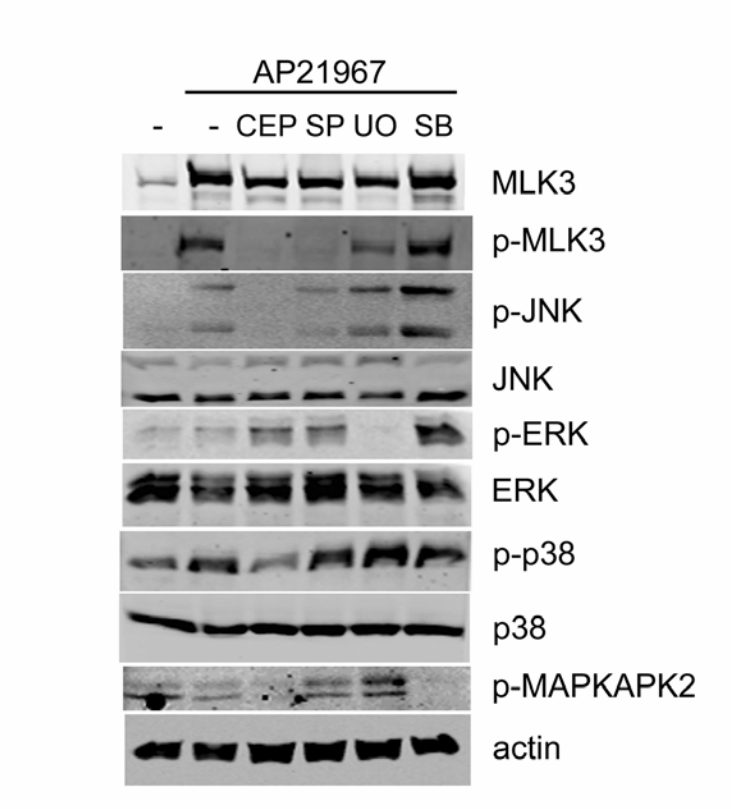

Supplementary figure 2.1 Effects of MLK3 silencing or overexpression on proliferation of MDA-MB-231 or MCF10A-MLK3 cells, respectively, grown in 2D on plastic. (a) 5000 MDA-MB-231 cells were seeded at Day 0 and proliferation was monitored by measuring absorbance at 450 nm using the CCK-8 kit according to manufacturer's instructions. (b) $1.5x10^4$ MCF10A-MLK3 cells were seeded at Day 0 and treated with vehicle or AP21967 at Day 1. Proliferation was monitored by measuring absorbance at 450 nm using the CCK-8 kit according to manufacturer's instructions. Experiments were done three times in triplicate wells.

Supplementary figure 2.2



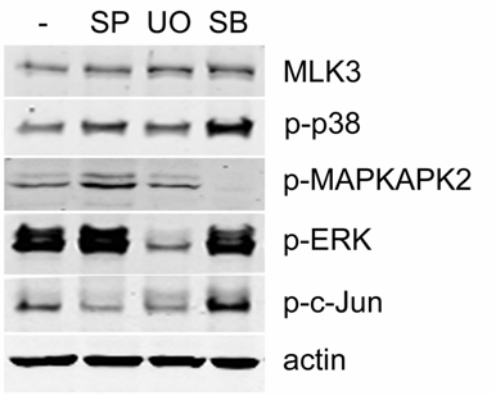

Supplementary figure 2.2 Effect of MLK3 expression on ERK5 activation. Subconfluent monolayers of MCF10A-MLK3 cells were treated with vehicle or 50 nM AP21967 in media containing 2% horse serum for 24 h. Cellular lysates were subjected to western blot analysis using the indicated antibodies. Data are representative of three independent experiments.

Supplementary figure 2.3



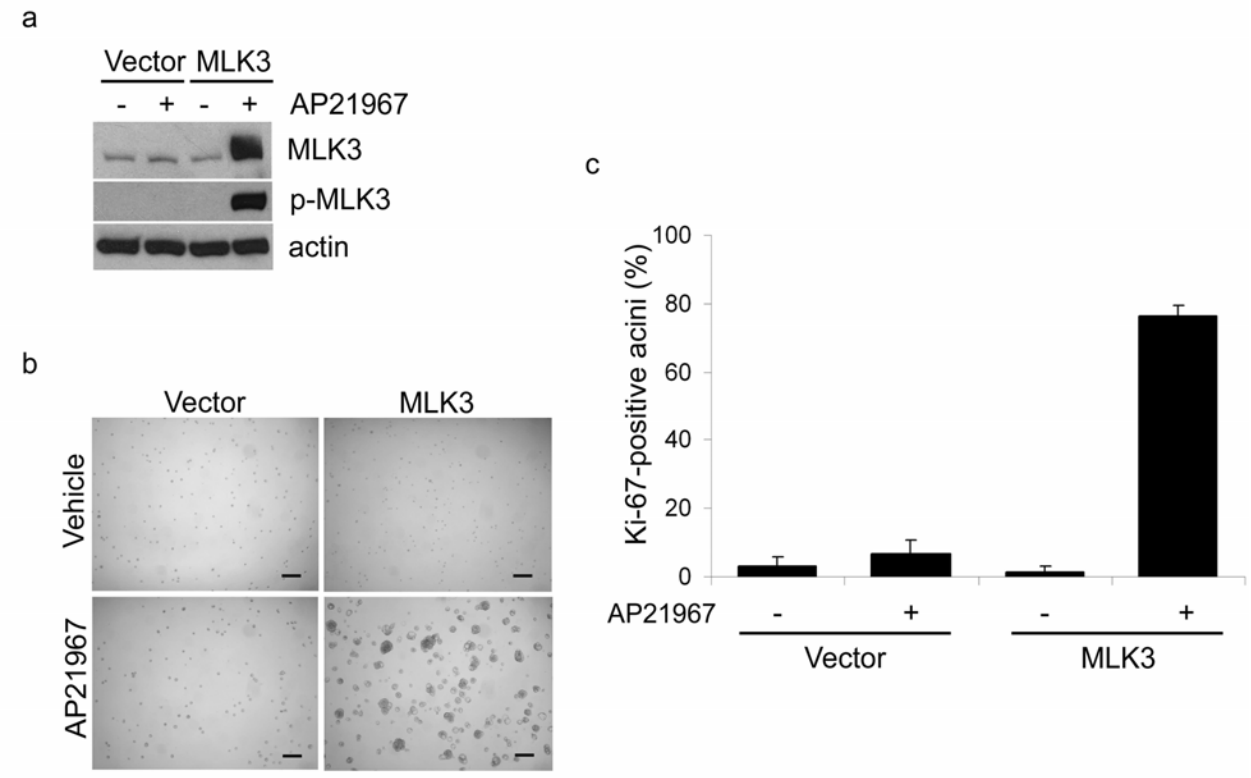

Supplementary figure 2.3 Effects of MAPK pathway inhibitors on signaling pathways in MCF10A-MLK3 cells. MCF10A-MLK3 cells were treated with vehicle or 50 nM AP21967 for 20 h and the indicated inhibitors: 400 nM CEP-11004, 15 μM SP600125, 10 μM U0126 or 10 μM SB203580 for an additional 24 h. Cellular lysates were analyzed by Western blotting with the indicated antibodies.

Supplementary figure 2.4




Supplementary figure 2.4 Effects of MAPK pathway inhibitors on signaling pathways in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or the indicated inhibitors: 400 nM CEP-11004, 15 μM SP600125, 10 μM U0126 or 10 μM SB203580 for 24 h. Cellular lysates were analyzed by Western blotting with the indicated antibodies.

Supplementary figure 2.5




Supplementary figure 2.5 Transcriptional inducer, AP21967, has no effect on MLK3 or acinar structures in MCF10A cells and vector control experiments. (a) MCF10A-vector and MCF10A-MLK3 cells were seeded in growth media. Medium was replaced with fresh assay media containing vehicle or 50 nM AP21967 for 20 h. Total cellular lysates were analyzed by Western blotting. (b) MCF10A-vector and MCF10A-MLK3 cells were seeded on Matrigel as described in Material and methods. Vehicle or 50 nM AP21967 was added to cultures on day 10 and images were acquired on day 20 of culture. Scale bar, 200 μm. (c) Cultures at day 20 were fixed and stained with anti-Ki-67 and then scored for the number of acini containing one or more Ki-67-positive cells. Column represents ~100 acini per condition. Error bar, S.D.

# REFERENCES

## REFERENCES


Bergers G, Graninger P, Braselmann S, Wrighton C and Busslinger M. (1995). Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. Mol Cell Biol 15: 3748-3758.

Blick T, Widodo E, Hugo H, Waltham M, Lenburg ME, Neve RM et al. (2008). Epithelial mesenchymal transition traits in human breast cancer cell lines. Clin Exp Metastasis 25: 629-642.

Bock BC, Vacratsis PO, Qamirani E, Gallo KA. (2000). Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation. J Biol Chem 275: 14231-14241.

Brown PH, Alani R, Preis LH, Szabo E, Birrer MJ. (1993). Suppression of oncogene-induced transformation by a deletion mutant of c-jun. Oncogene 8: 877-86.

Chadee DN, Kyriakis JM. (2004). MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. Nat Cell Biol 6: 770-776.

Chambers AF. (2009). MDA-MB-435 and M14 cell lines: identical but not M14 melanoma? Cancer Res 69: 5292-5293.

Ching YP, Leong VY, Lee MF, Xu HT, Jin DY, Ng IO. (2007). P21-activated protein kinase is overexpressed in hepatocellular carcinoma and enhances cancer metastasis involving c-Jun NH2-terminal kinase activation and paxillin phosphorylation. Cancer Res. 67: 3601-3608.

Cui X, Kim HJ, Kuiatse I, Kim H, Brown PH, Lee AV. (2006). Epidermal growth factor induces insulin receptor substrate-2 in breast cancer cells via c-Jun NH(2)-terminal kinase/activator protein-1 signaling to regulate cell migration. Cancer Res 66: 5304-5313.

Debnath J, Brugge JS. (2005). Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer 5: 675-688.

Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell 111: 29-40.

Debnath J, Muthuswamy SK, Brugge JS. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256-268.

Dhillon AS, Hagan S, Rath O, Kolch W. (2007). MAP kinase signalling pathways in cancer. Oncogene 26: 3279-3290.

Du Y, Bock BC, Schachter KA, Chao M, Gallo KA. (2005). Cdc42 induces activation loop phosphorylation and membrane targeting of mixed lineage kinase 3. J Biol Chem 280: 42984–42993.

Gallo KA, Mark MR, Scadden DT, Wang ZY, Gu QM, Godowski PJ. (1994). Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity. J Biol Chem 269: 15092-15100.

Gallo KA, Johnson GL. (2002). Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol 3: 663-672.

Goicoechea SM, Bednarski B, Garcia-Mata R, Prentice-Dunn H, Kim HJ, Otey CA. (2009). Palladin contributes to invasive motility in human breast cancer cells. Oncogene 28: 587-598.

Hall JP, Davis RJ. (2002). Inhibition of the p38 pathway upregulates macrophage JNK and ERK activities, and the ERK, JNK, and p38 MAP kinase pathways are reprogrammed during differentiation of the murine myeloid M1 cell line. J Cell Biochem 86: 1-11.

Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. Cell 100: 57-70.

Jacinto A, Woolner S, Martin P. (2002). Dynamic analysis of dorsal closure in Drosophila: from genetics to cell biology. Dev Cell 3: 9-19.

Johnson GL, Lapadat R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298: 1911-1912.

Johung K, Goodwin EC, DiMaio D. (2007) Human papillomavirus E7 repression in cervical carcinoma cells initiates a transcriptional cascade driven by the retinoblastoma family, resulting in senescence. J Virol 81:2102-2116.

Karin M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270: 16483-16486.

Khatlani TS, Wislez M, Sun M, Srinivas H, Iwanaga K, Ma L et al. (2007). c-Jun N-terminal kinase is activated in non-small-cell lung cancer and promotes neoplastic transformation in human bronchial epithelial cells. Oncogene 26: 2658-2666.

Lee GY, Kenny PA, Lee EH, Bissell MJ. (2007). Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 4: 359-365.

Lester RD, Jo M, Campana WM, Gonias SL. (2005). Erythropoietin promotes MCF-7 breast cancer cell migration by an ERK/mitogen-activated protein kinase-dependent

pathway and is primarily responsible for the increase in migration observed in hypoxia. J Biol Chem 280: 39273-7.

Leung IW, Lassam N. (1998) Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3. J Biol Chem 273: 32408-15.

Minet E, Michel G, Mottet D, Piret JP, Barbieux A, Raes M et al. (2001). c-JUN gene induction and AP-1 activity is regulated by a JNK-dependent pathway in hypoxic HepG2 cells. Exp Cell Res 265: 114-124.

Murakata C, Kaneko M, Gessner G, Angeles TS, Ator MA, O'Kane TM et al. (2002). Mixed lineage kinase activity of indolocarbazole analogues. Bioorg Med Chem Lett 12: 147-150.

Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol 3: 785-792.

Nielsen DL, Andersson M, Kamby C. (2009). HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. Cancer Treat Rev 35: 121-136.

Nihalani D, Meyer D, Pajni S, Holzman LB. (2001). Mixed lineage kinase-dependent JNK activation is governed by interactions of scaffold protein JIP with MAPK module components. EMBO J 20: 3447–3458.

Ory DS, Neugeboren BA, Mulligan RC. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 93: 11400-11406.

Polyak K, Weinberg RA. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 9: 265-273.

Radvanyi L, Singh-Sandhu D, Gallichan S, Lovitt C, Pedyczak A, Mallo G et al. (2005). The gene associated with trichorhinophalangeal syndrome in humans is overexpressed in breast cancer. Proc Natl Acad Sci U S A 102: 11005-11010.

Reginato MJ, Mills KR, Becker E, Bonni A, Lynch D, Muthuswamy SK et al. (2005). Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. Mol Cell Biol 25:4591-601.

Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB et al. (2007). Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia 9: 166-180.

Rittling SR, Coutinho L, Amram T, Kolbe M. (1989). AP-1/jun binding sites mediate serum inducibility of the human vimentin promoter. Nucleic Acids Res 17: 1619-1633.

Rizki A, Mott JD, Bissell MJ. (2007). Polo-like kinase 1 is involved in invasion through extracellular matrix. Cancer Res 67: 11106-11110.

Schachter KA, Du Y, Lin A, Gallo KA. (2006). Dynamic positive feedback phosphorylation of mixed lineage kinase 3 by JNK reversibly regulates its distribution to Triton-soluble domains. J Biol Chem 281: 19134-19144.

Shacka JJ, Sahawneh MA, Gonzalez JD, Ye YZ, D'Alessandro TL, Estévez AG. (2006) Two distinct signaling pathways regulate peroxynitrite-induced apoptosis in PC12 cells. Cell Death Differ 13:1506-1514.

Shaw KR, Wrobel CN, Brugge JS. (2004). Use of three-dimensional basement membrane cultures to model oncogene-induced changes in mammary epithelial morphogenesis. J Mammary Gland Biol Neoplasia 9: 297-310.

Simpson KJ, Selfors LM, Bui J, Reynolds A, Leake D, Khvorova A et al. (2008). Identification of genes that regulate epithelial cell migration using an siRNA screening approach. Nat Cell Biol 10: 1027-1038.

Sommers CL, Byers SW, Thompson EW, Torri JA, Gelmann EP. (1994a). Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 31: 325-335.

Sommers CL, Skerker JM, Chrysogelos SA, Bosseler M, Gelmann EP. (1994b). Regulation of vimentin gene transcription in human breast cancer cell lines. Cell Growth Differ 5: 839-846.

Stronach B, Perrimon N. (2002). Activation of the JNK pathway during dorsal closure in Drosophila requires the mixed lineage kinase, slipper. Genes Dev 16: 377-387.

Su S, Li Y, Luo Y, Sheng Y, Su Y, Padia RN et al. (2009). Proteinase-activated receptor 2 expression in breast cancer and its role in breast cancer cell migration. Oncogene 28: 3047-3057.

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K et al. (2008). MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Mol Cell 32: 43-56.

Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS. (1996). Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. J Biol Chem 271: 27225-8.

Timoshenko AV, Rastogi S, Lala PK. (2007). Migration-promoting role of VEGF-C and VEGF-C binding receptors in human breast cancer cells. Br J Cancer 97, 1090–1098.

Vivanco I, Palaskas N, Tran C, Finn SP, Getz G, Kennedy NJ et al. (2007). Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN. Cancer Cell 11: 555-569.

Wagner EF, Nebreda AR. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 9: 537-549.
Wilsbacher JL, Moores SL, and Brugge JS. (2006). An active form of Vav1 induces migration of mammary epithelial cells by stimulating secretion of an epidermal growth factor receptor ligand. Cell Commun Signal 4:5

Vacratsis PO, Gallo KA. (2000) Zipper-mediated oligomerization of the mixed lineage kinase SPRK/MLK-3 is not required for its activation by the GTPase cdc 42 but is necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not catalyze the activating phosphorylation of Thr258 of murine mitogen activated protein kinase kinase 4. J Biol Chem 275: 27893-900.

Yeh YT, Hou MF, Chung YF, Chen YJ, Yang SF, Chen DC et al. (2006). Decreased expression of phosphorylated JNK in breast infiltrating ductal carcinoma is associated with a better overall survival. Int J Cancer 118: 2678-2684.

Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D et al. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. (1997). J Biol Chem 272: 12116-21.

Zajchowski DA, Bartholdi MF, Gong Y, Webster L, Liu HL, Munishkin A et al. (2001). Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res 61: 5168-5178.

Zhang H, Gallo KA. (2001). Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain. J Biol Chem 276:45598-45603.

Zhang H, Wu W, Du Y, Santos SJ, Conrad SE, Watson JT et al. (2004). Hsp90/p50cdc37 is required for mixed-lineage kinase (MLK) 3 signaling. J Biol Chem 279: 19457-19463.

Zhang J, Yang PL, Gray NS. (2009). Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer 9: 28-39.

# CHAPTER III

## MLK3 REGULATES PAXILLIN PHOSPHORYLATION IN CHEMOKINE-MEDIATED MIGRATION AND INVASION AND IS REQUIRED FOR BREAST CANCER METASTASIS

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Part of this chapter has been submitted to *Cancer Research* and is currently in revision.

## Abstract

Mixed-lineage kinase 3 (MLK3, or MAP3K11) can activate multiple MAPKs and is critical in cancer cell migration and invasion. In the tumor microenvironment, prometastatic factors drive breast cancer invasion and metastasis, but their associated signaling pathways are not well-known. Herein, we provide evidence that MLK3 is required for chemokine (CXCL12)-induced invasion of basal breast cancer cells. Phosphorylation of the focal adhesion scaffold, paxillin, (by FAK/Src) is a key event leading to suppression of Rho activity, focal adhesion turnover and rapid cancer cell migration. We provide novel evidence that MLK3 induces robust phosphorylation of paxillin on Ser 178 and Tyr 118, whereas silencing or inhibition of MLK3-JNK blocks these phosphorylation events elicited by the prometastatic factors, CXCL12 and HGF. Our data suggest that MLK3 controls Tyr phosphorylation of paxillin through recruitment of FAK to paxillin. Silencing or inhibition of MLK3, inhibition of JNK, or expression of paxillin S178A all lead to enhanced Rho activity. These data indicate that the MLK3-JNK-paxillin axis limits Rho activity to promote focal adhesion turnover and migration. Consistent with this model, in breast cancer cells, MLK3 silencing results in increased focal adhesions and stress fibers. Finally, MLK3 silencing decreases formation of breast cancer lung metastases in a mouse xenograft model. In addition, breast cancer cells derived from mouse lung metastases show enhanced Ser 178 paxillin phosphorylation, which can be inhibited by blocking MLK activity. Our results reveal a novel MLK3-JNK-paxillin signaling axis as a potential therapeutic target and/or prognostic factor in breast cancer metastasis.

## Introduction

Significant decreases in breast cancer mortality have been achieved in recent years, due to improved diagnosis and treatment. However, approximately 40,000 deaths annually in the US are due to breast cancer (DeSantis et al., 2011), primarily from metastasis to distant organs. Metastasis is a multistep process requiring tumor cell migration, intravasation, survival in circulation, extravasation and colonization to a secondary site. Interrupting the metastatic process is key to reducing breast cancer mortality.

Chemokines and growth factors drive breast cancer migration, invasion and metastasis. For example, the chemokine CXCL12/SDF1-α, binds its cognate G-protein-coupled receptor, CXCR4, to promote cytoskeletal remodeling and migration in human breast cancer cells (Luker and Lucker, 2006); and the CXCL12-CXCR4 signaling is critical for breast cancer metastasis in mouse xenograft models (Luker and Lucker, 2006; Muller et al., 2001). Furthermore, high levels of CXCR4 are found in breast tumor cells isolated from pleural effusions (Dupont et al., 2007), and correlate with both lymph node metastases (Kato et al., 2003) and poor overall survival in patients (Kato et al., 2003; Li et al., 2004). Hepatocyte growth factor/scatter factor (HGF), through binding to its cognate tyrosine kinase receptor, c-met, promotes cell motility and invasion (Birchmeier et al., 2003). Aberrant c-met signaling and the MET oncogene have been implicated in breast cancer progression, particularly in the basal subtype (Gastaldi et al., 2010), and c-met overexpression predicts poor outcome in breast cancer patients (Kang et al., 2003; Lengyel et al., 2005).

MAPK signaling contributes to breast cancer cell migration, invasion, and metastasis. MLK3 is a MAPKKK that regulates the three major MAPK pathways (Chadee and Kyriakis, 2004). MLK3 contains an N-terminal SH3 domain, followed sequentially by a serine/threonine kinase domain, leucine zippers, a Cdc42/Rac interactive binding (CRIB) motif, and a C-terminal Pro-rich region. MLK3 is autoinhibited through its SH3 domain. Binding of GTP-bound Rac or Cdc42 through MLK3's CRIB motif disrupts SH3-mediated autoinhibition, promotes homodimerization through the leucine zippers, resulting in transautophosphorylation within the kinase domain and yielding the active kinase (Gallo and Johnson, 2002; Zhang and Gallo, 2001; Du et al., 2005). MLK3 regulates cancer cell migration and invasion (Simpson et al., 2008; Chen et al., 2010; Swenson-Fields et al., 2008; Zhan et al., 2011; Mishra et al., 2010). In our recent studies, we demonstrated that MLK3 signaling through JNK to the transcription factor AP-1 is required for MLK3-mediated migration and invasion in human mammary basal epithelial and breast cancer cells. MLK3-mediated up-regualtion of AP1 signaling induces expression of several genes associated with invasive breast cancer (Chen et al., 2010).

The mechanics of cancer cell migration involve cytoskeletal remodeling and focal adhesion dynamics (Ridley et al., 2003). Paxillin is a multi-domain adaptor protein which localizes to focal adhesions, the multiprotein complexes that bridge the extracellular matrix and cytoskeleton. The dynamics of focal adhesion assembly and disassembly are controlled by protein interactions and phosphorylation events within the paxillin signaling hub. Paxillin, itself, is phosphorylated at numerous sites (Brown and Turner, 2004). For example, recruitment of FAK to focal adhesions and subsequent

tyrosine phosphorylation of paxillin leads to focal adhesion disassembly (Brown and Turner, 2004; Webb et al., 2004; Zaidel-Bar et al., 2007). Phosphorylation of paxillin on Ser 178 by JNK is required for focal adhesion disassembly and migration in a basal breast cancer cell line (Huang et al., 2003).

Small Rho family GTPases include Rac, Cdc42 and Rho (Hall, 1998). Temporal and spatial activation of Rho GTPases is tightly controlled in migrating cells (Ridley et al., 2003). Disrupting the activity cycle of Rho family GTPases results in inefficient cell migration (Nobes and Hall, 1999). Cdc42 regulates the formation of filopodia, while Rac controls the formation of lamellipodia. Rho triggers the formation of stress fibers and regulates focal adhesion formation (Hall, 1998). Phosphorylation of paxillin on Tyr 31 and Tyr 118 leads to enhanced Rac activity and decreased RhoA activity ( Valles et al., 2004; Tsubouchi et al., 2002).

In this study, we demonstrate that MLK3-JNK signaling is required for migration and invasion in response to the prometastatic factor, CXCL12. Phosphorylation of the focal adhesion scaffold, paxillin, (by FAK/Src) leads to focal adhesion turnover and rapid cancer cell migration. We provide evidence that MLK3-activated JNK phosphorylates paxillin on Ser 178, which in turn recruits FAK to paxillin, resulting in tyrosine phosphorylation. Disruption of the MLK3-JNK-paxillin signaling pathway increases Rho activity, focal adhesions, and stress fibers in basal breast cancer cells. MLK3 silencing decreases formation of breast cancer lung metastases in a mouse xenograft model. In addition, breast cancer cells derived from mouse lung metastases show enhanced Ser 178 paxillin phosphorylation. Our results reveal a novel MLK3-JNK-paxillin signaling

axis as a potential therapeutic target and/or prognostic factor in breast cancer metastasis.

## Experimental procedures

*Cell lines, Chemicals, Antibodies, DNA constructs and siRNAs*- Human mammary epithelial and breast cancer cell lines were from ATCC. MDA-MB-231-luc2-tdTomato cells were from Caliper Life Sciences. Cell line authentication was performed using STR and amelogenin profiling. Antibodies against p-ERK (T202/Y204), p-JNK (T183/Y185), p-MLK3 (T277/S281), p-paxillin (Y118) were from Cell Signaling Biotechnology. ERK, JNK and FAK antibodies were from Santa Cruz Biotechnology. Anti-Flag M2, HA, vinculin and actin monoclonal antibodies were from Sigma. The antibody against p-paxillin (S178) was from Bethyl Laboratories. Anti-paxillin antibody was from Millipore. Additional antibodies were the MLK3 antibody (homemade or from Epitomics), CD44 antibody (Abcam), GFP (Clontech). Recombinant human CXCL12/SDF-α was from R&D systems. Collagen I and Matrigel were from Becton Dickinson. Pharmacological inhibitors SP600125, U0126 and SB203580 were from Calbiochem and CEP-11004, CEP-1347 were generously provided by Cephalon. Flag-MLK3 or MLK3 K144R constructs were described previously (Du et al., 2005). GFP-FAK was a gift from Dr. Jun-Lin Guan (University of Michigan). HA–PaxS178A construct was generated from wild type HA-Pax construct (a gift from Dr. Ravi Salgia, University of Chicago) using site-directed mutagenesis (Stratagene) following the manufacturer's instructions. MLK3 siRNAs #1 Mlk3 siRNA (5'-GGGCAGUGACGUCUGGAGUUU-3') and #2 Mlk3 siRNA (5'-CUGGAGGACUCAAGCAAUG-3') were from Dharmacon (Chadee and Kyriakis,

2004; Simpson et al., 2008). JNK1/2 siRNA (5'-AAA GAA UGU CCU ACC UUC U-3') was from Qiagen (Li et al., 2004). AP21967 was generously provided by Ariad Pharmaceuticals.

*Stable cell populations and transfections*- MCF10A-MLK3 cells (Chen et al., 2010) were maintained in growth media containing G418 (50 μg/ml) and hygromycin (12.5 μg/ml). MCF10A-MLK3 cells were treated with vehicle (ethanol) or 50 nM AP21967 (Ariad) to induce expression of MLK3. MDA-MB-231 cells expressing pSuper control or Mlk3 shRNA have been described (Chen et al., 2010). Transfection of DNA constructs (1-2 μg DNA in 6 cm dish) was performed using Lipofectamine 2000 (Invitrogen). siRNA transfection (30-100 nM) was performed using INTERFERin (Polyplus-transfection). Forty-eight hours posttransfection, the cells were subjected to migration, invasion assays, immunofluorescence or immunoblotting.

*Immunoblotting, co-immunoprecipitations, and Rho GTPase assays*- Preparation of cellular lysates and immunoblotting was as previously described (Chen et al., 2010). Western blots were developed by chemiluminescence method using x-ray film or visualized by fluorescence using the LI-COR Odyssey infrared scanner (LI-COR). Co-immunoprecipitation experiments were performed as previously described (Zhang and Gallo, 2001). Rho-GTP was measured using the Rhotekin-RBD pulldown assay (Cytoskeleton). Briefly, cells were lysed in ice-cold Triton X100 lysis buffer and cleared cellular extracts (500 μg) were incubated with the Rhotekin-RBD agarose beads (10 μg). Beads were pelleted, washed and resuspended in 1.5x SDS sample buffer. GTP-bound Rho was detected by immunoblotting.

*Migration and invasion assays*- Chemotactic migration was quantified using a Boyden chamber transwell assay as previously described (Chen et al., 2010). The chemoattractant in the lower chamber was 100 ng/ml CXCL12. For invasion assays, the chambers were coated with Matrigel (1:5 dilution in DMEM/F12).

*Immunofluorescence*- After formaldehyde fixation, the cells were permeabilized with 0.5% Triton X-100, blocked in 4% BSA, and stained with anti-vinculin antibody (1:200 dilution), followed by Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution). To visualize stress fibers, cells were stained with Alexa-Fluor conjugated phalloidin (1:50, Invitrogen), and nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml). Images were acquired using an Olympus FV1000 confocal laser scanning microscope. Focal adhesions were quantified using Image J software.

*Spontaneous metastasis model*- All the experiments involving animals were performed in accordance with standard protocols approved by the All University Committee on Animal Use and Care at Michigan State University. Female athymic nu/nu mice (6 week old, Harlan Laboratory) were maintained in microisolation cages under specific pathogen-free conditions. MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 ($2x10^6$ cells/site) were resuspended in a solution containing PBS and Matrigel (1:1 v/v) and surgically inoculated into No. 4 mammary glands. Tumors were measured using a caliper twice weekly. Mice were euthanized after 7 weeks, primary tumors were excised and lysed using RIPA buffer. The mouse lungs were removed, fixed in formalin overnight and paraffin sections were subjected to analysis using anti-CD44 specific human antibody and Vectastain Elite ABC kits and DAB Substrate (Vector laboratory).

Metastatic nodules were quantified in 10 lung sections per mouse and statistical analysis was performed using the GraphPad Prism 5 software.

*Experimental metastasis model*- MDA-MB-231-Luc2-tdTomato cells ($10^6$ in 100 µl saline) were injected into tail vein of athymic nu/nu mice. Weekly, following i.p injection with D-luciferin (150 mg/kg) mice were imaged using the Caliper IVIS Spectrum system. After 12 weeks, lungs containing MDA-MB-231 metastases were extracted, minced and cultured in puromycin (2 µg/ml). The recovered MDA-MB-231 cells were designated as MDA-MB-231 Lu cells.

## Results

**MLK3 is required for migration and invasion of triple-negative breast cancer cells towards CXCL12.** The chemokine CXCL12 plays a critical role in breast cancer cell migration and metastasis (Muller et al., 2001; Holland et al., 2006). To investigate whether MLK3 plays a role in CXCL12-induced migration and invasion, MLK3 was ablated in the highly invasive, basal-like breast cancer cell lines, MDA-MB-231 and BT549, both of which express high levels of CXCR4 (Holland et al., 2006). In a transwell migration assay, treatment with CXCL12 increased the migration of MDA-MB-231 cells expressing control vector by approximately 3-fold. MLK3 expression is efficiently ablated in this stable population of MDA-MB-231 shMlk3 cells (Chen et al., 2010). CXCL12-induced migration was completely blocked in MDA-MB-231 cells stably expressing shMlk3 or treated with CEP-1347, a selective MLK inhibitor (Figure 3.1A). As shown in Figure 3.1A, CXCL12 activated the JNK pathway. To confirm the efficacy of CEP-1347, we used phospho-JNK (p-JNK) as a readout for active MLK signaling.

Figure 3.1

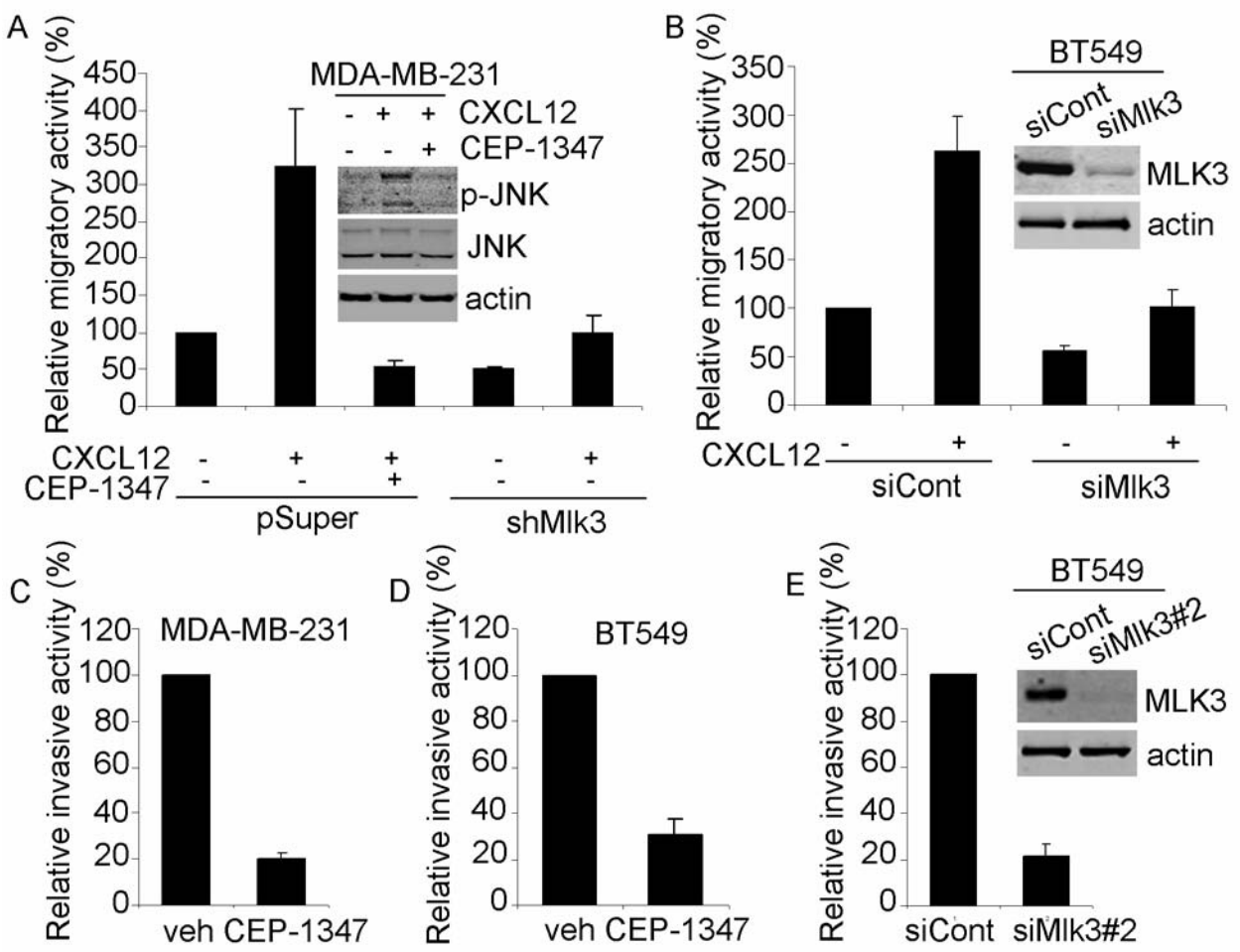
A
Relative migratory activity (%)
MDA-MB-231
CXCL12
CEP-1347
p-JNK
JNK
actin
CXCL12
CEP-1347
pSuper
shMlk3
B
Relative migratory activity (%)
BT549
siCont
siMlk3
MLK3
actin
CXCL12
siCont
siMlk3
C
Relative invasive activity (%)
MDA-MB-231
veh CEP-1347
D
Relative invasive activity (%)
BT549
veh CEP-1347
E
Relative invasive activity (%)
BT549
siCont
siMlk3#2
MLK3
actin
siCont
siMlk3#2

Figure 3.1 Silencing or inhibition of MLK3 blocks CXCL12-induced migration and invasion of basal breast cancer cells. (A) MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 were serum-deprived for 48 h and treated with CXCL12 (100 ng/ml) in the presence or absence of CEP-1347 (400 nM) for 30 min. Total cellular lysates were analyzed by western blotting using the indicated antibodies (upper panel). MDA-MB-231 cells stably expressing pSuper or pSuper-Mlk3 shRNA were subjected to a transwell migration assay. Cells were allowed to migrate towards CXCL12 (100 ng/ml) for 24 h. Migrated cells were counted in 5 random fields and the average cell number per field is presented. Column, mean of three experiments. Bar, SE. (B) BT549 cells were treated with control or Mlk3 siRNA for 48 h, serum-deprived overnight and then subjected to a transwell migration assay. Cells were allowed to migrate towards CXCL12 (100 ng/ml) for 48 h. (C) MDA-MB-231 cells were pretreated with DMSO or 400 nM CEP-1347 for 6 h in serum-free medium and subjected to a transwell invasion assay. Cells were allowed to invade towards CXCL12 (100 ng/ml) for 24 h. (D) BT549 cells were pretreated with DMSO or 400 nM CEP-1347 for 6 h in serum-free medium and subjected to a transwell invasion assay. Cells were allowed to invade towards CXCL12 (100 ng/ml) for 48 h. (E) BT549 cells were treated with control or Mlk3 siRNA for 48 h, serum-deprived overnight and subjected to a transwell invasion assay. Cells were allowed to invade towards CXCL12 (100 ng/ml) for 48 h. Column, mean of four experiments. Bar, SE.

Immunoblotting of total cellular lysates with a p-JNK antibody showed that CEP-1347 blocked CXCL12-induced JNK activation (Figure 3.1A). In addition, transient silencing of MLK3 in BT549 cells reduced CXCL12-induced migration (Figure 3.1B).

In a Matrigel invasion assay using CXCL12 as a chemoattractant, CEP-1347 reduced invasion of MDA-MB-231 cells by approximately 5-fold (Figure 3.1C). In addition, CEP-1347 blocked invasion of BT549 cells (Figure 3.1D). Silencing of MLK3 in BT549 cells also largely inhibited invasion (Figure 3.1E). Mlk3 silencing had negligible effect on proliferation of BT549 cells (Supplementary figure 3.1). Taken together, these results support the idea that MLK3 signaling is required for migration and invasion of invasive basal breast cancer cells in response to CXCL12.

**Active MLK3 promotes phosphorylation of paxillin through JNK**. We recently showed that induced expression of MLK3 promotes migration and invasion of MCF10A mammary epithelial cells, which requires the JNK-AP1 signaling axis (Chen et al., 2010). We hypothesized that, in addition to its impact on gene expression, MLK3-JNK signaling might act upon cytoskeletal or focal adhesion proteins to regulate cell migration and invasion. Phosphorylation of Ser 178 of paxillin by JNK is necessary for focal adhesion turnover and cell migration ( Huang et al., 2003).

To investigate whether MLK3 can promote phosphorylation of paxillin, MCF10A cells engineered to inducibly express MLK3 were used (Chen et al., 2010). Upon induction of MLK3, JNK was activated and robust phosphorylation of Ser 178 of paxillin was observed. Inhibition of MLK3 with K252a, or of JNK with SP600125, blocked phosphorylation of paxillin at Ser 178. In contrast, inhibition of ERK signaling with U0126, or p38 with SB203580, had no effect on MLK3-induced paxillin phosphorylation

(Figure 3.2A). Like K252a, its derivative CEP-1347 blocked MLK3-induced paxillin phosphorylation (Supplementary figure 3.2). Consistent with these findings, in MDA-MB-231 cells, transient expression of wildtype MLK3, but not kinase inactive, MLK3 K144R, induced JNK activation and phosphorylation of paxillin at Ser 178 (Figure 3.2B). Furthermore, silencing of JNK1/2 in MDA-MB-231 cells decreased phosphorylation of paxillin at Ser 178 (Figure 3.2C), confirming the requirement for JNK in paxillin phosphorylation. Thus the MLK3-JNK signaling axis promotes paxillin phosphorylation at Ser 178.

**Chemokine and growth factor can induce paxillin phosphorylation in an MLK3-dependent manner.** To determine whether MLK3 is required for phosphorylation of paxillin at Ser 178, MDA-MB-231 cells stably expressing control vector or shMlk3 were serum-deprived, followed by treatment with 10% serum and paxillin phosphorylation was assessed. Serum treatment led to JNK activation and maximal phosphorylation of paxillin at Ser 178 at 30 min, both of which were largely abrogated in cells expressing shMlk3 (Figure 3.3A). These data point to MLK3 is a major mediator of JNK signaling to paxillin in breast cancer cells in response to serum. Since phosphorylation of paxillin on Ser 178 is associated with breast cancer migration, we investigated the impact of the promigratory factors, CXCL12 and HGF, on paxillin phosphorylation. As shown in Figure 3.3B, both CXCL12 and HGF induced JNK activation and phosphorylation of paxillin at Ser 178 in MDA-MB-231 cells; and both JNK activation and paxillin Ser 178 phosphorylation were reduced by treatment with the MLK inhibitor, CEP-1347.

Figure 3.2

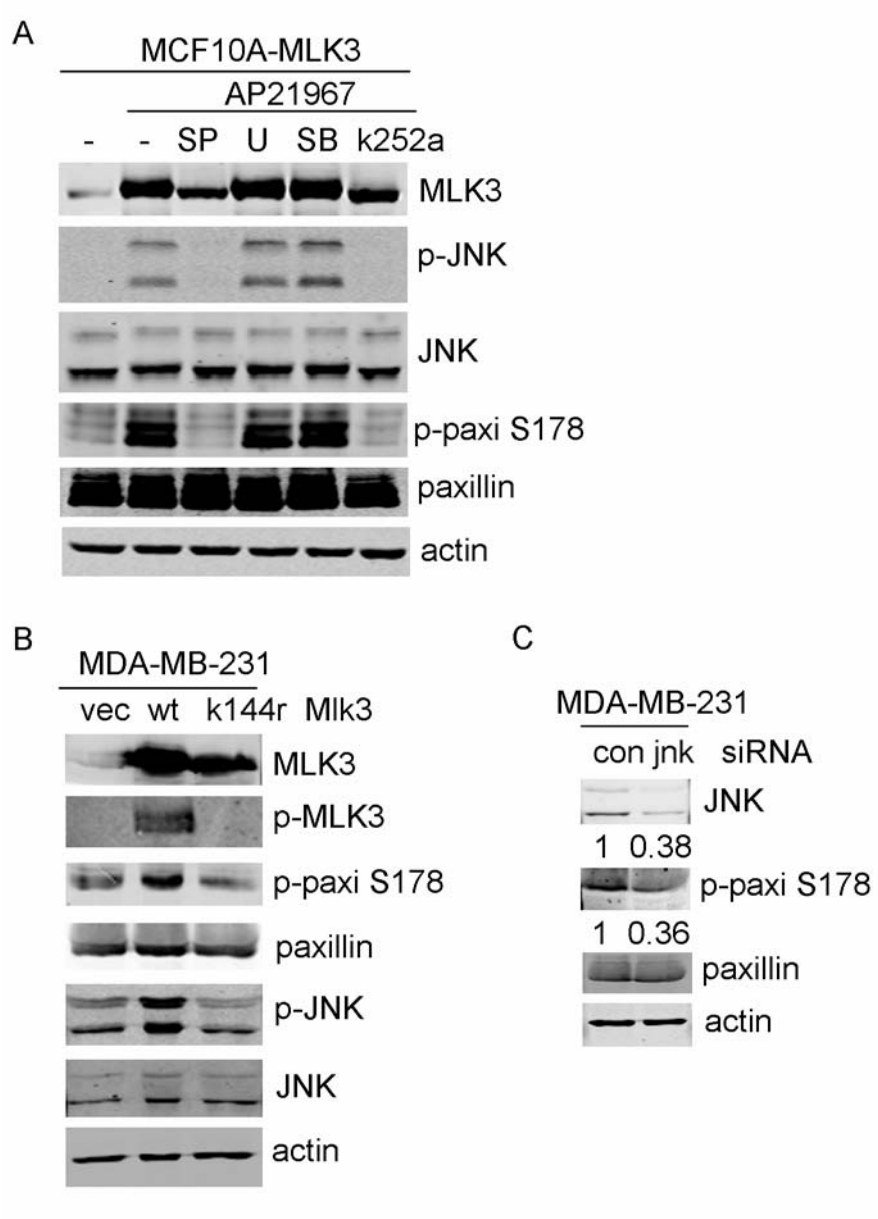
A
MCF10A-MLK3
AP21967
- - SP U SB k252a
MLK3
p-JNK
JNK
p-paxi S178
paxillin
actin
B
MDA-MB-231
vec wt k144r Mlk3
MLK3
p-MLK3
p-paxi S178
paxillin
p-JNK
JNK
actin
C
MDA-MB-231
con jnk siRNA
JNK
1 0.38
p-paxi S178
1 0.36
paxillin
actin

Figure 3.2 MLK3-JNK signaling promotes phosphorylation of paxillin at Ser 178. (A) Effects of MAPK pathway inhibitors on signaling pathways in MCF10A-MLK3 cells. MCF10A-MLK3 cells were treated with vehicle or 50 nM AP21967 for 20 h along with the indicated inhibitors: 15 μM SP600125, 10 μM U0126, 10 μM SB203580 or 400 nM K252a, for an additional 24 h. Cellular lysates were analyzed by western-blotting with the indicated antibodies. (B) MDA-MB-231 cells were transfected with empty vector, or expression vectors for wildtype MLK3 or kinase inactive mutant MLK3 K144R for 24 h. Cellular lysates were analyzed by western-blotting. (C) MDA-MB-231 cells were treated with control or JNK1,2 siRNA for 48 h. Cellular lysates were analyzed by western-blotting with the indicated antibodies. Quantitation of blots normalized to actin was performed using LI-COR Odyssey software V3.0.

Figure 3.3

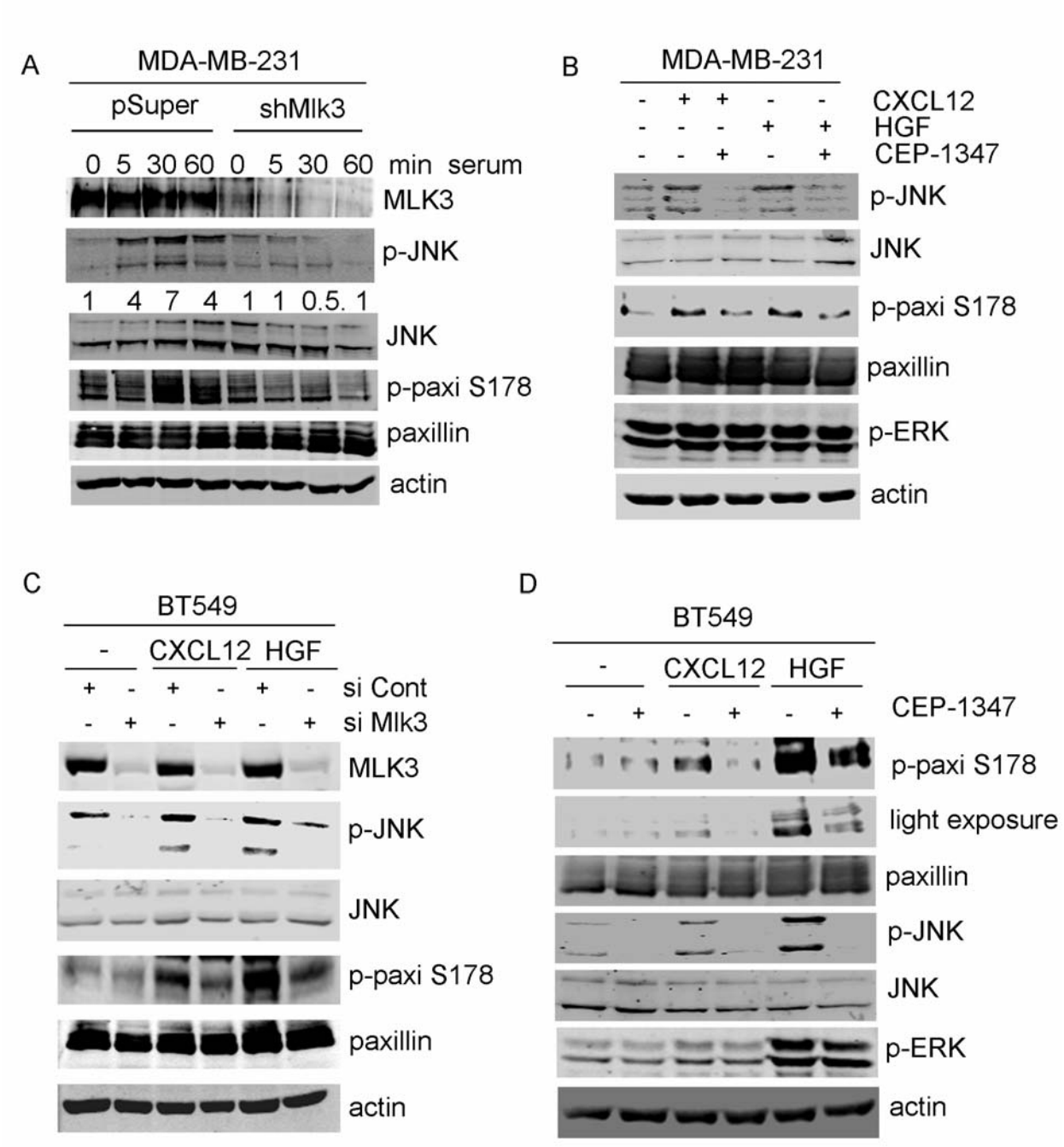
A
MDA-MB-231
pSuper
shMlk3
0 5 30 60 0 5 30 60 min serum
MLK3
p-JNK
1 4 7 4 1 1 0.5. 1
JNK
p-paxi S178
paxillin
actin
B
MDA-MB-231
CXCL12
HGF
CEP-1347
p-JNK
JNK
p-paxi S178
paxillin
p-ERK
actin
C
BT549
- CXCL12 HGF
si Cont
si Mlk3
MLK3
p-JNK
JNK
p-paxi S178
paxillin
actin
D
BT549
- CXCL12 HGF
CEP-1347
p-paxi S178
light exposure
paxillin
p-JNK
JNK
p-ERK
actin

Figure 3.3 MLK3 silencing or an MLK inhibitor impairs paxillin phosphorylation at Ser 178. (A) MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 were serum-deprived for 72 h, followed by addition of medium containing 10% serum for the indicated times. Total cellular lysates were analyzed by western blotting using the indicated antibodies. Quantitation of p-JNK/JNK determined by LI-COR Odyssey software V3.0 is shown. (B) MDA-MB-231 cells were serum-deprived for 72 h and pretreated with CEP-1347 (400 nM) for 6 h, followed by treatment of CXCL12 (100 ng/ml) or HGF (100 ng/ml) for 30 min. Cellular lysates were analyzed by western blotting. (C) BT549 cells were transfected with control or Mlk3 siRNA for 24 h and serum-deprived overnight, followed by treatment of CXCL12 (100 ng/ml) or HGF (100 ng/ml) for 30 min. Cellular lysates were analyzed by western blotting. (D) BT549 cells were serum-deprived overnight and pretreated with CEP-1347 (400 nM) for 6 h, followed by treatment of CXCL12 (100 ng/ml) or HGF (100 ng/ml) for 30 min. Cellular lysates were analyzed by western blotting.

Likewise, in BT549 cells, based on 4 independent experiments, both CXCL12 and HGF induced JNK activation (3 and 3.4-fold, respectively) and paxillin phosphorylation at Ser 178 (3.2 and 4.4-fold, respectively), which was attenuated by silencing with Mlk3 siRNA (Figure 3.3C). MLK inhibition with CEP-1347 also reduced both JNK activation and Ser 178 paxillin phosphorylation (Figure 3.3D). The requirement of MLK3 in paxillin phosphorylation was confirmed using a different siRNA sequence (Supplementary figure 3.3 A and B). In BT549 cells, HGF potently activated ERK, whereas only a small increase in ERK activation was observed in response to CXCL12. ERK activation was refractory to the MLK inhibitor, consistent with the proposed scaffolding role of MLK3 in ERK activation (Chadee et al., 2004). Notably, MDA-MB-231 cells harbor activating mutations in both Ras and Raf (Hollestelle et al., 2007), resulting in constitutive ERK activation. Thus, it is not too surprising that CXCL12 and HGF have relatively little effect on ERK activity in MDA-MB-231 cells. In summary, these data show a critical role for active MLK3 in JNK activation and paxillin phosphorylation at Ser 178, triggered by either a prometastatic chemokine or growth factor in basal breast cancer cells.

**MLK3 controls Tyr 118 phosphorylation of paxillin and its association with focal adhesion kinase.** Cell migration requires efficient assembly and disassembly of focal adhesion complexes. Paxillin undergoes phosphorylation at multiple sites to modulate protein-protein interactions in focal adhesions (Brown and Turner, 2004). The role of Ser 178 phosphorylation of paxillin is not completely clear. In corneal epithelial cells, Ser 178 phosphorylation of paxillin recruits FAK leading to tyrosine phosphorylation of paxillin (Huang et al., 2008).

Since we have shown that MLK3 is critical for Ser 178 phosphorylation of paxillin, we tested whether MLK3 indirectly modulates tyrosine phosphorylation of paxillin and regulates interactions among focal adhesion proteins. Ectopic expression of MLK3 in BT549 cells promoted both Ser 178 and Tyr 118 paxillin phosphorylation, demonstrating that active MLK3 drives Tyr 118 phosphorylation of paxillin (Figure 3.4A). In response to serum stimulation of MDA-MB-231 cells, the MLK inhibitor, CEP-1347, reduced Tyr 118 phosphorylation of paxillin by 2-fold at 5 min and 3.5-fold at 20 min, paralleling the effects of JNK inhibition (Figure 3.4B). Consistent with these data, HGF-induced Tyr 118 phosphorylation of paxillin was dramatically reduced in MDA-MB-231 cells in which Mlk3 had been stably silenced (Figure 3.4C). From these data we conclude that MLK3 is critical for Tyr 118 phosphorylation of paxillin. To test whether MLK3 influences association of paxillin with FAK, we took advantage of 293T cells as an efficient cotransfection system. Ectopically expressed GFP-FAK and HA-paxillin showed weak association in coimmunoprecipitations from serum-deprived 293T cells (Figure 3.4D). EGF has previously been shown to facilitate the interaction between FAK and paxillin (Huang et al., 2008). Our data confirm that the association between GFP-FAK and HA-paxillin is enhanced by stimulation with EGF (Figure 3.4D). However, pre-treatment with the MLK inhibitor, CEP-1347, abrogated the EGF-induced association of GFP-FAK and HA-paxillin (Figure 3.4D). We were able to detect endogenous paxillin in a FAK immunoprecipitate from MDA-MB-231 cells in growth medium, which was reduced by CEP-1347. In the immunoprecipitated FAK complex, levels of Ser 178 phosphorylated paxillin and total paxillin correlate directly, consistent with the idea that Ser 178 phosphorylation drives association of FAK with paxillin (Figure 3.4E).

Conversely, Flag-MLK3 was ectopically expressed in MDA-MB-231 cells and FAK association with paxillin was assessed using a co-immunoprecipitation assay. Ectopic expression of Flag-MLK3 in MDA-MB-231 cells robustly increased the interaction of endogenous paxillin with endogenous FAK as well as the phosphorylation of paxillin at both Ser 178 and Tyr 118 (Figure 3.4F). These data, taken together, provide strong evidence that MLK3 regulates both paxillin phosphorylation and protein interactions.

**MLK3 silencing results in accumulation of focal adhesions at the cell periphery.** MLK3 modulates phosphorylation of Ser 178 and Tyr 118 of paxillin, which is required for focal adhesion disassembly (Zaidel-Bar et al., 2007; Huang et al., 2003). Consistent with this, silencing of MLK3 in BT549 cells increased the number of focal adhesions at the cell periphery, which were quantified as vinculin-staining focal adhesions (Figure 3.5A, B). Similar effects were observed using two different Mlk3 siRNA sequences. Silencing of MLK3 had no effect on total vinculin levels as shown by immunoblotting of cellular lysates(Figure 3.5C). Our data suggest that MLK3 is important for focal adhesion turnover.

**MLK3-JNK-paxillin signaling negatively regulates Rho activity**. Phosphorylation of Tyr 118 of paxillin leads to decreased Rho activity, enhancing focal adhesion turnover and cell migration (Tsubouchi et al., 2002). Ectopic expression of MLK3 and wildtype paxillin in 293T cells resulted in a high phospho Ser 178 –paxillin signal in immunoblots of cellular lysates. As expected, no phospho Ser 178-paxillin signal was detected upon coexpression of the phosphorylation-defective mutant of paxillin S178A with MLK3 (Supplementary figure 3.4A). In vector control 293T cells or in 293T cells expressing wildtype paxillin, Tyr 118 phosphorylation of paxillin was

Figure 3.4

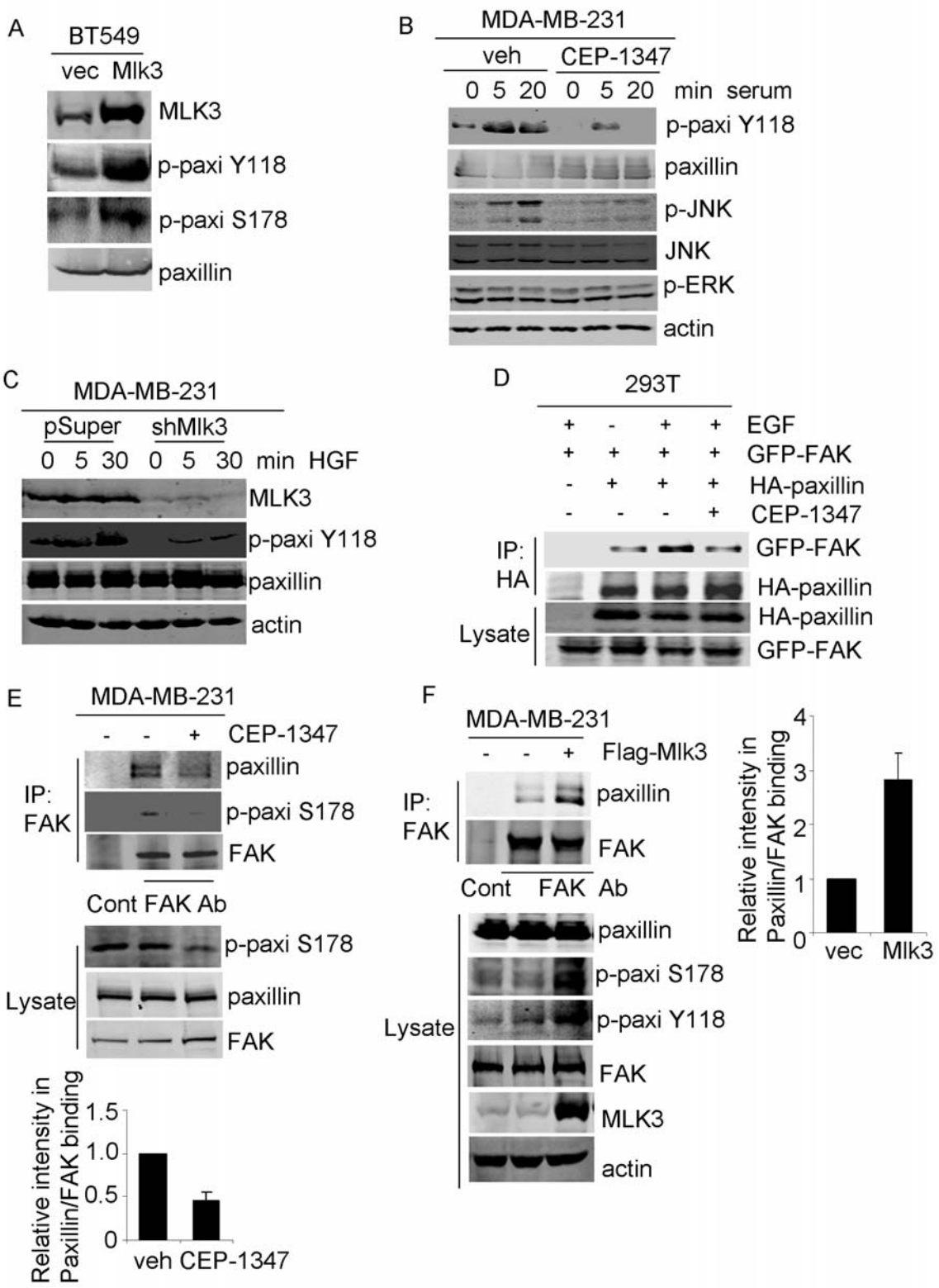
A
BT549
vec Mlk3
MLK3
p-paxi Y118
p-paxi S178
paxillin
B
MDA-MB-231
veh CEP-1347
0 5 20 0 5 20 min serum
p-paxi Y118
paxillin
p-JNK
JNK
p-ERK
actin
C
MDA-MB-231
pSuper shMlk3
0 5 30 0 5 30 min HGF
MLK3
p-paxi Y118
paxillin
actin
D
293T
+ - + + EGF
+ + + + GFP-FAK
- + + + HA-paxillin
- - - + CEP-1347
IP: HA
GFP-FAK
HA-paxillin
Lysate
HA-paxillin
GFP-FAK
E
MDA-MB-231
- - + CEP-1347
IP: FAK
paxillin
p-paxi S178
FAK
Cont FAK Ab
Lysate
p-paxi S178
paxillin
FAK
Relative intensity in Paxillin/FAK binding
1.5
1.0
0.5
0
veh CEP-1347
F
MDA-MB-231
- - + Flag-Mlk3
IP: FAK
paxillin
FAK
Cont FAK Ab
Lysate
paxillin
p-paxi S178
p-paxi Y118
FAK
MLK3
actin
Relative intensity in Paxillin/FAK binding
4
3
2
1
0
vec Mlk3

Figure 3.4 MLK3 promotes Tyr 118 phosphorylation of paxillin and the interaction of FAK with paxillin. (A) BT549 cells were transiently transfected with control vector or Flag-Mlk3 vector. Total cellular lysates were analyzed by western blotting with the indicated antibodies. (B) MDA-MB-231 cells were serum-deprived for 72 h and pretreated with CEP-1347 (400 nM) for 6 h, followed by treatment with serum-containing medium for the indicated times. Cellular lysates were analyzed by western blotting. (C) MDA-MB-231 control cells or cells depleted of MLK3 were serum-deprived for 72 h, followed by addition of HGF (100 ng/ml). Cellular lysates were analyzed by western blotting. (D) 293T cells were cotransfected, as indicated, with vectors expressing GFP-FAK and HA-paxillin for 24 h. Cells were treated with serum-free medium and CEP-1347 for 6 h, followed by treatment with EGF (100 ng/ml) for the indicated times. Total cellular lysates were incubated with anti-HA antibody-conjugated agarose beads. Lysates and immunoprecipitates were subjected to western-blotting analysis. (E) MDA-MB-231 cells were treated with DMSO or CEP-1347 (400 nM) overnight. Cellular lysates were subjected to immunoprecipitation using a rabbit control IgG or an anti-FAK antibody, followed by western-blotting analysis of the lysates and immunoprecipitates. The intensities of western blotting signals were quantified using Odyssey software V3.0. The results are expressed as the ratio of the relative intensities of FAK to paxillin with control (=1). Column, mean of three experiments. Bar, SE. (F) MDA-MB-231 cells were transiently transfected with control vector or Flag-Mlk3. After 24-48 h, cellular lysates were subjected to immunoprecipitation using control IgG or anti-FAK antibody, followed by western-blotting analysis of the lysates and immunoprecipitates. Quantitation of FAK and paxillin association was as described in (E).

Figure 3.5

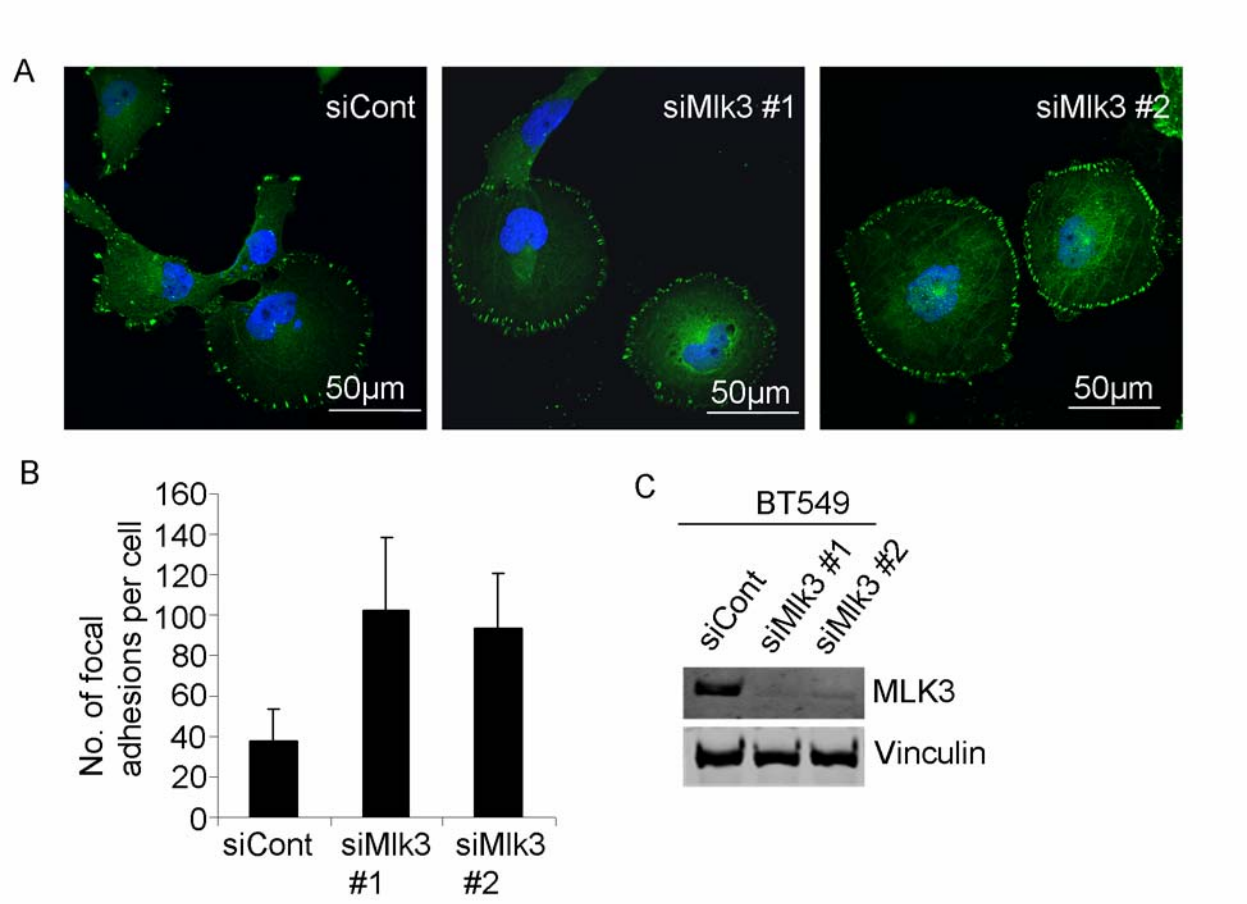


Figure 3.5 MLK3 knockdown increases the number of vinculin-containing focal adhesions. (A) BT549 cells were transfected with control siRNA or two different Mlk3 siRNAs for 48 h. Cells on the coverslips were fixed, stained with anti-vinculin antibody (green) and nuclei were stained with DAPI (blue). Images were taken using Olympus FluoView confocal microscope. Bar, 50 μm. (B) Quantitation of numbers of vinculin-containing focal adhesions. Vinculin-positive focal adhesions were quantified using Image J software. Over 20 cells were quantified in each group. Column, mean of two experiments. Bar, SE. (C) BT549 cells were transfected with control siRNA or Mlk3 siRNAs for 48 h. Cellular lysates were analyzed by western-blotting.

observed. However, the paxillin mutant S178A was refractory to serum-induced Tyr 118 phosphorylation, consistent with the idea that Ser 178 phosphorylation of paxillin is a prerequisite to Tyr 118 phosphorylation (Supplementary figure 3.4B). Likewise, in BT549 breast cancer cells, ectopically expressed wildtype paxillin, but not paxillin S178A, was phosphorylated on Tyr 118 (Figure 3.6A).

An increase in cellular stress fibers is observed in keratinocytes upon expression of the phosphorylation-defective mutant of paxillin S178A (Huang et al., 2003). As shown in Figure 3.6B, a similar phenotype was observed upon silencing of MLK3 in BT549 breast cancer cells. Since Rho promotes stress fiber formation, we assessed whether silencing or inhibition of MLK3 and its signaling to JNK affects Rho activity in breast cancer cells. In both MDA-MB-231 and BT549 cells, Rho activity was increased upon silencing of MLK3 (Figure 3.6C). Furthermore, inhibition of MLK3 catalytic activity, using CEP-1347, as well as inhibition of downstream signaling to JNK with SP600125, increased Rho activity in MDA-MB-231 cells (Figure 3.6D, E).

If the mechanism by which MLK3-JNK suppresses Rho activity is through Ser 178 phosphorylation of paxillin, then expression of a phosphorylation-defective mutant of paxillin, S178A, should enhance Rho activity in MDA-MB-231 cells. As shown in Figure 3.6F, expression of paxillin S178A, which fails to undergo Tyr 118 phosphorylation, resulted in a marked increase of Rho activity and decreased migration of MDA-MB-231 cells (Figure 3.6G). These data reveal the MLK3-JNK-paxillin signaling axis as a negative modulator of Rho activity in basal breast cancer cells.

Figure 3.6

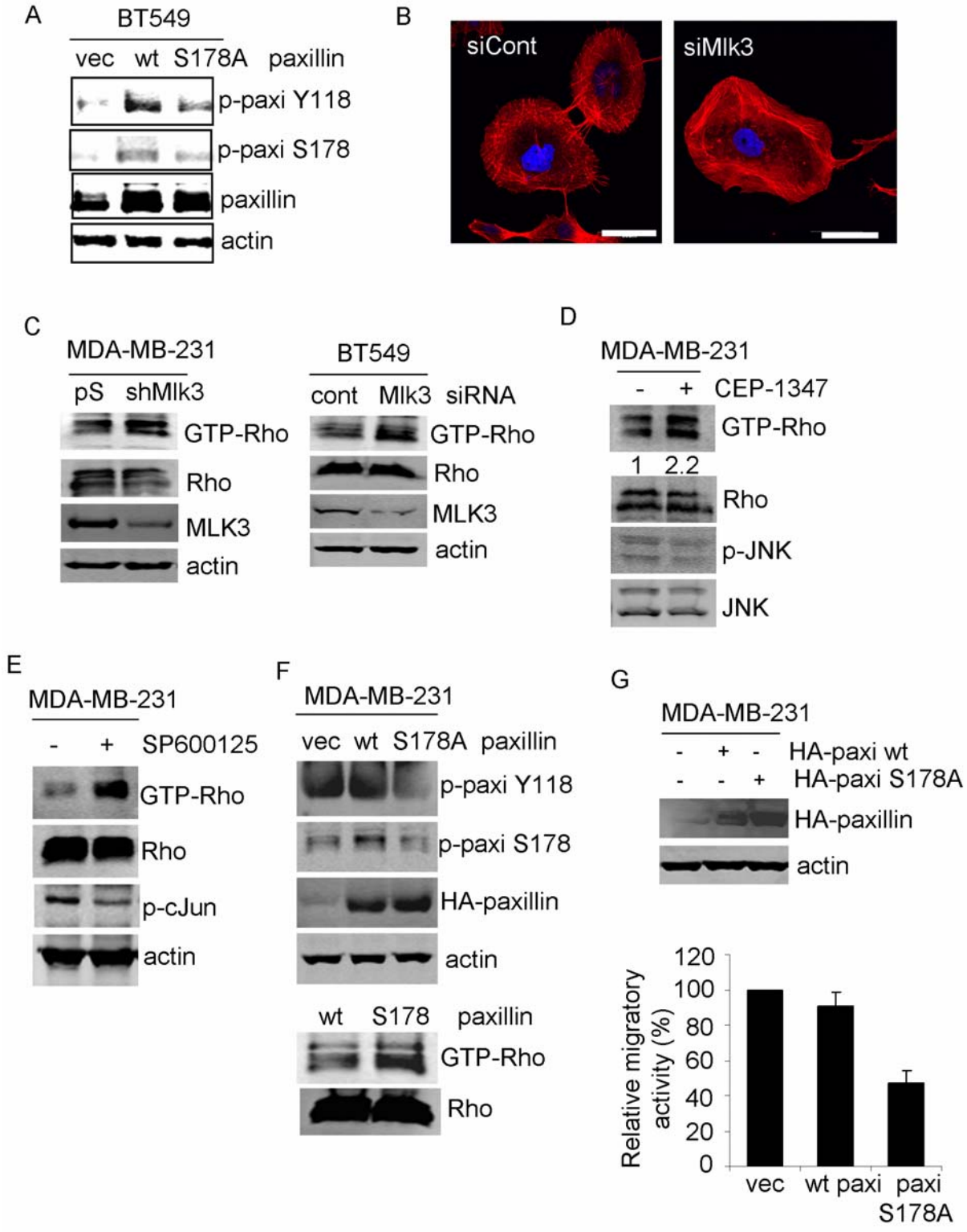
A
BT549
vec wt S178A paxillin
p-paxi Y118
p-paxi S178
paxillin
actin
B
siCont
siMlk3
C
MDA-MB-231
pS shMlk3
GTP-Rho
Rho
MLK3
actin
BT549
cont Mlk3 siRNA
GTP-Rho
Rho
MLK3
actin
D
MDA-MB-231
- + CEP-1347
GTP-Rho
1 2.2
Rho
p-JNK
JNK
E
MDA-MB-231
- + SP600125
GTP-Rho
Rho
p-cJun
actin
F
MDA-MB-231
vec wt S178A paxillin
p-paxi Y118
p-paxi S178
HA-paxillin
actin
wt S178 paxillin
GTP-Rho
Rho
G
MDA-MB-231
- + - HA-paxi wt
- - + HA-paxi S178A
HA-paxillin
actin
Relative migratory activity (%)
120
100
80
60
40
20
0
vec
wt paxi
paxi S178A

Figure 3.6 MLK3-JNK-mediated Ser 178 phosphorylation of paxillin is necessary for Tyr 118 phosphorylation of paxillin and inhibits Rho activity. (A) BT549 cells were transfected with vectors expressing HA-paxillin wt or HA-paxillin S178A mutant for 24 h. Cells were then serum-deprived for 48 h and treated with serum-containing medium for 30 min. Cellular lysates were analyzed by western blotting. (B) BT549 cells were treated with control or Mlk3 siRNA for 48 h, stained with phalloidin (F-actin) and DAPI (nucleus) and imaged using confocal microscopy. Bar, 50 μm. (C) MDA-MB-231 cells stably expressing pSuper or pSuper-Mlk3 were subjected to a Rhotekin pull down assay. Lysates and pulldowns were analyzed by western blotting using the indicated antibodies (left panel). BT549 cells were treated with control or Mlk3 siRNA and extent of Rho activation was determined using a Rhotekin pull down assay (right panel). (D) MDA-MB-231 cells were treated with DMSO or CEP-1347 (400 nM) for 6 h. Rho activity was determined using a Rhotekin pulldown assay. (E) MDA-MB-231 cells were treated with DMSO or SP600125 (15 μM) for 6 h. Activated Rho was assessed using a Rhotekin pulldown assay. (F) MDA-MB-231 cells were transiently transfected with wildtype HA-paxillin (Wt) or HA-paxillin S178A mutant for 24-48 h. Lysates were analyzed by western blotting with indicated antibodies. Simultaneously, the total cellular lysates were subjected to a Rhotekin pull down assay. (G) MDA-MB-231 cells expressing HA-paxillin wt or HA-paxillin S178A mutant were subjected to a transwell migration assay. Cellular lysates were analyzed by western blotting.

**MLK3 silencing decreases formation of lung metastases of human breast cancer cells.** To determine whether silencing of MLK3 is sufficient to prevent metastases, we inoculated MDA-MB-231 cells stably expressing a control vector or an Mlk3 shRNA vector into the mammary gland of athymic nu/nu mice. Both MDA-MB-231 shControl and MDA-MB-231 shMlk3 formed primary tumors. The MDA-MB-231 shControl tumors were slightly larger than MDA-MB-231 shMlk3 tumors, but this did not reach statistical significance (Figure 3.7A). Seven weeks post-inoculation, mice were euthanized, primary tumors were excised, and lung sections were prepared. MLK3 silencing was maintained in the primary tumors over the course of the experiment as shown in immunoblots of tumor lysates (Supplementary figure 3.5).

Lung micrometastases were detected by immunohistochemistry of lung sections using a human-specific CD44 antibody. Numerous micrometastases were observed in the lung sections of the mice inoculated with the MDA-MB-231 control cells, whereas only very few micrometastases were found in the lung sections of mice which had been inoculated with MDA-MB-231 shMlk3 cells (Figure 3.7B). Confirming the species specificity of the CD44 antibody, lung and liver tissue lysates from athymic nu/nu mice showed no human CD44 immunoreactivity. Furthermore, silencing of MLK3 in MDA-MB-231 cells had no impact on CD44 protein levels (Supplementary figure 3.6). These data provide evidence for a critical role of MLK3 in breast cancer metastasis. Ser 178 phosphorylation of paxillin is associated with the metastatic phenotype. In this study we have shown that prometastatic factors signal through MLK3-JNK to promote Ser 178 phosphorylation of paxillin (Figure 3.7C) and migration/invasion. To assess whether

Figure 3.7

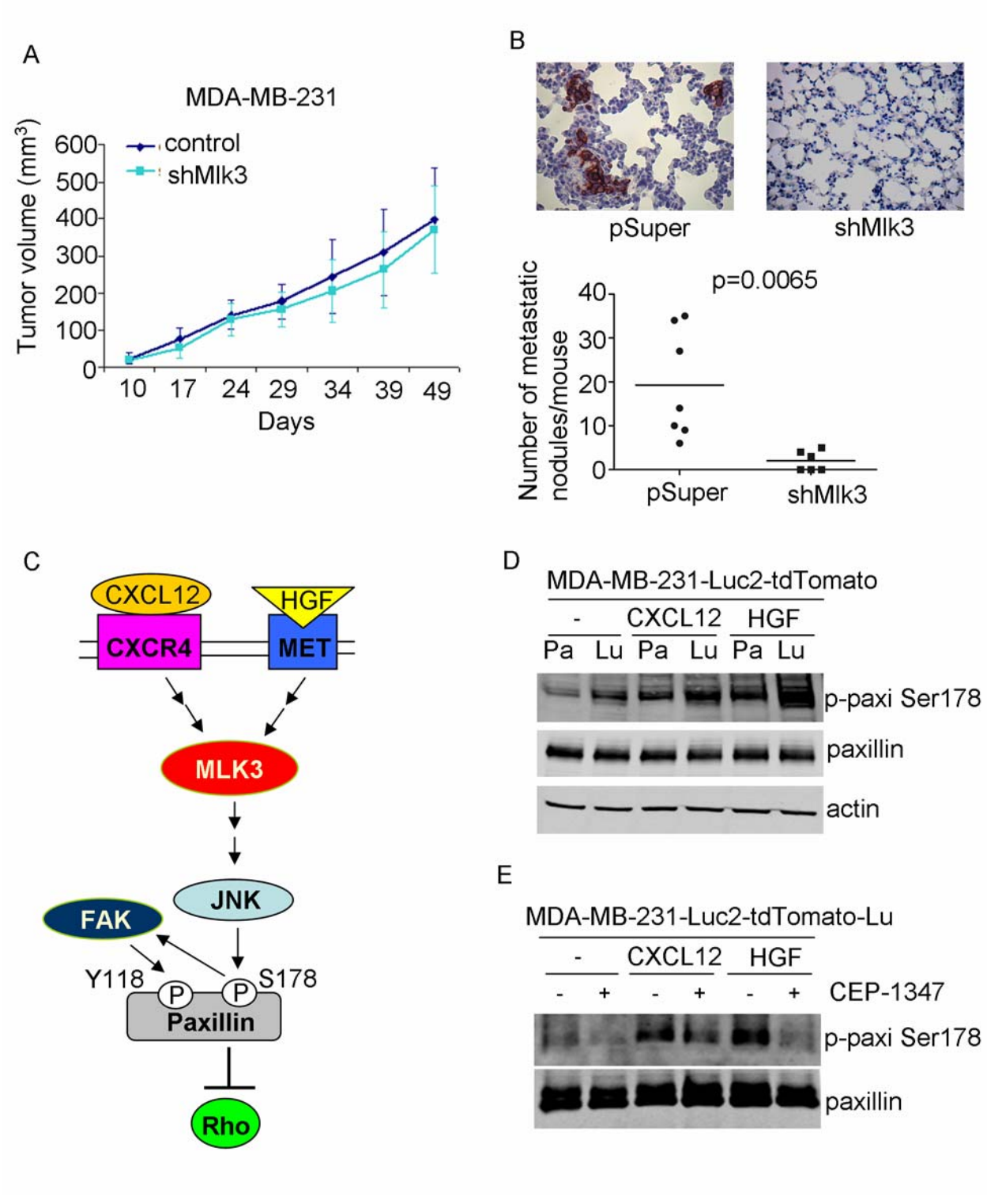
A
MDA-MB-231
Tumor volume (mm3)
600
500
400
300
200
100
0
control
shMlk3
10 17 24 29 34 39 49
Days
B
pSuper
shMlk3
Number of metastatic nodules/mouse
40
30
20
10
0
p=0.0065
pSuper
shMlk3
C
CXCL12
HGF
CXCR4
MET
MLK3
JNK
FAK
Y118
S178
P
P
Paxillin
Rho
D
MDA-MB-231-Luc2-tdTomato
-
CXCL12
HGF
Pa Lu Pa Lu Pa Lu
p-paxi Ser178
paxillin
actin
E
MDA-MB-231-Luc2-tdTomato-Lu
-
CXCL12
HGF
- + - + - +
CEP-1347
p-paxi Ser178
paxillin

Figure 3.7 Depletion of MLK3 prevents formation of lung metastases in a xenograft model. (A) MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 were inoculated into the mouse mammary gland fat pads. Tumor growth was monitored and tumor volume was determined as (0.5 x length x width$^2$). (B) Immunohistochemistry was performed on mouse lung sections using a human specific antibody anti-CD44 antibody. Images were acquired using light microscopy. Magnification, 400x. Quantitation was performed by counting the CD44-positive nodules in 10 sections per mouse. The statistical analysis was done using the GraphPad Prism 5 software. (C) Schematic model showing that the MLK3-JNK-pSer 178 paxillin signaling axis, activated through prometastatic factors CXCL12 and HGF, enhances FAK-paxillin interaction and FAK-mediated Tyr118 phosphorylation of paxillin, leading to suppression of Rho activity. (D) Cultured cells derived from lung metastases of the MDA-MB-231-Luc2-tdTomato were designated as Lu. Serum-deprived parental MDA-MB-231-Luc2-tdTomato cells (designated as Pa) and Lu cells were treated with CXCL12 and HGF (100 ng/ml) for 20 min and the cellular lysates were analyzed by immunoblotting with the indicated antibodies. (E) Serum-deprived Lu cells treated -/+CEP-1347 overnight, and treated with CXCL12 or HGF as in (D).

Ser 178 is associated with metastatic potential, we isolated MDA-MB-231 cells from lung metastases using an experimental metastasis model. MDA-MB-231 cells expressing luciferase (luc2) were injected into the tail vein of athymic nu/nu mice. Using bioluminescence imaging, overt metastases were observed 12 weeks post injection. The MDA-MB-231 cells isolated from the lung metastases (Lu) show higher paxillin phosphorylation on Ser 178 compared with parental MDA-MB-231 cells (Pa), both at the basal level as well as in response to either CXCL12 or HGF (Figure 3.7D). Furthermore, CEP-1347 inhibits both CXCL12- and HGF- induced Ser 178 phosphorylation of paxillin in Lu cells (Figure 3.7E), indicating these cells are still sensitive to an MLK inhibitor. Screening of a panel of human mammary epithelial and breast cancer cell lines revealed a correlation between phospho Ser 178 paxillin and the metastatic potential of several human breast cancer cell lines (Supplementary figure 3.7). These data, taken together, suggest that phosphorylation of paxillin on Ser 178 may be a predictor of lung metastatic potential.

## Discussion

Deciphering key signaling pathways underlying breast cancer cell migration and invasion may reveal novel therapeutic targets for effectively treating or preventing metastatic breast cancer. We previously demonstrated that the MLK3-JNK signaling axis plays a critical role in breast cancer cell migration and invasion. MLK3-JNK signaling upregulates multiple AP-1-driven invasion genes and promotes a malignant phenotype in mammary epithelial cells grown in 3D Matrigel culture (Chen et al., 2010). JNK is important in breast cancer cell migration and invasion, and with progression of

breast cancer (Huang et al., 2003; Cui et al., 2006; Yeh et al., 2006). How the MLK3-JNK signaling axis regulates cell migration machinery remains largely unknown.

In this study, we report, for the first time, that in response to prometastatic factors, including CXCL12 and HGF, MLK3 signals to JNK to control the phosphorylation of paxillin on both Ser 178 and Tyr 118 (Figures 3.2-4), phosphorylation events that are essential in cell migration (Huang et al., 2003; Petit et al., 2000). We present evidence that MLK3 modulates the interaction between the two key focal adhesion proteins, paxillin and FAK (Figure 3.4). Furthermore, we show that the MLK3-JNK-paxillin signaling axis negatively regulates Rho activity to promote focal adhesion turnover in cell migration (Figures 3.5, 3.6). Finally, MLK3 is critical for the formation of breast cancer lung metastases in a mouse xenograft model (Figure 3.7). The importance of paxillin phosphorylation is highlighted by the finding that cells derived from MDA-MB-231 lung metastases show higher phosphorylation of paxillin at Ser 178, compared with parental MDA-MB-231 cells, implicating this phosphorylation site in breast cancer metastasis. Taken altogether, our data reveal a novel MLK3-JNK-paxillin signaling pathway that regulates breast cancer cell migration and invasion. This investigation underscores the potential of MLK3 as a therapeutic target in breast cancer metastasis.

Paxillin undergoes dynamic phosphorylation during cell migration ( Brown and Turner, 2004). Phosphorylation of Ser 178 on paxillin is essential for cell migration (Huang et al., 2003). The prometastatic factors that signal to paxillin in breast cancer cell migration are unknown. Here, we present the novel finding that both CXCL12 and HGF signal through MLK3 to paxillin, consistent with our data showing that MLK3 is required for CXCL12-induced breast cancer cell migration (Figure 3.1). Induced

expression of MLK3 robustly increases phosphorylation of paxillin on Ser 178 whereas silencing or inhibition of MLK3 blocks paxillin phosphorylation in basal breast cancer MDA-MB-231 and BT549 cells (Figures 3.2, 3.3). These data identify MLK3 as an important signaling node that relays extracellular cues to JNK-mediated phosphorylation of paxillin. It is possible that other MAPKKKs may contribute to paxillin phosphorylation through JNK. However, at least in triple negative, basal breast cancer cells, MLK3 appears to play a dominant role. Because chemokines like CXCL12 and growth factors like HGF are consistently linked with invasion and metastasis, our findings provide a strong rationale for targeting MLK3 in the context of breast cancer metastasis. In agreement with our findings, localized JNK activation and Ser 178 phosphorylation of paxillin is observed during the migration of rat kidney epithelial cells, which involves the aPKC-Exocyst complex (Rosse et al., 2009). Interestingly, PKC is important for activation of MLK3 in response to free fatty acids (Jaeschke and Davis, 2007). Whether PKC plays a role in CXCL12- or HGF- induced MLK3 activation remains to be determined.

Rapid assembly and disassembly of focal adhesions is a well-described property of many migrating cancer cells. Experimental disruption of focal adhesion turnover typically results in migratory defects in cancer cells (Xu et al., 2010). Phosphorylation of paxillin at Ser 178 by JNK (Huang et al., 2003) and Tyr 118 by FAK/Src (Bellis et al., 1995; Schaller and Parsons, 1995) is critical for focal adhesion turnover and cell migration ( Huang et al., 2003; Petit et al., 2000; Su et al., 2009; Vindis et al., 2004). For instance, a tyrosine phosphomimetic mutant of paxillin enhances focal adhesion turnover, whereas a non-phosphorylatable mutant shows defective focal

adhesion turnover and migration (Webb et al., 2004; Zaidel-Bar et al., 2007). Furthermore, phosphorylation of Tyr 118 on paxillin is implicated in cancer invasion and metastasis (Azuma et al., 2005). We show herein that ectopic expression of MLK3 robustly enhances phosphorylation of paxillin at Tyr 118, whereas silencing or inhibition of MLK impairs Tyr 118 phosphorylation (Figure 3.4). These data support a model in which MLK3 is required for focal adhesion turnover in cell migration through controlling the tyrosine phosphorylation of paxillin. Indeed, MLK3 silencing increases the number of focal adhesions in breast cancer cells (Figure 3.5). Furthermore, experiments using a nocodazole-based assay (Ezratty et al., 2005), in which nocodazole washout promotes microtubule formation and focal adhesion turnover, reveal a defect in focal adhesion disassembly upon MLK3 silencing in MDA-MB-231 cells (data not shown), consistent with the idea that MLK3 is necessary for turnover of focal adhesions.

Elevated levels and kinase activity of FAK are found in high grade human cancers, including breast cancer, and correlate with invasive phenotypes, metastatic disease and poor prognosis (McLean et al., 2005). FAK regulates proliferation, survival and migration in a wide range of cancer cells; and FAK inhibitors are currently in clinical trials for treating human solid tumors (Parsons et al. 2008). FAK is activated through release of its intramolecular autoinhibition, followed by autophosphorylation within its kinase domain, which recruits Src to form an active FAK/Src complex. Our data showing that MLK3 promotes the interaction of FAK with paxillin, may explain how MLK3 controls Tyr 118 phosphorylation of paxillin and promotes focal adhesion turnover, since association of FAK with paxillin promotes tyrosine phosphorylation of paxillin and is correlated with less stable focal adhesions (Shan et al., 2009). Ablation of the Ser 178

phosphorylation site on paxillin decreased phosphorylation of Tyr 118 (Figure 3.6), suggesting that, at least in this experimental context, Ser 178 phosphorylation is a prerequisite for Tyr 118 phosphorylation. This is consistent with the finding that the paxillin S178A mutant has decreased affinity for FAK (Huang et al., 2008). In our working model, MLK3-JNK-Ser 178 paxillin phosphorylation regulates both the association of FAK with paxillin and indirectly controls subsequent tyrosine phosphorylation of paxillin (Figure 3.7C).

Focal adhesion dynamics are tightly controlled by Rho GTPases ( Ridley et al., 2003; Hall, 1998). Active Rho increases stress fibers and focal adhesion maturation and decreases focal adhesion turnover (Ren et al., 2000). While Rho activity is required for cell migration, aberrantly high Rho activity also impairs cell migration (Nobes and Hall, 1999). FAK promotes focal adhesion turnover, in part through suppression of Rho activity (Ren et al., 2000). In particular, FAK/Src-mediated Tyr 118 phosphorylation of paxillin has been proposed to release p190 Rho-GAP from its sequestration with Ras-GAP, leading to downregulation of Rho activity (Tsubouchi et al., 2002). We demonstrate that inhibition of MLK3, JNK, or Ser 178 paxillin phosphorylation, using an MLK inhibitor, a JNK inhibitor or the paxillin S178A mutant, respectively, led in all cases to increased Rho activity (Figure 3.6). Our experimental evidence supports a model in which the MLK3-JNK-paxillin Ser 178 signaling axis negatively regulates Rho activity, through FAK-mediated tyrosine phosphorylation of paxillin. Indeed, MLK3 silencing in breast cancer cells resulted in increased focal adhesions (Figure 3.5) and stress fibers (Figure 3.6), two Rho-associated phenotypes. In A549 lung carcinoma cells, a role for MLK3 in limiting Rho activity through an interaction with p63 RhoGEF has also been

described (Swenson-Fields et al., 2008). In summary, we have identified in basal breast cancer cells, a distinct pathway involving an active MLK3-JNK-paxillin axis that functions to negatively control Rho activity.

To assess the requirement for MLK3 in the formation of lung metastases, control or shMlk3-expressing MDA-MB-231 cells were introduced into the mammary fat pad of athymic nu/nu mice (Valastyan et al., 2009). Whereas control MDA-MB-231 cells formed numerous micrometastases in the lung, lung micrometastases were nearly absent in mice transplanted with MLK3 knockdown MDA-MB-231 cells (Figure 3.7). In agreement with our findings, MLK3 knockdown has been shown to reduce lymph node metastases of MDA-MB-231 cells (Cronan et al., 2011). Our findings support a critical role for MLK3 in breast cancer lung metastasis. Although MLK3 has been shown to promote cell survival ( Chen et al., 2010; Cronan et al., 2011), which might contribute to formation of metastases, we propose that an important mechanism through which MLK3 promotes metastasis is through facilitating cancer cell migration and invasion.

Our study demonstrates that MLK3 controls Ser 178 phosphorylation of paxillin, which is required for cell migration. Interestingly, phospho-Ser 178 paxillin correlates with metastatic potential of breast cancer cells, suggesting that Ser 178 phosphorylation of paxillin might be a predictive biomarker for metastasis. Our novel findings demonstrate that prometastatic factors found in the tumor microenvironment converge on MLK3 to promote breast cancer cell migration and invasion. Since the MLK inhibitor, CEP-1347, efficaciously blocks invasion in response to such factors, we are currently testing the effect of this compound in a preclinical study using a mouse xenograft model. Taken together, our findings indicate that prometastatic factors signal to MLK3

to regulate phosphorylation of paxillin and its interaction with FAK, promoting basal breast cancer migration and invasion. We also provide evidence that the MLK3-JNK-paxillin axis negatively regulates Rho activity and focal adhesion turnover. Finally, we demonstrate a critical role of MLK3 in breast cancer metastasis. Thus, targeting MLK3 could be a promising therapeutic strategy for treatment or prevention of metastatic disease in breast cancer.

## Acknowledgements

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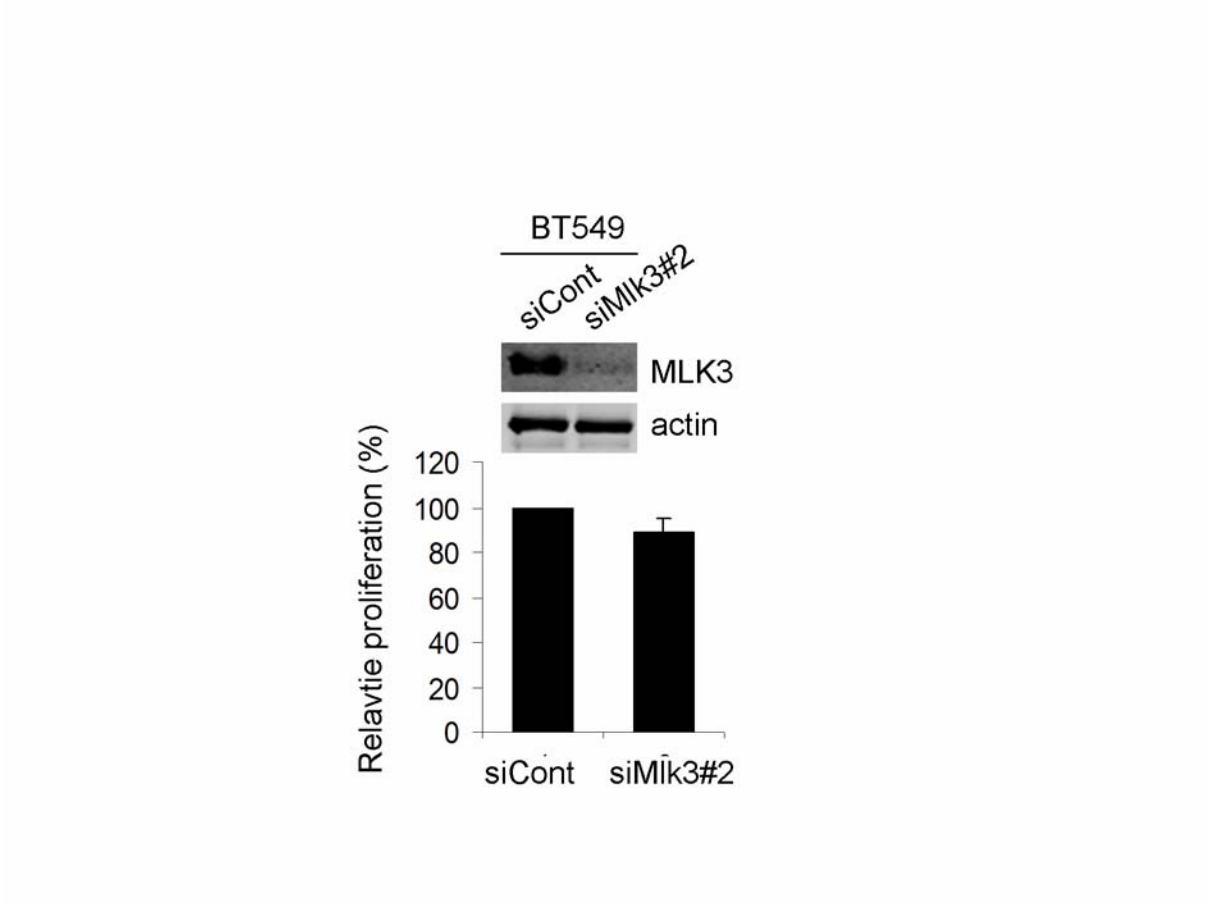
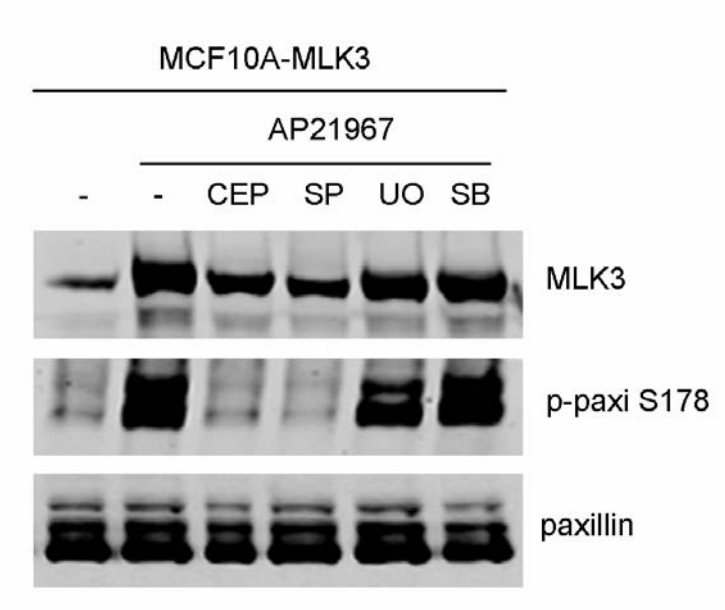
Supplementary figure 3.1



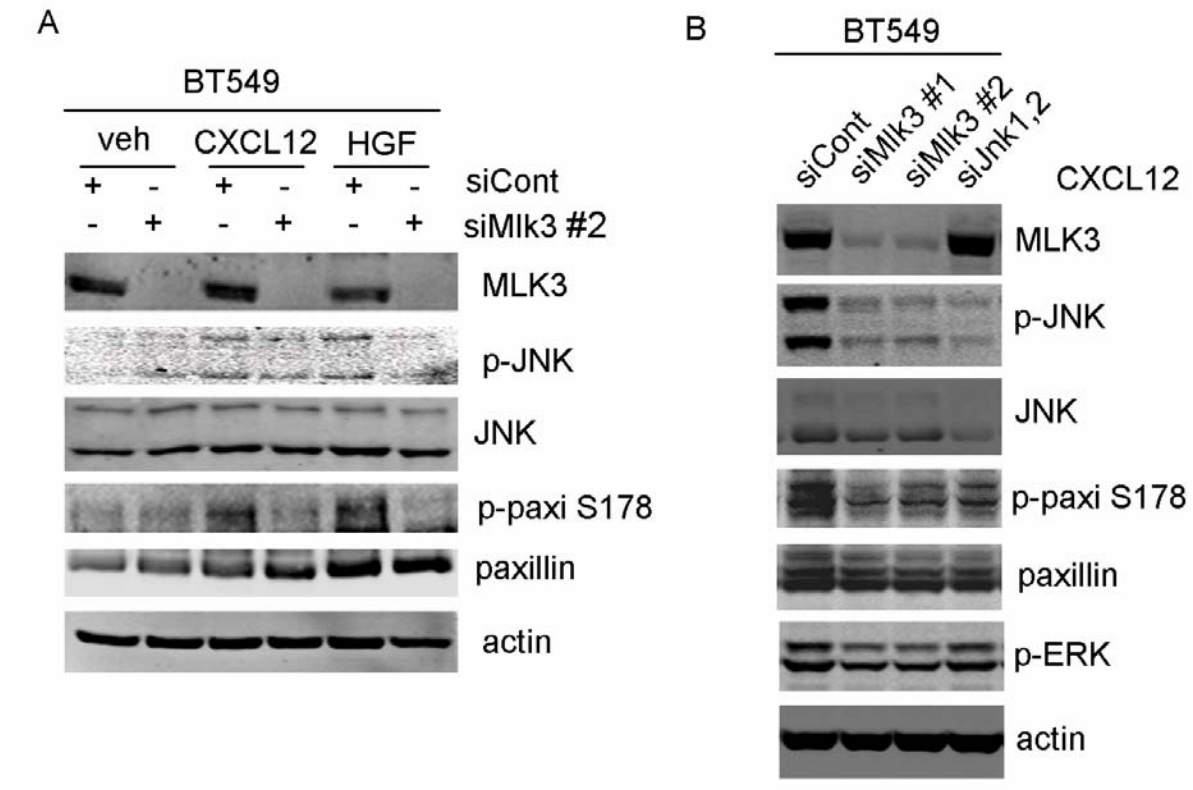

Supplementary figure 3.1 Effect of MLK3 silencing on proliferation of BT549 cells. BT549 cells were treated with control or Mlk3 siRNA for 48 h, serum-deprived overnight and then subjected to a proliferation assay in growth medium for 48 h using the CCK-8 kit. The results are expressed relative to proliferation of control cells (=100%). Column, mean of three experiments. Bar, SE. Cell lysates were harvested at the end of the experiment and subjected to immunoblotting.

Supplementary figure 3.2



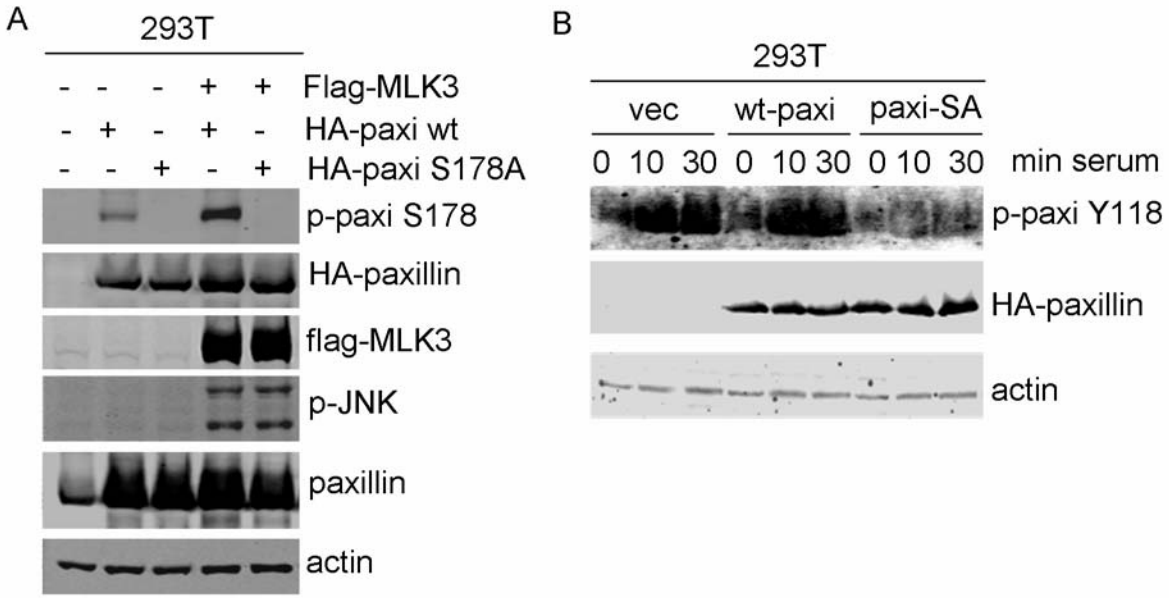

Supplementary figure 3.2 Effects of MAPK pathway inhibitors on signaling pathways in MCF10A-MLK3 cells. MCF10A-MLK3 cells were treated with vehicle or 50 nM AP21967 for 20 h and the indicated inhibitors: 15 μM SP600125, 10 μM U0126, 10 μM SB203580 or 400 nM CEP-1347, for an additional 24 h. Cellular lysates were analyzed by western blotting with the indicated antibodies.

Supplementary figure 3.3



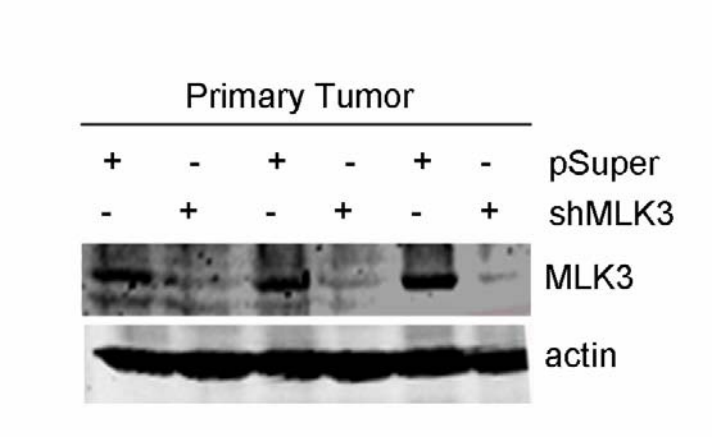

Supplementary figure 3.3 Effect of MLK3 silencing or JNK silencing on paxillin phosphorylation on S178. (A) BT549 cells were transfected with control or Mlk3 siRNA for 24 h and serum-deprived overnight, followed by treatment with CXCL12 (100 ng/ml) or HGF (100 ng/ml) for 30 min. Cellular lysates were analyzed by western blotting with the indicated antibodies. (B) BT549 cells were transfected with control, two different siRNAs for Mlk3 (30 nM), or JNK1,2 siRNA (100 nM) for 24-48 h, followed by serum deprivation for 12 h and then treatment with CXCL12 (100 nM) for 30 min. Cellular lysates were analyzed by western blotting using the indicated antibodies.

Supplementary figure 3.4



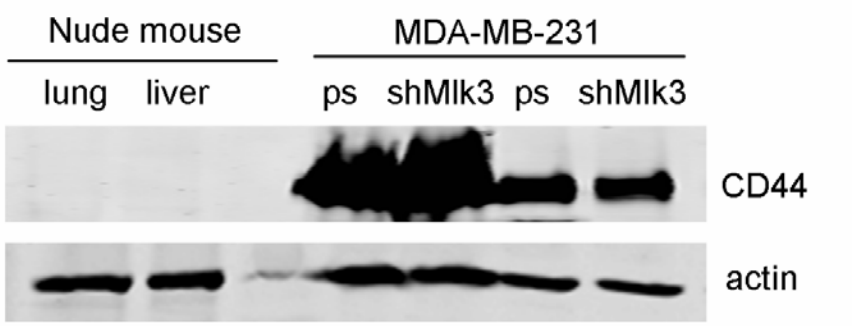

Supplementary figure 3.4 Generation of paxi S178A mutant and its effect on paxillin Y118 phosphorylation. (A) 293T cells were transfected with vectors expressing HA-paxillin wt or HA-paxillin S178A mutant with Flag-Mlk3 for 24 h. Cellular lysates were analyzed by western blotting using the indicated antibodies. (B) 293T cells were transfected with vectors expressing either HA-paxillin wt or HA-paxillin S178A mutant for 24 h. Cells were then serum-deprived overnight, followed by readdition of 10% serum at the indicated times. Cellular lysates were analyzed by western-blotting.

Supplementary figure 3.5



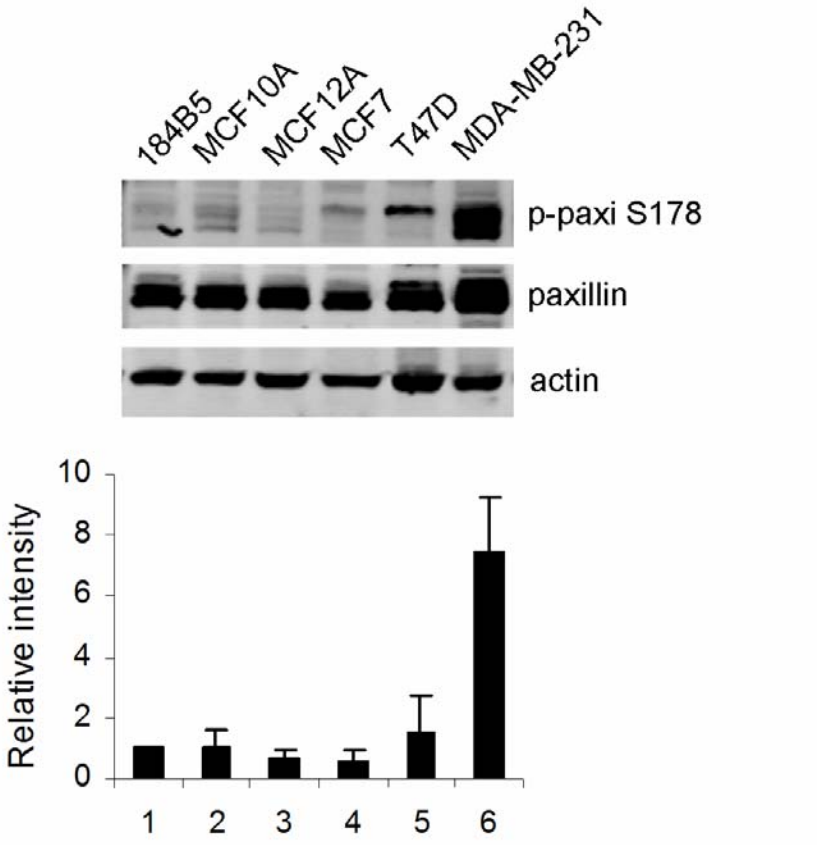

Supplementary figure 3.5 MLK3 silencing is maintained in primary tumors. Liver and lung were dissected from nude mice and lysed in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS). Mouse tissue lysates and lysates of MDA-MB-231 control or depleted of MLK3 were analyzed by western blotting using the indicated antibodies.

Supplementary figure 3.6




Supplementary figure 3.6 Confirmation of species specificity of the anti-human CD44 antibody. Liver and lung were dissected from nude mice and lysed in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS). Mouse tissue lysates and lysates of MDA-MB-231-pSuper or MDA-MB-231-shMlk3 (equal protein loading) were analyzed by western blotting using the indicated antibodies. Additional lanes were underloaded with MDA-MB-231 lysates for clarity.

Supplementary figure 3.7




Supplementary figure 3.7 Phosphorylation of paxillin at S178 is higher in metastatic breast cancer cell lines. Non-tumorigenic mammary epithelial and breast cancer cell lines were cultured in growth media and harvested at approximately 70-80% confluence according to Materials and Methods. 40 µg total lysates were resolved in 12% SDS-PAGE and analyzed by western blotting. Phosph-Ser 178 paxillin intensity was quantified using Odyssey software 3.0 and normalized to actin intensity.

# REFERENCES

## References


Azuma K, Tanaka M, Uekita T, Inoue S, Yokota J, Ouchi Y, et al. Tyrosine phosphorylation of paxillin affects the metastatic potential of human osteosarcoma. Oncogene 2005;24:4754-4764.

Bellis SL, Miller JT, Turner CE. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. J Biol Chem, 1995;270:17437-17441.

Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003;4:915-925.

Brown MC, Turner CE. Paxillin: adapting to change. Physiol Rev 2004;84:1315-1339.

Chadee DN, Kyriakis JM. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. Nat Cell Biol 2004;6:770-776.

Chen J, Miller EM, Gallo KA. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. Oncogene 2010; 29:4399-4411.

Cronan MR, Nakamura K, Johnson NL, Granger DA, Cuevas BD, Wang JG, et al. Defining MAP3 kinases required for MDA-MB-231 cell tumor growth and metastasis. Oncogene 2011;10.1038/onc.2011.544.

Cui X, Kim HJ, Kuiatse I, Kim H, Brown PH, Lee AV. Epidermal growth factor induces insulin receptor substrate-2 in breast cancer cells via c-Jun NH(2)-terminal kinase/activator protein-1 signaling to regulate cell migration. Cancer Res 2006;66:5304-5313.

DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics. CA Cancer J Clin 2011;61:409-418.

Du Y, Bock BC, Schachter KA, Chao M, Gallo KA. Cdc42 induces activation loop phosphorylation and membrane targeting of mixed lineage kinase 3. J Biol Chem 2005;280:42984-42993.

Dupont VN, Gentien D, Oberkampf M, De Rycke Y, Blin NA. Gene expression signature associated with metastatic cells in effusions of breast carcinoma patients. Int J Cancer 2007;121:1036-1046.

Ezratty EJ, Partridge MA, Gundersen GG. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat Cell Biol 2005;7: 581-590.

Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol 2002;3:663-672.

Gastaldi S, Comoglio PM, Trusolino L. The Met oncogene and basal-like breast cancer: another culprit to watch out for? Breast Cancer Res 2010;12:208.

Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279:509-514.

Holland JD, Kochetkova M, Akekawatchai C, Dottore M, Lopez A, McColl SR. Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells. Cancer Res 2006;66:4117-4124.

Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW, Schutte M. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. Mol Cancer Res 2007;5:195-201.

Huang Z, Yan DP, Ge BX. JNK regulates cell migration through promotion of tyrosine phosphorylation of paxillin. Cell Signal 2008;20:2002-2012.

Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. Nature 2003;424:219-223.

Jaeschke A, Davis RJ. Metabolic stress signaling mediated by mixed-lineage kinases. Mol Cell 2007;27:498-508.

Kang JY, Dolled-Filhart M, Ocal IT, Singh B, Lin CY, Dickson RB, et al. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. Cancer Res 2003;63:1101-1105.

Kato M, Kitayama J, Kazama S, Nagawa H. Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. Breast Cancer Res 2003;5:R144-150.

Lengyel E, Prechtel D, Resau JH, Gauger K, Welk A, Lindemann K, et al. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer 2005;113:678-682.

Li G, Xiang Y, Sabapathy K, Silverman RH. An apoptotic signaling pathway in the interferon antiviral response mediated by RNase L and c-Jun NH2-terminal kinase. J Biol Chem 2004;279:1123-31.

Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell 2004;6:459-469.

Luker KE, Luker GD. Functions of CXCL12 and CXCR4 in breast cancer. Cancer Lett 2006;238:30-41.

McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. Nat Rev Cancer 2005;5: 505-515.

Mishra P, Senthivinayagam S, Rangasamy V, Sondarva G, Rana B. Mixed lineage kinase-3/JNK1 axis promotes migration of human gastric cancer cells following gastrin stimulation. Mol Endocrinol 2010;24:598-607.

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50-56.

Nobes CD, Hall A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol 1999;144:1235-1244.

Parsons JT, Slack-Davis J, Tilghman R, Roberts WG. Focal adhesion kinase: targeting adhesion signaling pathways for therapeutic intervention. Clin Cancer Res 2008;14:627-632.

Petit V, Boyer B, Lentz D, Turner CE, Thiery JP, Valles AM. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J Cell Biol 2000;148:957-970.

Ren XD, Kiosses WB, Sieg DJ, Otey CA, Schlaepfer DD, Schwartz MA. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. J Cell Sci 2000;113 (Pt 20):3673-3678.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science 2003;302:1704-1709.

Rosse C, Formstecher E, Boeckeler K, Zhao Y, Kremerskothen J, White MD et al. An aPKC-exocyst complex controls paxillin phosphorylation and migration through localised JNK1 activation. PLoS Biol 2009;7:e1000235.

Schaller MD, Parsons JT. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol 1995;15:2635-2645.

Shan Y, Yu L, Li Y, Pan Y, Zhang Q, Wang F, et al. Nudel and FAK as antagonizing strength modulators of nascent adhesions through paxillin. PLoS Biol 2009;7:e1000116.

Simpson KJ, Selfors LM, Bui J, Reynolds A, Leake D, Khvorova A, et al. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. Nat Cell Biol 2008;10:1027-1038.

Su S, Li Y, Luo Y, Sheng Y, Su Y, Padia RN, et al. Proteinase-activated receptor 2 expression in breast cancer and its role in breast cancer cell migration. Oncogene 2009;28:3047-3057.

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K,et al. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Mol Cell 2008;32:43-56.

Tsubouchi A, Sakakura J, Yagi R, Mazaki Y, Schaefer E, Yano H, et al. Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration. J Cell Biol 2002;159:673-683.

Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, Wang ZC, et al. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell 2009;137:1032-1046.

Valles AM, Beuvin M, Boyer B. Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. J Biol Chem 2004;279:44490-44496.

Vindis C, Teli T, Cerretti DP, Turner CE, Huynh-Do U. EphB1-mediated cell migration requires the phosphorylation of paxillin at Tyr-31/Tyr-118. J Biol Chem 2004;279:27965-27970.

Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004;6:154-161.

Xu Y, Bismar TA, Su J, Xu B, Kristiansen G, Varga Z, et al. Filamin A regulates focal adhesion disassembly and suppresses breast cancer cell migration and invasion. J Exp Med 2010;207:2421-2437.

Yeh YT, Hou MF, Chung YF, Chen YJ, Yang SF, Chen DC, et al. Decreased expression of phosphorylated JNK in breast infiltrating ductal carcinoma is associated with a better overall survival. Int J Cancer 2006;118:2678-2684.

Zaidel-Bar R, Milo R, Kam Z, Geiger BA. paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. J Cell Sci 2007;120:137-148.

Zhan Y, Modi N, Stewart AM, Hieronimus RI, Liu J, Gutmann DH, et al. Regulation of mixed lineage kinase 3 is required for Neurofibromatosis-2-mediated growth suppression in human cancer. Oncogene 2011;30:781-789.

Zhang H, Gallo KA. Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain. J Biol Chem 2001;276:45598-45603.

# CHAPTER IV

# CONCLUSIONS AND PERSPECTIVES

Development of distant metastases is a major cause of mortality in breast cancer patients. Despite intensive study by many researchers, the process by which cancer cells from a primary breast tumor seed in secondary sites and form metastatic lesions is still poorly understood. Because breast cancer metastasis involves multiple inefficient steps, elucidation of the mechanisms which control discrete steps of this process may provide the opportunity to identify potential targets for treatment and prevention of metastasis.

The aim of this thesis research is to study the role of MLK3 signaling in breast cancer cell proliferation, migration, invasion and metastasis. This work describes identification and characterization of a signaling pathway in which MLK3 signals to its downstream kinase, c-Jun N-terminal kinase (JNK), and the transcription factor AP-1 to promote breast cancer cell migration and invasion, with a requirement for expression of AP-1 target invasion genes. In addition, experimental evidence presented herein demonstrates that MLK3 directly regulates focal adhesion components of the cell migration machinery, to facilitate breast cell migration and invasion. MLK3 signaling to JNK controls phosphorylation of paxillin, a key event in focal adhesion turnover during cell migration. MLK3 promotes phosphorylation of paxillin at Ser 178 through JNK, which lead to recruitment of FAK/Src and FAK/Src-mediated phosphorylation of paxillin on Tyr 118. Tyr 118 phosphorylation of paxillin is a critical event in inhibiting Rho activity to allow turnover of focal adhesions of a migrating cell. Our results show that the MLK3-JNK-paxillin signaling axis functions as a negative regulator for Rho activity. Inhibition of each component of this signaling pathway causes an aberrantly high Rho activity,

defective focal adhesion turnover and impaired cell motility. A model for MLK3 in the regulation of cancer cell migration is proposed (Figure 4.1).

MLK3 silencing in breast cancer cells increases the numbers of focal adhesions, suggesting that MLK3 is required for focal adhesion turnover. Using a nocodazole-based assay, in which washout of nocodazole and regrowth of microtubules promotes turnover of focal adhesions, preliminary data was obtained suggesting that MLK3-silenced breast cancer cells have a defect in focal adhesion turnover (Chen J and Misek S, unpubllished data). An important future question needs to be addressed.is the role of MLK3 in dynamics of formation, maturation and turnover of focal adhesions in migrating cells. To address this question, time-lapse microscopy could be used to monitor the effect of MLK3 inhibition, silencing, or overexpression on the real time changes of focal adhesions in live cells expressing fluorescent tagged focal adhesion proteins such as paxillin; the constitutive focal adhesion protein vinculin; and the mature focal adhesion marker zyxin.

Data presented in Chapter 3, shows that MLK3 negatively regulates Rho activation through the JNK-paxillin pathway. Furthermore, overexpression of MLK3 was found to activate Rac in HEK293 cells and MCF-10A cells and stable silencing MLK3 in MDA-MB-231 cells can reduce Rac activity (Chen J., unpublished data). An antagonism between Rac and Rho activity has been widely observed. Is MLK3-mediated Rac activation a consequence resulting from Rho inhibition by MLK3? To address this question, selective Rho inhibitor, C3 transferase, which catalyzes ADP-ribosylation on Rho to block its activation, could be used to investigate whether a Rho inhibitor can rescue Rac inactivation caused by silencing of MLK3. Furthermore, it will be interesting

Figure 4.1

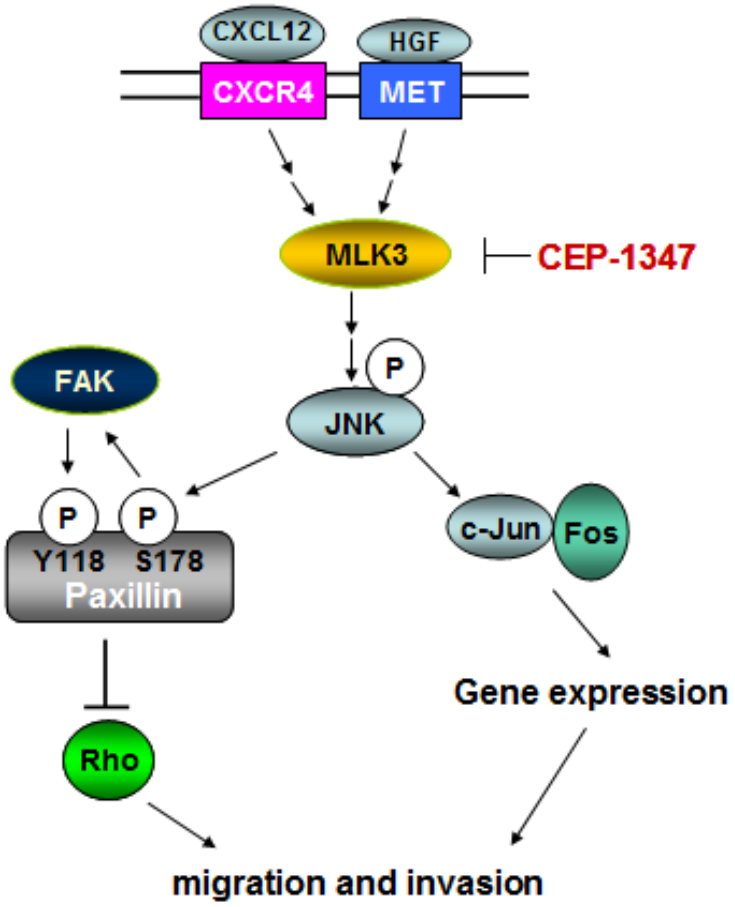



Figure 4.1 Schematic model of MLK3 signaling in cell migration and invasion. In response to prometastatic factors CXCL12 and HGF, MLK3 activates the JNK pathways. Active JNK triggers phosphorylation of the transcription factor c-Jun enhancing transcriptional activity of AP-1, which results in expression of invasion genes. In addition, MLK3 signaling to JNK can modulate phosphorylation of the focal adhesion protein paxillin as well as FAK recruitment to paxillin resulting in inhibition of Rho activity and enhanced cell migration and invasion.

to investigate the components of the signaling pathway responsible for Rac activation by MLK3. In mammalian cells, many GEFs have been identified for Rac activation. It will be interesting to screen these GEFs to identify which GEF specifically mediates MLK3-induced Rac activation.

Investigations in Chapter 2 demonstrate that MLK3 is sufficient to induce invasion of nontumorigenic mammary epithelial cells. Along with this invasive phenotype, MLK3 can activate expression of AP-1 target invasion genes. I hypothesize that MLK3 can reprogram a signature of invasion genes to promote malignant phenotypes. Global gene expression analysis is being performed using in our established mammary epithelial cells inducibly expressing MLK3 to determine the MLK3 gene signature. Establishing the signature of MLK3-induced invasion genes in mammary epithelial cells may help to identify which types of breast tumors might respond to MLK inhibitors.

MLK3 has a profound migratory phenotype in many cancer cells. In a mouse xenograft model, MLK3 was necessary for breast cancer dissemination to the lung, since metastatic breast cancer cells depleted of MLK3 failed to form pulmonary micrometastases. It will be interesting to investigate further the impact of MLK3 in the process of metastasis formation. Do cancer cells in a primary tumor fail to invade the stroma when MLK3 is silenced? Or is MLK3 necessary for extravasation of metastatic cells? To study first question, a close examination of cancer cells, stroma and vascular structures in primary tumors by immunostaining would be appropriate. For the second question, it may be possible to intravenously inject luciferase-labeled cancer cells in mice and monitor the kinetics of extravasation by in vivo bioluminescence imaging. There may, however, be issues of sensitivity and ability to pinpoint the location of the

tumor cells at the dissemination site. Furthermore, since an MLK inhibitor can inhibit migration and invasion in breast cancer cells, a xenograft mouse model is being used to test whether an MLK inhibitor can prevent the development of metastases. Taken together, these experiments will provide insight into the role of MLK3 in breast cancer metastasis and provide important preclinical data for evaluating the potential of an MLK inhibitor as a novel therapeutic strategy for metastatic breast cancer.

In an experimental metastasis model, metastatic breast cancer cells labeled with luciferase were injected into the tail vein of athymic mice and the formation of lung metastases was monitored by in vivo bioluminescence imaging. Metastatic cells colonizing the lung were isolated and grown in culture. Interestingly, these cells show markedly elevated paxillin phosphorylation on Ser 178 compared with the parental cells, suggesting that S178 phosphorylation of paxillin may predict the metastatic potential of breast cancer cells. It would be informative to examine the level of paxillin phosphorylation on S178 in human breast cancer specimens as a function of stage and grade. These specimens include primary tumors from patients who developed distant metastases compared with those who remained metastasis-free during a given time period. We hypothesize that higher level of paxillin S178 phosphorylation in primary tumors may associate with higher risk to develop metastasis in breast cancer patients. Such a study would test whether paxillin phosphorylation on S178 could be a intrinsic feature of metastatic cells and be useful as a clinical biomarker for predicting metastasic potential and determining how aggressively to treat patients.