DISSECTING THE ROLE OF MIXED LINEAGE KINASE 3 (MLK3) IN BREAST CANCER INVASION AND METASTASIS

By

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ABSTRACT

DISSECTING THE ROLE OF MIXED LINEAGE KINASE 3 (MLK3) IN BREAST CANCER INVASION AND METASTASIS

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MLK3 is a mitogen-activated protein kinase kinase kinase (MAP3K) protein which can activate multiple MAPK pathways. MLK3 has been recently shown to be critical for breast cancer metastasis. In this thesis, the mechanisms through which MLK3 signaling controls discrete steps of cancer invasion and metastasis have been investigated. MLK3 was found to regulate the expression of FOS-related antigen-1 (FRA-1), a member of activator protein-1 (AP-1) transcription factor family. Overexpression of catalytically active MLK3, but not catalytically inactive MLK3, induced expression of FRA-1, suggesting that MLK3 utilizes its kinase activity rather than scaffolding function to control FRA-1 levels. In addition, MLK3 overexpression was sufficient to drive cancer cell migration in non-invasive, estrogen receptor positive (ER+) breast cancer cells. Conversely, deletion of MLK3 in highly metastatic, triple-negative breast cancer (TNBC) cells decreased FRA-1 expression at both the transcript and protein levels. Similarly, FRA-1 level was reduced upon treatment of TNBC cells with CEP-1347 or URMC-099, two structurally distinct, adenosine triphosphate (ATP)-competitive, MLK inhibitors. These data, together with the findings in ER+ breast cancer cells, indicate that MLK3 kinase activity is required for FRA-1 expression. MLK3 is capable of activating several MAPK pathways. Experiments utilizing small molecule inhibitors targeting specific MAPK pathways identified JNK and ERK pathways as mediators for MLK3-induced FRA-1 expression.

FRA-1 is an oncogenic transcription factor controlling a subset of invasion genes. The work described in this thesis demonstrates a role for MLK3 in regulating matrix

metalloproteinase (MMP) levels in breast cancer cells. In ER+ breast cancer cells, MLK3 overexpression induced expression of MMP-1 and MMP-9, two well-characterized, FRA-1 target genes. Silencing of FRA-1 in MLK3-overexpressing ER+ breast cancer cells attenuated MMP-1 and MMP-9 expression indicating that FRA-1 is a required intermediary in MLK3-induced MMP regulation. Furthermore, MLK3 deletion or MLK inhibitor treatment was sufficient to decrease both MMP-1 and MMP-9 levels in TNBC cells.

MMPs are known to facilitate cancer invasion. Overexpression of MLK3 in ER+ breast cancer cells, which induces both MMP-1 and MMP-9, enhanced the ability of these cells to invade through a thin layer of Matrigel and this invasion was attenuated upon FRA-1 silencing. Moreover, MLK3 deletion or MLK inhibitor treatment also blocked Matrigel invasion of highly invasive TNBC cells. Consistent with a role for MMP-1 in vascular intravasation, deletion of MLK3 which downregulates MMP-1 expression rendered TNBC cells defective in both endothelial permeability and transendothelial migration. Upregulation of FRA-1 and MMP-1 was observed in circulating tumor cells (CTCs) derived from TNBC-bearing mice when compared with parental TNBC cells, further supporting the role of MLK3/ MMP-1 axis in vascular intravasation. High levels of MMP-1 in breast cancer patients are strongly associated with poor prognosis, including increased distant metastases, shortened recurrence, and poorer overall survival. This investigation has deciphered key components of the MLK3 signaling pathways that control the transcription factor FRA-1 and MMP target genes during cancer invasion and intravasation. These studies support the idea that targeting MLK3 is a promising therapeutic strategy for combating TNBC metastasis.

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KEY TO ABBREVIATIONS

3D	Three dimensional
6-FAM	5(6)-Carboxyfluorescein
ADAM12	a disintegrin and metalloproteinase 12
ADORA2B	adenosine receptor A_{2B}
ALDH1	aldehyde dehydrogenase-1
Angptl4	angiopietin-like 4
AP-1	activating protein-1
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
BC	breast cancer
BMP	bone morphogenetic protein
C	carboxyl
C.L	cellular lysate
C.M	concentrated conditioned medium
Cas9	CRIPR-associated protein 9
Cdc42	cell division cycle 42
CHIP	carboxyl terminus of Hsc70-interacting protein
COMP	cartilage oligomeric matrix protein
COX-2	cyclooxygenases-2
CRIB	Cdc42-/Rac1-interactive binding
CRISPR	clustered regularly interspaced short palindromic repeats

CTC	circulating tumor cell
CXCL	C-X-C ligand
CXCR	C-X-C chemokine receptor
DAPI	4’, 6-Diamidino-2-Phenylindole
DDR1	discoidin domain receptor 1
DLK	dual-leucine zipper –bearing kinase
DMFS	distant Metastasis Free Survival
DMSO	dimethyl sulfoxide
DTCs	disseminated tumor cells
E-cadherin	epithelial cadherin
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescence protein
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
EREG	epiregulin
ERK	Extracellular-signaling regulated kinase
FA	focal adhesion
Fas-L	Fas ligand
FBS	fetal bovine serum
FGFR1	fibroblast growth factor receptor 1
FRA-1	Fos-related antigen 1

GAP	GTPase-activating protein
GBM	glioblastoma
gDNA	genomic deoxyribonucleic acid
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
GPI	glycosyl-phosphatidyl inositol
GTP	guaniosine-5'- triphosphate
GTPase	guaniosine triphosphatase
Her2	human epidermal growth factor receptor
HGF	hepatocyte growth factor
HR	hazardous ratio
HSP	heat shock protein
IGF	Insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
IKC	I-kappa-B kinase complex
IKK	I-kappa-B kinase
IL1-β	Interleukin 1 beta
JDP	Jun dimerization protein
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
JSAP	JNK/stress-activated protein kinase-associated protein
KO	knockout
LZ	leucine zipper

LZK	leucine zipper-bearing kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinase
MCP-3	monocyte chemoattractant protein-3
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MKK	mitogen-activated protein kinase kinase
MLCK	myosin light chain kinase
MLK	mixed lineage kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
N	amino
n.s.	not statistically significant
N-cadherin	neural cadherin
NF2	neurofibromatosis type
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	non-small cell lung carcinoma
OS	overall Survival
PAI-1	plasminogen activator inhibitor-1
PI3K	phosphoinositide 3-kinase
PR	progesterone receptor
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction

Rac1	Ras-related C3 botulinum toxin substrate 1
Rap1	rhoptery associated protein 1
RFS	relapse Free Survival
RhoA	Ras homologous A
RNAi	ribonucleic acid interference
ROCK	Rho-associated protein kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
SAM	sterile-alpha-motif
SAPK	stress-activated protein kinase
SD	standard deviation
SDF-1	stromal-derived factor
SH	Src-homology
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SPARC	secreted Protein Acidic and Rich in Cysteine
Stat3	signal transducer and activator of transcription 3
TAK	TGFβ-activated kinase
TGFβ	transforming growth factor beta
TNBC	triple-negative breast cancer
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor alpha
TPL	tumor progression locus

TRAF	TNFR-associated factor
u-PA	urokinase-type plasminogen activator
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
WDR62	WD40-repeat protein 62
ZAK	zipper and sterile-alpha motif kinase
α1-PI	α1-protease inhibitor
α2-M	alpha 2-Macroglobulin

CHAPTER I

LITERATURE REVIEW

A portion of this chapter (p. 16-p. 38) has been published in an open access manuscript for *Cancers*, 2016, Volume 8, Issue 5 (Rattanasinchai and Gallo, 2016).

1. Breast cancer

Breast cancer is the most frequently diagnosed cancer in women worldwide and is a major health concern in the US. Each year, over 200,000 new cases are expected in the US. Due to this prevalence of breast cancer, it is estimated that about one in every eight US women will develop breast cancer during her lifetime (Siegel et al., 2015; Siegel et al., 2016).

Prognosis and treatment strategies for breast cancer patients are partly determined by breast cancer subtypes. Breast cancer can be classified into four major molecular subtypes: luminal A, luminal B, Human epidermal growth factor receptor (Her2) amplified, and triple-negative breast cancer (TNBC)(Dai et al., 2015). The majority of breast cancer patients (50-60%) present with luminal A subtype, which is characterized by the presence of hormone receptors: estrogen receptors (ERs) and/or progesterone receptors (PRs) (Dai et al., 2015). This breast cancer subtype generally has the best prognosis. The luminal B subtype comprises 15-20% of all breast cancer patients. Although luminal B subtype displays hormone receptor positive similar to luminal A, it is typically more aggressive and is associated with higher proliferation rates as judged by increased proliferation marker (Ki-67) and/or Her2 expression (Dai et al., 2015). The Her2 subtype, which includes about 15-20% of all breast cancer patients, is characterized by the presence of Her2 gene amplification and/or protein overexpression with the absence of hormone receptors. Her2 breast cancer tends to be aggressive and highly metastatic (Dai et al., 2015). The last subtype is TNBC which shows no expression of ERs, PRs, and Her2 and is detected in about 10% of breast cancer patients. This subtype is considered to be the most aggressive and highly metastatic breast cancer and has a very poor prognosis (Dai et al., 2015).

The treatment of breast cancer involves combination of surgical resection, radiation therapy and systemic therapy, which includes hormonal therapy, targeted therapy and

chemotherapy. The first-line systemic therapy of ER+ breast cancer is hormonal therapy that targets either ER or the production of its ligands (Lumachi et al., 2013). While patients with high Her2 expression will benefit from the treatment with antibodies against Her2 itself or small molecule inhibitors that block its signaling (Figueroa-Magalhães et al., 2014). Unlike the other subtypes, no targeted therapy is currently available for TNBC and the standard systemic therapy is chemotherapy (Yersal and Barutca, 2014).

Improvements in early diagnosis and therapeutics over the past decades have caused a decline in mortality rate of breast cancer patients. Nonetheless, over 40,000 deaths are still expected each year in the US, making breast cancer the second leading cause of cancer-related deaths in US women. Importantly, the majority of breast cancer-related deaths are due to the metastatic disease, which is the formation of secondary tumors from cancer cells that have disseminated form the primary tumor to the distant sites, most commonly to bone, lung, and brain. Therefore, understanding molecular mechanisms that control the metastatic process may reveal the key signaling molecules that can be used as promising drug targets to combat breast cancer metastasis.

In this chapter, a signaling molecule named Mixed-Lineage Kinase 3 (MLK3) will be introduced and its involvement in cancer invasion and metastasis in several types of cancers will be discussed. The overall goal of this dissertation is to identify a novel downstream signaling modules of MLK3 that are critical for breast cancer invasion and metastasis and to assess the potential of MLK3 as a promising druggable target for a treatment of breast cancer metastasis.

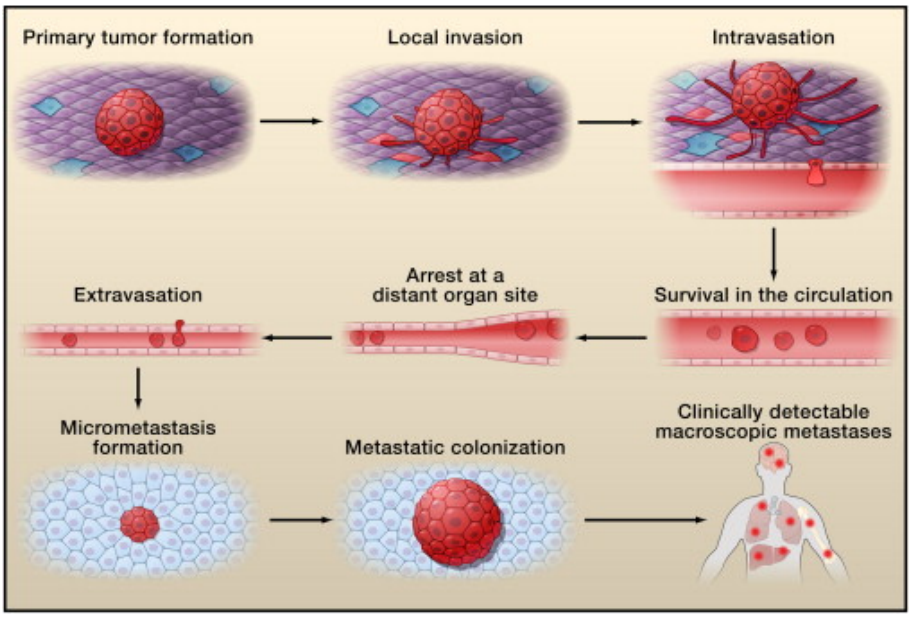

Figure 1.1 Illustration of the metastatic cascade. Metastasis is an extremely complex, multi-step event involving several biological processes that requires distinct cellular alterations. Cancer cells must first dissociate from the primary tumor, invade through the surrounding extracellular matrix (ECM), and enter into the blood or lymphatic vessel (intravasation). Then these cancer cells must survive in circulation, exit to the distant site (extravasation), adapt to the new microenvironment, and initiate an outgrowth (colonization). Only cancer cells that accomplish all of these steps will give rise to the metastatic tumor (Valastyan and Weinberg, 2011).[1]

2. Metastatic cascade

Metastasis is the major cause of death in many cancer types. Despite improvements in cancer therapeutics, which have proven effective in early stage cancer, metastatic disease remains incurable and patients with metastatic cancer have a poorer outcome (Siegel et al., 2015). The metastasis cascade consists of multiple steps and is considered fairly inefficient, as failure to complete any step of this process will prevent metastatic disease (Bacac and Stamenkovic, 2008; Talmadge and Fidler, 2010). Acquisition of genetic and/or epigenetic alterations in primary tumors, which causes a subset of cancer cells to gain invasive capability, is thought to initiate the metastatic process. Only a subset of cancer cells capable of degrading the

[1] Reprinted from Cell, Vol 127, S. Valastyan and R.A. Weinberg, Tumor Metastasis: Molecular Insights and Evolving Paradigms, page 275-279, Copyright (2011), with permission from Elsevier.

extracellular matrix (ECM) can disseminate into the ECM surrounding the primary tumor. To form distant metastases, cancer cells must be able to intravasate into blood or lymphatic vessels, survive and circulate within the bloodstream, and extravasate out at a distant site. Only if the new microenvironment is favorable will these cancer cells colonize and form a distant metastatic tumor (Fidler, 2003).

2.1 Cancer invasion/ Local invasion

Cancer invasion is the process in which tumor cells break away from a well-confined primary tumor into surrounding tissues or ECMs. Depending upon the microenvironment, tumor plasticity and cell-type specificity, there are at least three known mechanisms for cancer invasion: mesenchymal, amoeboid, and collective invasion (**Figure 1.2**) (Clark and Vignjevic, 2015; Friedl and Alexander, 2011; Sahai and Marshall, 2003).

Mesenchymal invasion involves a transition of epithelial-like cancer cells to mesenchymal-like cancer cells. Epithelial-to-mesenchymal transition (EMT) is a process that involves a loss of cell-cell adhesion and epithelial polarity and a shift towards a mesenchymal phenotype which enhances migration capacity and invasiveness of cancer cells. Depending upon cell types and contexts, many signaling molecules such as growth factors, *e.g.* epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF), and cytokines, *e.g*. transforming growth factor beta (TGFβ) and tumor necrosis factor alpha (TNFα) are known induce EMT (Christiansen and Rajasekaran, 2006). Through several signal transduction mediators, this reprogramming is eventually orchestrated by a subset of transcription factors including FOS-related antigen-1 (FRA-1) (Bakiri et al., 2015; Cheng et al., 2016; Diesch et al., 2014; Lemieux et al., 2009), Snail (Mani et al., 2008), Slug (Vuoriluoto et al., 2011; Wang et al., 2016), ZEB1 (Bakiri et al., 2015), ZEB2 (Bakiri et al., 2015), and Twist

(Mani et al., 2008; Wang et al., 2016) that function to repress epithelial markers and induce expression of mesenchymal-associated genes. Some of these transcription factors such as ZEB 1/2 can directly suppress transcription of E-cadherin, a major cell-cell adhesion molecule as well as induce several mesenchymal genes (Wong et al., 2014). FRA-1, another transcription factor, functions to regulate expression of the other transcription factors including ZEB 1/2 and Slug, and the other EMT-associated signaling factor such as TGF-β (Bakiri et al., 2015).

Another important characteristic of mesenchymal-like cancer cells is the ability to degrade ECM. In a context of carcinomas in situ, the primary tumors are well-confined within the thin layer basement membrane which is an organized composite of several ECMs with collagen type IV being the most abundant protein (LeBleu et al., 2007). In order to become invasive, these tumors must first overcome such constraints. Derailing the tightly-controlled protease activities is a major mechanism cancer cells utilize to degrade the basement membrane and remodel the surrounding stromal ECM (Valastyan and Weinberg, 2011) (see **Table 1.1** for MMPs and their substrates). Several matrix metalloproteinases are overexpressed in various types of cancer and have been shown to play a critical role in cancer invasion and metastasis (Cathcart et al., 2015). In breast cancer, the transcript levels of MMP-1, -9, -11, -12 and -13 are found to be upregulated in higher grade breast tumors compared to normal breast tissues (Merdad et al., 2014), and high levels of MMP-1 also predict the progression of atypical ductal hyperplasia to invasive breast cancer (Poola et al., 2005).

Although amoeboid invasion is observed in many types of cancer, *i.e.* breast cancer (Wolf et al., 2003) and glioblastomas (Seol et al., 2012), little is known about this type of invasion. In contrast to mesenchymal invasion, cancer cells utilizing amoeboid invasion do not rely on protease activity (Wolf et al., 2003). Instead, this type of invasion relies on Rho/ROCK

pathway-mediated contractility. As a result, these actin-rich pseudopod-like cancer cells invade through surrounding stroma by squeezing themselves through the gaps between ECMs rather than degrading this matrix (Friedl and Alexander, 2011; Wolf et al., 2003).

Collective invasion/collective cell invasion is predominantly observed in tumor specimens from many types of cancer including breast cancer and prostate cancer (Friedl et al., 2012). Collective invasion is described as the invasion of cancer cells that maintain their cell-cell interactions and moves as a cohort (Friedl and Alexander, 2011; Giampieri et al., 2009). Similar to amoeboid invasion, little is known about the mechanism behind this type of cancer invasion.

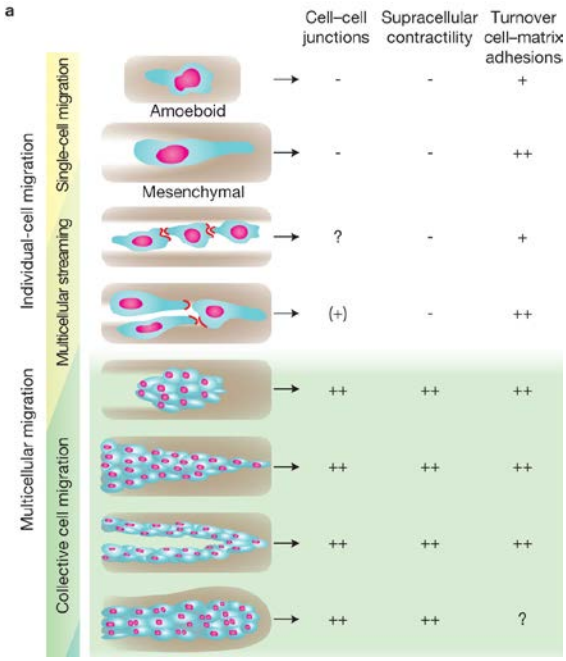

Figure 1.2 Modes of cancer cell migration/ invasion. Cancer cells can invade as single cells or as a cohort. Single cell migration/invasion refers to the invasion of individual cells with no contacts with adjacent cells while collective cell migration/ invasion refers to the invasion of a small group of cells that still maintain cell-cell contact. Multicellular streaming refers to invasion pattern with features of both single cell and collective cell invasion (Friedl et al., 2012).[2]

[2] Reprinted by permission from Macmillan Publishers Ltd: Nature Cell Biology Friedl, P., J. Locker, E. Sahai, and J.E. Segall. 2012. Classifying collective cancer cell invasion. *Nat Cell Biol*. 14:777-783.), copyright (2012).

Table 1.1 Matrix metalloproteinases and their substrates: extracellular matrices and others.

Enzyme	MMP	Common name	ECM Substrates	Other substrates
Collagenase	MMP-1	Interstitial collagenase, collagenase 1	Collagens (type I, II, III, VII, VIII, X, and XI), gelatin, fibronectin, vitronectin, laminin, entactin, tenascin, aggrecan, link protein, myelin basic protein, versican	Autolytic, C1q, α2-M, ovostatin, α1- PI, α1-antichymotrypsin, IL1-β, proTNFα, IGFBP-2, IGFBP- 3, casein, serum amyloid A, proMMP-1, proMMP-2, proMMP-9,
	MMP-8	Neutrophil collagenase, collagenase 2	Collagens (type I, II, and III), aggrecan	Autolytic, C1q, α2-M, ovostatin, α1-PI, substance P, fibrinogen2, angiotensin I, angiotensin II, bradykinin, plasmin C1-inhibitor
	MMP-13	Collagenase 3	Collagens (type I, II, III, IV, VI, IX, X, and XIV), collagen telopeptides, gelatin, fibronectin, SPARC, aggrecan, perlecan , large tenascin-C	Autolytic, C1q, α2-Macroglobulin, casein, fibrinogen, factor XII, α1-antichymotrypsin, proMMP-9
	MMP-18	Collagenase 4 (*Xenopus*)	Collagen type I, gelatin	
Gelatinase	MMP-2	Gelatinase A	Collagens (type I, II, III, IV, V, VII, X, and XI), gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, galectin-3, versican, decorin, myelin basic protein	Autolytic, α1-PI, α2-M, α1-antichymotrypsin, IL1-β, proTNFα, IGFBP-3, IGFBP-5, substance P, serum amyloid A8, proMMP-1, proMMP-2, proMMP-9, proMMP-13, latent TGFβ9, MCP-3, FGFR1, big endothelin-1, plasminogen
	MMP-9	Gelatinase B	Collagens (type IV, V, XI, and XIV), gelatin, elastin, vitronectin, laminin, SPARC, aggrecan, link protein, galectin-3, versican, decorin, myelin basic protein,	Autolytic, α2-M, ovostatin, α1-PI, IL1-β, proTNFα, substance P, casein, carboxymethylatedtransferrin, angiotensin I, angiotensin II, plasminogen, proTGFβ, IL-2Rα, release of VEGF (substrate not known)

Modified from Nagase H (Nagase, 2001), Sohail A *et al.* (Sohail et al., 2008), and Visse R *et al.* (Visse and Nagase, 2003); α2-M, α2-Macroglobulin; α1-PI, α1-protease inhibitor; IL1-β, Interleukin 1 beta; SPARC, secreted Protein Acidic and Rich in Cysteine; TNFα, tumor necrosis factor alpha; IGFBP, insulin-like growth factor-binding protein; TGFβ, transforming growth factor beta; MCP-3, monocyte chemoattractant protein-3; FGFR1, fibroblast growth factor receptor 1; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; COMP, cartilage oligomeric matrix protein ; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; Fas-L, Fas ligand; GPI, glycosyl-phosphatidyl inositol.

Table 1.1 (cont'd)

Enzyme	MMP	Common name	ECM Substrates	Other substrates
Stromelysin	MMP-3	Stromelysin 1	Collagens (type III, IV, V, VII, IX, X, and XI), collagen telopeptides, gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC	Autolytic, α2-M, ovostatin, , α1- PI, α2−antiplasmin, α1-antichymotrypsin, IL1-β, proTNFα, IGFBP-3, substance P, T-kininogen, casein, carboxymethylated transferrin, antithrombin-III, serum amyloid A, fibrinogen, plasminogen, osteopontin, proMMP-1, proMMP-3, proMMP-7, proMMP-8, proMMP-9, proMMP-13, IGFBP-3, E-cadherin, pro-HB-EGF, u-PA, fibrin, PAI-1
	MMP-10	Stromelysin 2	Collagens (type III, IV, and V), gelatin, elastin, fibronectin, aggrecan, link protein	Autolytic, casein, proMMP-1, proMMP-7, proMMP- 8, proMMP-9
	MMP-11	Stromelysin 3	Gelatin, fibronectin, collagen type IV, laminin	α1-PI, α2-M, ovostatin, IGBFP-1, casein, α2−antiplasmin, plasminogen activator inhibitor-2, casein, carboxymethylated-transferrin
Matrilysin	MMP-7	Matrilysin-1	Collagen IV, gelatin, aggrecan, link protein, elastin, fibronectin, vitronectin, laminin, SPARC, enactin, decorin, myelin basic protein, tenascin,	Autolytic, α1-PI, α2-M, proTNFα, casein, carboxymethylated transferrin, osteopontin18, proMMP-1, proMMP-2, pro-MMP-7 proMMP-9, plasminogen, pro-α-defensin, Fas-L, β4 integrin, E-cadherin
	MMP-26	Matrilysin-2	Collagen IV, gelatin, fibronectin, vitronectin	α1-PI, α2-M, fibrinogen, proMMP-9
Membrane-type MMP	MMP-14	MT1-MMP	Collagens (type I, II, and III), gelatin, fibronectin, tenascin, vitronectin, laminin, entactin, aggrecan, perlecan	α2-M, ovostatin, α1-PI, proTNFα, fibrinogen, factor XII, fibrin, CD44, tissue transglutaminase, proMMP-2, proMMP-13
	MMP-15	MT2-MMP	Fibronectin, tenascin, entactin, laminin, aggrecan, perlecan	proTNFα, tissue transglutaminase, proMMP-2
	MMP-16	MT3-MMP	Collagen type III, gelatin, fibronectin, vitronectin, laminin,	α1-PI, α2-M, casein, proMMP-2, tissue transglutaminase
	MMP-17	MT4-MMP (GPI -anchored)	Gelatin	Fibrinogen, fibrin, proTNFα, proMMP-2
	MMP-24	MT5-MMP	Fibronectin, Gelatin, chondroitin sulphate proteoglycan, dermatan sulphate proteoglycan	ProMMP-2, tissue transglutaminase
	MMP-25	MT6-MMP (GPI -anchored)	Collagen type IV, gelatin, fibronectin, chondroitin sulphate proteoglycan, dermatan sulphate proteoglycan	Fibrinogen, fibrin, α1-PI, proMMP-2

Table 1.1 (cont'd)

Enzyme	MMP	Common name	ECM Substrates	Other substrates
Metalloelastase	MMP-12	Macrophage elastase	Collagen IV, elastin, gelatin, fibronectin, vitronectin, laminin, entactin, aggrecan, myelin basic protein	α2-M, α1-PI, proTNFα, fibrinogen, factor XII, casein, plasminogen
Enamelysin	MMP-20	Enamelysin	Amelogenin aggrecan, COMP	Autolysis
Others	MMP-19	N/A	Collagen IV, gelatin, laminin, entactin, tenascin-C, fibronectin, aggrecan	Autolysis, fibrinogen, fibrin
	MMP-21	XMMP (Xenopus)	Gelatin	Casein
	MMP-22	CMMP (Chicken)	N/A	N/A
	MMP23	CA-MMP	Gelatin	
	MMP-27	CMMP (Gallus)	Gelatin	Autolysis, casein
	MMP-28	Epilysin	N/A	Casein

2.2 Intravasation

Intravasation, in the context of cancer metastasis, refers to a process by which cancer cells enter either lymphatic vessels (lymphatic intravasation) or blood vessels (hematogenous intravasation) which is required for dissemination to other organs of the body. Once invading cancer cells reach the endothelial cell layers which line the surface of blood or lymphatic vessels, cancer cells must enter into the vasculature in a process called "transendothelial migration" which involves the disruption of endothelial cell barrier and transmigration across endothelial cell layers. Despite being so important, this remains the least studied step of the metastatic cascade, possibly due to limited experimental models that recapitulate this event (Juncker-Jensen et al., 2013). Nevertheless, at least two mechanisms: paracellular and transcellular intravasation, have been proposed.

Paracellular intravasation describes the transmigration of cancer cells across endothelial cell layer through path generated by disrupting endothelial cell-cell junctions. In a study using an *in ovo* tumor grafting[3], MMP-1 functions to cleave and activate the endothelial protease-activated receptor-1 (PAR-1) which results in weakening the endothelial cell junctions, thus, allowing cancer cells to transmigrate across the endothelial cell layers (**Figure 1.3**). In breast cancer patients, a disintegrin and metalloproteinase 12 (ADAM12) is overexpressed specifically in the tumor vasculature of infiltrating ductal carcinoma compared with normal breast tissue vasculature. The results from several *in vitro* assays suggest that high levels of either tumor- or macrophage-secreted cytokines, such as tumor necrosis factor alpha (TNFα) and transforming

[3] An *in ovo* tumor grafting involves grafting of human tumor cells onto chorioallantoic membrane (CAM) of chicken embryos. Chicken embryos at the early stage, when the lymphoid organs are not well developed, are naturally immunodeficient and are well-vascularized; their CAMs also provide a unique supporting microenvironment for primary tumor formation and a source of angiogenic blood vessel Deryugina, E.I., and J.P. Quigley. 2008. Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell metastasis. *Histochemistry and cell biology*. 130:1119-1130.. This assay can also be used to study the process of intravasation, survival in circulation, and extravasation.

growth factor beta (TGFβ) in tumor microenvironment, induce expression of ADAM12 which, in turn, promotes cleavage of several membrane-anchored endothelial proteins including vascular endothelial cadherin (VE-cadherin) and angiopoietin receptor TIE-2, resulting in disruption of endothelial cell junctions (Frohlich et al., 2013). In addition, many tumors are also known to induce neoangiogenesis. These newly formed blood vessels generally have weaker cell-cell junctions, thus providing an easy escape route for opportunistic cancer cells (Reymond et al., 2013).

Conversely, transcellular intravasation describes the transmigration of cancer cells directly through individual endothelial cells. In this mechanism, cancer cells regionally trigger endothelial myosin light chain kinase (MLCK) activation at the invasion site, causing the localized endothelial myosin contraction and, ultimately, cytoskeletal rearrangement which generate path for cancer cells to transmigrate across endothelial cells (Khuon et al., 2010).

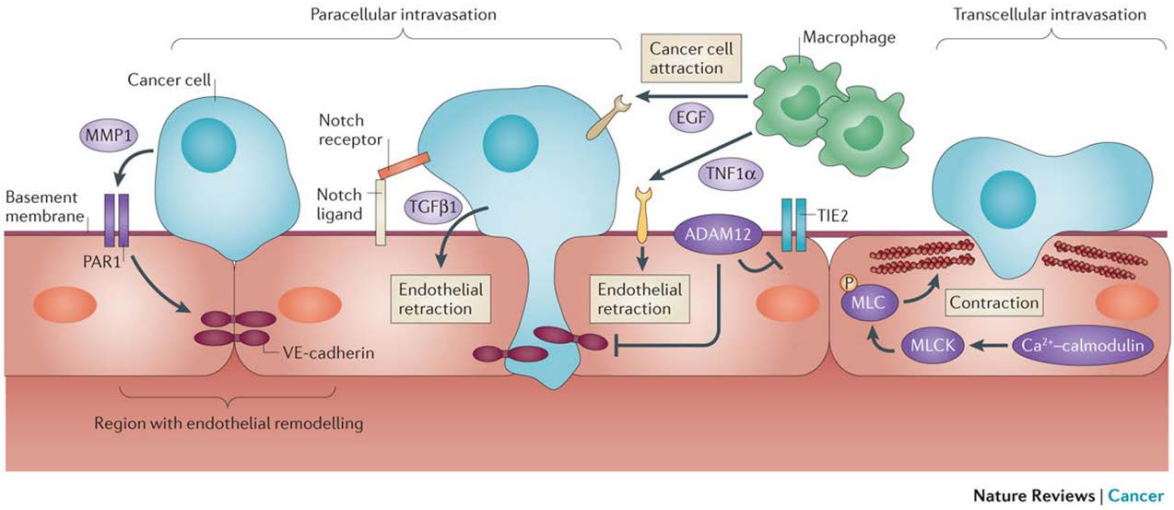

Figure 1.3 Illustration of cancer intravasation. Cancer cells enter the vasculature through path generated from disrupting endothelial cell-cell junctions (paracellular intravasation) or directly through individual endothelial cells (transcellular intravasation) (Reymond et al., 2013)[4]. See text for more detail.

[4] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer Reymond, N., B.B. d'Água, and A.J. Ridley. 2013. Crossing the endothelial barrier during metastasis. *Nature Reviews Cancer*. 13:858-870..copyright (2013)

2.3 Survival in circulation: circulating tumor cells (CTCs)

Cancer cells that have successfully intravasated into the vasculature and remain circulating in the bloodstream are called circulating tumor cells (CTCs); they are considered as the population responsible for the formation of distant metastases (Budd et al., 2006; Cristofanilli et al., 2007; Cristofanilli et al., 2004; Cristofanilli et al., 2005; Hayes et al., 2006). Indeed, the number of CTCs is used as a prognostic marker for disease progression, overall survival, and to assess the treatment responses in patients with several types of cancer including breast (Budd et al., 2006; Cristofanilli et al., 2004; Cristofanilli et al., 2005; Giuliano et al., 2014; Giuliano et al., 2011), prostate (Danila et al., 2007; De Bono et al., 2008; Scher et al., 2009) and colorectal cancer (Cohen et al., 2008; Tol et al., 2010). Notably, not all CTCs are capable of giving rise to disseminated tumors (Aceto et al., 2015). First of all, the majority of CTCs are destroyed in the bloodstream. Upon entering the vasculature, CTCs must first endure the shear force generated from the blood flow and, later, they must escape the immune surveillance in order to survive in the circulation. Direct interaction of CTCs with platelets is thought to be a survival mechanism for CTCs as platelets serve as a shield to reduce the shear force and make cancer cells inaccessible to destruction by immune cells (Joyce and Pollard, 2009; Lou et al., 2015). Secondly, not all CTCs possess the characteristic of stemness[5]. Only those that express stem cell markers (*e.g.* aldehyde dehydrogenase-1 (ALDH1) and CD44), EMT markers (*e.g.* Twist, Vimentin, N-cadherin, and ZEB1), or both markers seem to be competent for disease progression (Aktas et al., 2009; Barriere et al., 2014). Indeed, patients whose CTCs possess stem cell and EMT markers are prone to relapse and have higher metastatic burden; they are also poorly responsive to chemotherapy (Mitra et al., 2015).

[5] Stemness is the characteristic found in normal stem cells as well as in cancer stem cells (CSCs). It is the ability of the cells to self-renew and differentiate into different cell types.

2.4 Extravasation

Extravasation refers to the process of cancer cells exiting the circulation. Although the process of extravasation appears to closely resemble the process of intravasation, their mechanisms may not be fully identical, partly due to the distinct microenvironments inside and outside of the vasculature (Valastyan and Weinberg, 2011). For example, cancer intravasation can be facilitated by the tumor-promoting macrophages that reside within or near primary tumors; such macrophages may not present during extravasation. In addition, the newly-formed blood vessels near or within primary tumors where most intravasation takes place are considered leaky, while, at the site of extravasation, the vasculature are fully-functioned and less permeable. Nevertheless, these two processes involve the same concept – disruption of endothelial cell barrier followed by transmigration across this cell layer. During extravasation, cancer cells must first induce vascular hyperpermeability. It is thought that cancer cells secrete several factors including. angiopietin-like 4 (Angptl4), epiregulin (EREG), cyclooxygenases-2 (COX-2), MMP-1, and MMP-2 to disrupt the integrity of endothelial cell barrier (Gupta et al., 2007). Interestingly, this vasculature permeability can also occur prior to the arrival of the CTCs, through the secreted factors from the primary tumors (Huang et al., 2009).

2.5 Colonization: disseminated tumor cells (DTCs)

Cancer cells that have accomplished the step of extravasation and infiltrated into secondary sites are called disseminated tumor cells (DTCs)(Braun and Naume, 2005). After organ infiltration, these DTCs must first overcome host tissue defense in order to survive. It is thought that some DTCs undergo dormancy/latency to escape the stressful microenvironment in the host tissues (Sosa et al., 2013). This idea is supported by the clinical findings that, in multiple cancers including breast cancer, prostate cancer, and melanomas), some patients develop

metastatic recurrence years or even decades after primary tumor removal, suggesting a long term survival of disseminated tumor cells in the absence of clinical metastases (Sosa et al., 2013).

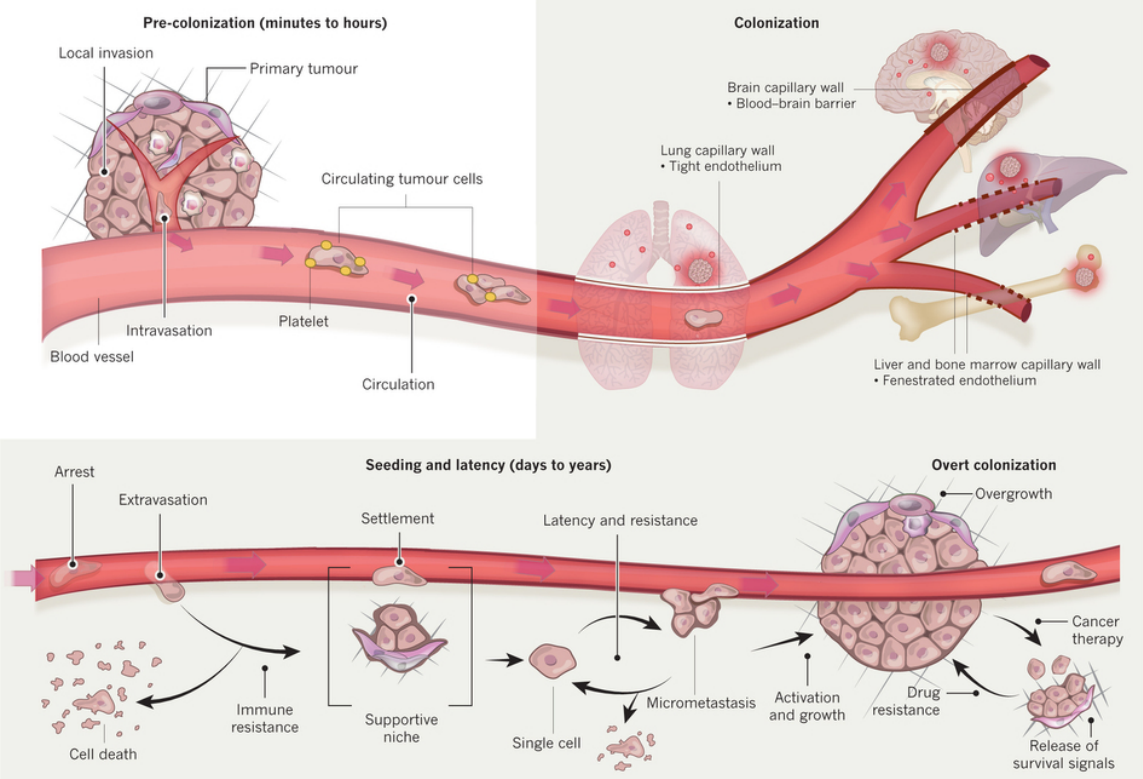

Figure 1.4 Illustration for metastatic colonization. Colonization is considered the final step in the metastatic cascade. After organ infiltration, disseminated tumor cells (DTCs) must thwart immune destruction and other obstacles in the new microenvironment to initiate the formation of metastases. Some DTCs or micrometastases can also enter a latent stage and become dormant for many years before they reinitiate the outgrowth and form metastases (Massagué and Obenauf, 2016).[6]

At least three categories of dormancy mechanisms have been proposed: 1) cellular dormancy caused by downregulation of proliferative signal, extracellular signal-regulated kinase (ERK), and upregulation of stress-induced signal, P38, 2) angiogenic dormancy caused by the poor vascularization which maintains a constant number of cells by cell division and turnover,

[6] Reprinted by permission from Macmillan Publishers Ltd: Nature Massagué, J., and A.C. Obenauf. 2016. Metastatic colonization by circulating tumour cells. *Nature*. 529:298-306..copyright (2016).

and 3) immune-mediated dormancy (Sosa et al., 2014). Only if the host microenvironment provides adequate cues for tumor growth will these DTCs awake from the dormant/latent stage and initiate the metastatic outgrowth (**Figure 1.4**). Many of the pro-metastatic cues, such as EGF, present in the microenvironment are produced by stromal cells; only a subset of DTCs capable of utilizing these local factors will give rise to metastases. Some of these factors, such as Wnt, TGFβ, bone morphogenetic protein (BMP), Notch, are known to stimulate stem-cell supporting pathways including the signal transducer and activator of transcription 3 (Stat3) pathways, while others are required for cell metabolism, proliferation and survival pathways, such as phosphoinositide 3-kinase (PI3K), and mitogen activated protein kinase (MAPK) (Massagué and Obenauf, 2016). Notably, some cancer cells are also capable of amplifying these pathway outputs through the sustained autocrine production in order to survive in the microenvironment with low levels of these factors (Massagué and Obenauf, 2016).

3. The mixed lineage kinase (MLK) family

Dysregulation of mitogen-activated protein kinase (MAPK) pathways has been reported in several types of cancer and is involved in multiple aspects of cancer progression (Dhillon et al., 2007; Kim and Choi, 2010). The MAPK signaling cascade is an evolutionarily conserved pathway and is important for many biological processes such as cell survival, cell proliferation, cell differentiation and cell motility (Cargnello and Roux, 2011; Gallo and Johnson, 2002; Goldsmith et al., 2010; Seger and Krebs, 1995). Members of the MAPK cascade function as signal mediators, transmitting extracellular signals to intracellular substrates. In normal cells, MAPK signaling, triggered upon mitogen stimulation, is tightly controlled by a three-tiered phosphorelay of protein kinases. Once stimulated, MAP kinase kinase kinases (MAP3Ks) phosphorylate and activate specific MAP kinase kinases (MAP2Ks, MKKs), which in turn

phosphorylate and activate specific MAP kinases (MAPKs). Depending upon the context and cell type, MAPK proteins can either phosphorylate cytosolic substrates or translocate into the nucleus to phosphorylate and regulate transcription factors. Overexpression and constitutively-active mutated MAPK proteins as well as their upstream regulators are commonly present in various types of cancers. Notably, these alterations lead to upregulation of MAPK pathway and sustained signaling for cell survival, proliferation, migration and invasion of cancer cells.

The MLK family belongs to a large family of MAP3Ks and the name "mixed lineage kinase" arose from the fact that the amino acid sequence of their catalytic domains resembles both tyrosine kinases and serine/threonine kinases. Despite the name, biochemical analyses of MLKs has demonstrated only serine/threonine kinase function (Gallo et al., 1994). Indeed, this family resides within the "tyrosine kinase-like" branch of the human kinome. Of the seven mammalian MLK family members, all have been shown to function as MAP3Ks in various contexts. Based on functionally conserved domain arrangements and sequence similarity, the MLK family can be clustered into three subfamilies: the MLKs, the dual-leucine zipper –bearing kinases (DLKs), and zipper and sterile-alpha motif kinases (ZAKs) (Gallo and Johnson, 2002) (**Figure 1.5**).

3.1 Mixed lineage kinases (MLKs)

The mammalian MLK subfamily consists of 4 members: MLK1 (MAP3K9), MLK2 (MAP3K10), MLK3 (MAP3K11), and MLK4, which has two alternatively spliced isoforms known as MLK4α and MLK4β (Kashuba et al., 2011). In Drosophila, only one MLK subfamily homolog, named Slipper, exists (Gallo and Johnson, 2002).

All four members were demonstrated to possess MAP3K function *in vitro* (Marusiak et al., 2014). These MLKs share several functional domains including an amino (N)-terminal Src-

homology 3 (SH3) domain, a catalytic domain, leucine/isoleucine zipper (LZ) motifs, and a Cdc42-/Rac1-interactive binding (CRIB) motif (Ezoe et al., 1994; Gallo et al., 1994; Ing et al., 1994). MLK1-4 share 75% sequence similarity in their kinase domains and 65% in their SH3 domains. However, all members of the MLK subfamily have proline-rich carboxyl (C)-terminal regions with divergent amino acid sequences and poorly understood functions.

Figure 1.5 Schematic representation of the MLK family. The MLK family consists of three subfamilies based upon their functional domain arrangements. The MLK subfamily contains an N-terminal Src-homology 3 (SH3) domain followed by a kinase domain, a leucine zipper (LZ) motif, a Cdc42-/Rac1-interactive binding (CRIB) motif, and a proline-rich C-terminal region. The DLK subfamily contains a kinase domain followed by two LZ motifs that are separated by a short amino acid spacer, and a proline-rich C-terminal region. The ZAK subfamily consists of two isoforms: ZAKα contains a kinase domain followed by a LZ motif and a Sterile-α motif (SAM) while the N-terminus though LZ motif of ZAKβ is identical to ZAKα but lacks the SAM and extended C-terminal region (Rattanasinchai and Gallo, 2016).

3.2 Dual leucine zipper kinases (DLKs)

The DLK subfamily consists of two members: DLK (MAP3K12) and LZK (MAP3K13). Unlike MLKs, DLKs lack an N-terminal SH3 domain and a centrally-located CRIB motif. Instead, the catalytic domains are followed by two LZ motifs that are separated by a 31-amino acid spacer. Similar to MLKs, the C-termini of DLKs are sequence-divergent proline-rich regions of unknown regulatory function.

3.3 Zipper and sterile-alpha motif kinases (ZAKs)

ZAK is the sole member of the third MLK subfamily. It contains a unique functional domain, the sterile-α-motif (SAM), which distinguishes it from the other subfamilies (Liu et al., 2000). So far, two splice variants have been identified: ZAKα and ZAKβ. ZAKα contains a kinase domain followed by a short LZ motif and SAM domain, while ZAKβ is identical to ZAKα from the N-terminus to LZ motif but then diverges and lacks a SAM domain.

4. MLK3

MLK3, also known as MAP3K11, SPRK, PTK1, slipper (*Drosophila*), was first identified in 1994 (Ezoe et al., 1994; Gallo et al., 1994; Ing et al., 1994). This 847-amino acid protein is the only member in the MLK subfamily that has been extensively studied in terms of regulation, functions and implications in human diseases (Leung and Lassam, 1998; Liou et al., 2010; Schachter et al., 2006; Song et al., 2010; Vacratsis and Gallo, 2000; Zhan et al., 2011; Zhang and Gallo, 2001; Zhang et al., 2004). MLK3, as its name MAP3K11 implies, functions as a MAP3K to phosphorylate and activate specific MAP2Ks, which in turn activate specific MAPKs. Three major MAPKs are well described: c-Jun N-terminal kinase (JNK)/Stress-activated protein kinase (SAPK), Extracellular-signaling regulated kinase (ERK), and P38 MAPK. Depending upon context, MLK3 is capable of activating each of these MAPKs (Chadee

and Kyriakis, 2004b; Chadee et al., 2006; Gallo and Johnson, 2002). MLK3 can activate the JNK pathway by phosphorylating and activating MKK4/7 (Rana et al., 1996; Zhang et al., 2004), or on MKK3/4/6 to activate P38 MAPK (Kim et al., 2004; Tibbles et al., 1996). MLK3 has been reported to activate ERK in both a kinase-independent and kinase-dependent manner. MLK3 can function as a scaffold protein bridging Raf-1 to ERK-specific MAP3K B-Raf. This binding results in Raf-1-dependent B-Raf phosphorylation and activation, which leads to activation of MAPK/ERK kinase 1 and 2 (MEK1/2 also known as MAP2K 1/2 or MKK1/ 2) and consequently ERK phosphorylation and activation. A recent study has shown that MLK1-4 are enzymatically capable of RAF-independent MEK phosphorylation which, ultimately, can lead to reactivation of ERK signaling in melanoma cells with acquired resistance to a B-Raf inhibitor (Vemurafenib) in B-Raf V600E tumors (Ascierto et al., 2012b).

Similar to MLK3-mediated ERK activation, MLK3 has also been shown to negatively regulate the GTPase RhoA in both a kinase-dependent and kinase-independent manner. In a kinase-independent mechanism, MLK3 can directly bind to and sequester the RhoA specific guanine nucleotide exchange factor (GEF), p63RhoGEF thereby preventing its activation by $G_{\alpha q}$, thus suppressing or spatial regulating the activation of RhoA (Swenson-Fields et al., 2008). In breast cancer cells, catalytic activity of MLK3 is required to activate JNK, which in turn phosphorylates paxillin, a focal adhesion (FA) complex scaffold protein, on serine 178. This phosphorylation event of paxillin recruits FAK, which in turn promotes further phosphorylation of paxillin on tyrosine 31 and 118 (Chen and Gallo, 2012). This phosphorylated paxillin is thought to compete with the RhoA-specific GTPase-activating protein (GAP), p190RhoGAP, for binding to SH2 domains of p120RasGAP. Thus, upon phosphorylation, paxillin binds p120RasGAP, releasing p190RhoGAP from p120RasGAP, allowing p190RhoGAP to suppress

RhoA activity (Tsubouchi et al., 2002).

How MLK3 signaling results in diverse cellular outcomes is still unclear and requires further investigation. It has been shown that subcellular localization of MLK3 may determine how MLK3 regulates its downstream effectors. While MLK3 localization at the plasma membrane is required for maximal JNK activation (described later in MLK3 regulation), MLK3 distribution at the centrosome and on microtubules appears to regulate microtubule organization during mitosis in a JNK-independent fashion (Swenson et al., 2003). One possible explanation for various cellular localization and function is the ability of MLK3 to interact with several scaffolding proteins forming localized signalosomes. Hence scaffolding proteins may dictate signaling specificity as well as subcellular localization. At least three members of JNK-interacting proteins (JIPs), JIP-1, -2 and, -3, have been demonstrated to serve as scaffold proteins for a MLK3-MKK7-JNK signaling module (Whitmarsh, 2006; Yasuda et al., 1999). JIPs have been recognized as the cargos for a molecular motor kinesin, the motor protein that moves along microtubule filaments (Verhey et al., 2001). The association of MLK3 with JIPs may provide further explanation for the dynamic cellular distribution of MLK3. Interestingly, the JIP-3 variant, JNK/stress-activated protein kinase-associated protein 1 (JSAP1) is also reported to facilitate JNK activation via an MLK3-independent MEKK1-MKK4-JNK module (Ito et al., 1999). Additionally, JIP-2 has been shown to facilitate MLK3-dependent P38 MAPK activation by serving as a docking site for recruitment of MLK3, MKK3, and either P38α or P38δ isoforms. Of note, JIP-4 is the only member of the JIP family that does not form a complex with MLK3 (Kelkar et al., 2005). Additionally, a recent study has identified WD40-repeat protein 62 (WDR62) as a novel MLK3 scaffold protein. Interestingly, although WDR62 is known to recruit JNK 1/ 2 as well as MKK4/7, the JNK specific MAP2Ks, the recruitment of MLK3 to this

complex does not appear to enhance JNK phosphorylation. Rather, the association of MLK3 with this scaffold protein is thought to maintain MLK3 in its inactive state (Hadad et al., 2015) thus, theoretically, preventing MLK3 from phosphorylating and activating its downstream effectors.

Another explanation of how MLK3 might stimulate a wide spectrum of biological responses is its ability to turn on a subset of genes through controlling the transcription factor activation. The AP-1 transcription factor is a protein complex comprised of JUN and FOS protein dimers. In some contexts, members of the activating transcription factor (ATF) or Jun dimerization protein (JDP) subfamilies can also substitute as partners within these AP-1 dimers. MLK3 can regulate activation of JNK (Chen and Gallo, 2012; Chen et al., 2010; Hartkamp et al., 1999; Sathyanarayana et al., 2002; Tibbles et al., 1996; Zhan et al., 2012a) and ERK (Chadee and Kyriakis, 2004a; Chadee and Kyriakis, 2004b; Zhan et al., 2012a), two major modulators of AP-1 transcription factors (Hess et al., 2004). JNK phosphorylates and activates c-Jun, while ERK acts through FOS family members. Of note, a positive feedback loop exists among AP-1 members. For example, increased signaling through MLK3-JNK axis upon induced overexpression of MLK3 in non-tumorigenic mammary epithelial cells results in elevated levels of c-JUN and FRA-1 (Chen et al., 2010). Similarly, ERK has been shown to phosphorylate and stabilize FRA-1, which indirectly stabilizes c-JUN through its interaction with FRA-1(Talotta et al., 2010). Depending upon transcriptional cofactors, AP-1 activation can result in several biological responses including cell differentiation, cell cycle progression, and invasion (Hess et al., 2004).

In addition to its regulation of the AP-1 transcription factor, the role of MLK3 as an activator of the NF-kappaB (NF-κB) transcription factor has also been established (Hehner et al.,

2000). MLK3 can directly phosphorylate and activate both IκB kinase α (IKKα) and IκB kinase β (IKKβ). It also can form a complex with two other IκB kinase kinases, MEKK1 and NF-κB-inducing kinase, via association with its leucine zipper region; thus MLK3 is critical for activation of the IκB kinase complex (IKC). In unstimulated T cells, the activity of NF-κB is suppressed via its interaction with an inhibitory protein IκB. Upon T cell stimulation, MLK3 is required for activation of IKC which, in turn, phosphorylates IκB. Phosphorylation of IκB promotes its ubiquitination and proteasome-dependent degradation thereby releasing NF-κB from IκB and allowing NF-κB to translocate to the nucleus where it initiates NF-κB-dependent gene expression (Hehner et al., 2000). Interestingly, a pan-MLK inhibitor was found to block nuclear translocation of p65, a component of NF-κB complex, in estrogen receptor positive (ER+) breast cancer cells. This suggests a critical role for MLKs in regulating NF-κB signaling in cancer cells (Wang et al., 2013).

5. MLK3 regulation

5.1 MLK3 contains intrinsic autoinhibitory function

Protein kinases activate their downstream effectors through phosphorylation. To prevent undesired activation, the activities of protein kinases must be tightly controlled. Many protein kinases utilize multiple mechanisms to regulate their activity. MLK3 is no exception. Autoinhibition is commonly used as a repression mechanism for negative regulation of the catalytic activity of protein kinases (Pufall and Graves, 2002). MLK3 is one such protein kinase that contains an intrinsic autoinhibitory function that keeps it in its inactive, “closed” conformation, thereby limiting its activity in cells. The intrinsic inhibition of MLK3 is mediated by the N-terminal SH3 domain which serves as an autoinhibitory domain (Pufall and Graves, 2002). Indeed, mutation of the conserved tyrosine residue required for SH3-ligand binding to

alanine (Y52A) increases MLK3 kinase activity. A typical SH3 domain recognizes and interacts with a tandem PxxP motif on its ligand (Aitio et al., 2010). However, biochemical assays have shown that MLK3's autoinhibitory function is mediated through the interaction between its SH3 domain and a region containing a single proline residue at amino acid 469, which is located between LZ domain and CRIB motif, rather within the C-terminal proline-rich region (Zhang and Gallo, 2001). This critical proline residue is evolutionarily conserved among MLK subfamily members as well as Slipper, MLK3 homolog in Drosophila, suggesting they all utilize this common regulatory mechanism. To release MLK3 from this intrinsic autoinhibition, an additional extrinsic factor is required. Site- directed mutagenesis studies coupled with binding and activity analyses support a model wherein the association of the GTP-bound cell division cycle protein 42 (Cdc42) or Ras-related C3 botulinum toxin substrate 1 (Rac1) with MLK3 through its CRIB motif disrupts the SH3-mediated autoinhibition resulting in a conformational change that leads to autophosphorylation and activation of MLK3 (Böck et al., 2000; Burbelo et al., 1995; Teramoto et al., 1996) (**Figure 1.6**).

5.2 Dimerization and autophosphorylation of MLK3

When MLK3 is in its "open" conformation, MLK3 activity is still tightly controlled by the other mechanisms mediated through additional protein-protein interaction domains. Dimerization of MLK3 is mediated through the LZ motif, supported by the fact that MLK3 mutants lacking the LZ motif fail to form homodimers. Dimerization of MLK3 is a prerequisite for MLK3 autophosphorylation and activation of its downstream c-Jun N-terminal kinase (JNK) pathway (Leung and Lassam, 1998) (**Figure 1.6**). Co-expression of the MLK3 upstream regulator Cdc42 with MLK3 is sufficient to induce homodimerization and autophosphorylation of MLK3 as well as kinase-dependent activation of its downstream signaling effectors. Cdc42 is

also required for subcellular targeting of active MLK3 at the plasma membrane leading to maximal activation of the MLK3-MKK7-JNK-c-Jun signaling axis (Du et al., 2005). When LZ-mediated dimerization of MLK3 is destabilized by substitution of a conserved leucine residue with an α-helix-disrupting proline residue, Cdc42 can still induce autophosphorylation of monomeric MLK3 protein. However this monomeric MLK3 is unable to phosphorylate MKK4 on one of its activation specific phosphorylation sites, threonine 258, suggesting that homodimerization of MLK3 is required for proper activation of MAP2K and subsequent activation of the JNK pathway (Vacratsis and Gallo, 2000).

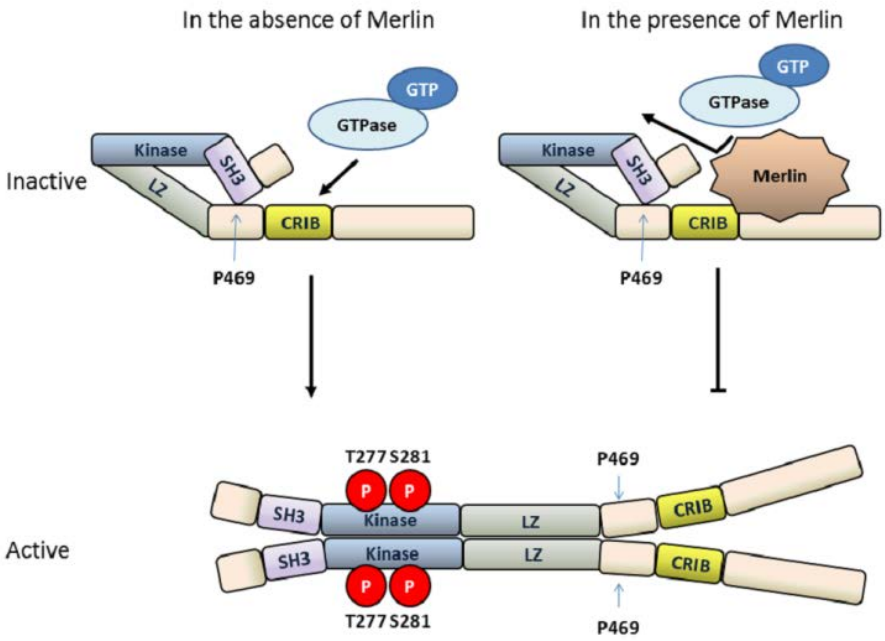

Figure 1.6 Model for MLK3 autoinhibition, Merlin-dependent inhibition and GTPase-mediated activation. MLK3 is autoinhibited through intramolecular interactions between its N-terminal Src-homology 3 (SH3) domain and a sequence containing proline 469 that is located between the leucine zipper (LZ) and Cdc42-/Rac1-interactive binding (CRIB) motif. Upon binding to an active (GTP-bound) Cdc42/Rac1 through the CRIB motif, MLK3 undergoes conformational change, dimerization and autophosphorylation at threonine 277 and serine 281. In the presence of Merlin, direct association between MLK3 and Merlin inhibits MLK3 activation by preventing the activated GTPase from binding to the CRIB motif (Rattanasinchai and Gallo, 2016).

5.3 MLK3 activity is regulated by the tumor suppressor protein Merlin

Tumor suppressor genes encode proteins that are capable of preventing cancer development by inhibiting cell division and promoting cell apoptosis (Sun and Yang, 2010). Merlin is a tumor suppressor protein encoded by Neurofibromatosis type 2 (NF2) gene. Deletion and loss-of-function mutations of NF2 are associated with development and/or invasiveness of several cancers such as schwannomas, meningiomas, ependymomas, mesothelioma, endometrial cancer, ovarian cancer, glioblastoma, and breast cancer (Petrilli and Fernandez-Valle, 2016; Schroeder et al., 2014). In ovarian cancer, Merlin has been shown to negatively regulate MLK3 activity (Chadee et al., 2006; Zhan and Chadee, 2010; Zhan et al., 2011). Binding of MLK3 to the C-terminus of Merlin inhibits the association of MLK3 with Cdc42 (Zhan et al., 2011) as well as disrupts the MLK3/B-Raf complex (Chadee et al., 2006). This interaction therefore prevents both kinase-dependent and independent functions of MLK3 and, as a consequence, inhibits activation of MLK3 downstream effectors: B-Raf, JNK and ERK (Chadee et al., 2006; Zhan et al., 2011) (**Figure 1.6**). Indeed, knockdown of MLK3 is sufficient to block JNK and ERK activation in a human schwannoma cell line bearing an NF2 loss-of–function mutation and, as a consequence, suppresses proliferation of these cells (Chadee et al., 2006). Likewise, increased cell proliferation observed upon Merlin silencing in ovarian cancer cells can be reversed by MLK3 knockdown (Zhan et al., 2011).

5.4 Heat shock protein-mediated MLK3 stability

Heat shock protein (HSP) 90 is a ubiquitously expressed molecular chaperone that plays pivotal roles in cancer progression (Neckers, 2006; Whitesell and Lindquist, 2005). HSP90 in concert with its co-chaperone $p50^{cdc37}$ functions to assist protein folding and maturation of a number of oncogenic protein kinases, including Akt, B-RAF, and Src. Thus this chaperone

complex is important for the stability and function of several key signaling molecules in cancer (Jackson, 2013; Whitesell and Lindquist, 2005). In MCF7 breast cancer cells engineered to inducibly express FLAG-tagged MLK3, HSP90 as well as its kinase-specific co-chaperone $p50^{cdc37}$ were identified by liquid chromatography/ tandem mass spectrometry in affinity complexes with MLK3 (Zhang et al., 2004). Similar to other protein kinase clients, the HSP90/$p50^{cdc37}$ complex interacts with the catalytic domain of MLK3; and is required for MLK3 stability and function. In MCF7 cells, inhibition of HSP90 chaperone activity with geldanamycin, an ansamycin antibiotic, caused downregulation of MLK3 protein levels as well as blocked TNF-induced activation of MLK3 downstream effectors, MKK7, JNK and c-Jun (Zhang et al., 2004). It was later shown in ovarian cancer cells that HSP90-MLK3 dissociation induced by geldanamycin facilitated association of MLK3 with HSP70 and its co-chaperone E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP), followed by E2 ubiquitin-conjugating enzyme UbcH5a, -b, -c and -d, resulting in MLK3 ubiquitination and degradation via proteasome pathway (Blessing et al., 2014) (**Figure 1.7**). CHIP-mediated degradation of MLK3 is important for suppression of ovarian cancer cell invasion (Blessing et al., 2014).Of note, HSP90 inhibitors are capable of disrupting the function of various oncogenic kinases and are being examined as potential anticancer therapeutics. Given the findings discussed above, it is possible that the anti-tumor activities of HSP90 inhibitors may result in part from promoting degradation of MLK3.

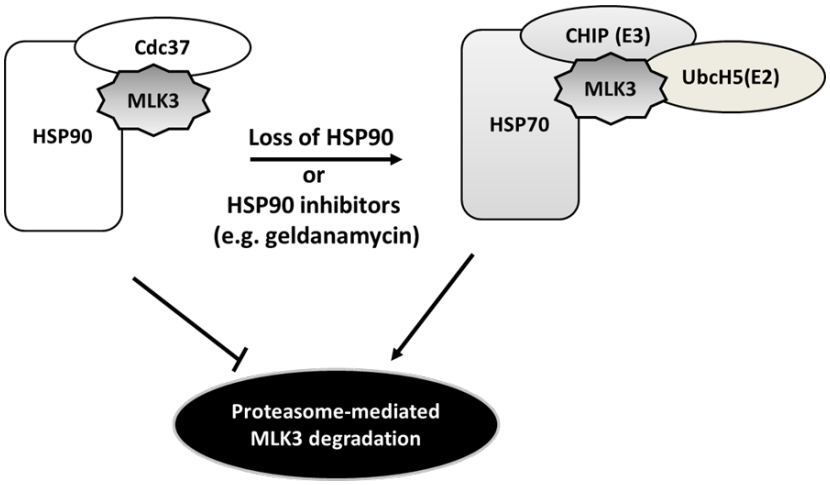

Figure 1.7 Illustrated model for MLK3 stability. Association of MLK3 with HSP90 as well as its co-chaperone Cdc37 is required for MLK3 stability. Loss of HSP90 or inhibition of its activity results in dissociation of MLK3 from the HSP90 chaperone complex and promotes interaction of MLK3 with HSP70 along with its co-chaperone E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP). This association, in turn, recruits E2 ubiquitin-conjugating enzyme UbcH5a, -b, -c and -d, resulting in MLK3 ubiquitination and proteasome-dependent degradation (Rattanasinchai and Gallo, 2016).

6. MLK3 transduces signals from several cell-surface receptors

The ability to transmit signals from various classes of cell surface receptors to multiple signaling cascades makes MLK3 one of the network hubs in cellular signaling (Swenson-Fields et al., 2008) (**Figure 1.8**). Notably, most cell surface receptors that signal through MLK3 have been shown to play crucial roles in multiple "hallmarks of cancer" including cell proliferation, invasion and cell survival (Hanahan and Weinberg, 2011b). Likewise, overexpression or mutation of intracellular downstream targets of these receptors commonly occurs in cancer. Indeed, MLK3 has been shown to be overexpressed in breast (Chen et al., 2010), ovarian (Zhan et al., 2012b) and pancreatic (Chandana, 2010) cancer. In addition, more than 20% of tumors from patients presenting with microsatellite unstable gastrointestinal cancers harbor the MLK3 P252H mutation, which increases MLK3 activity (Velho et al., 2010; Velho et al., 2014).

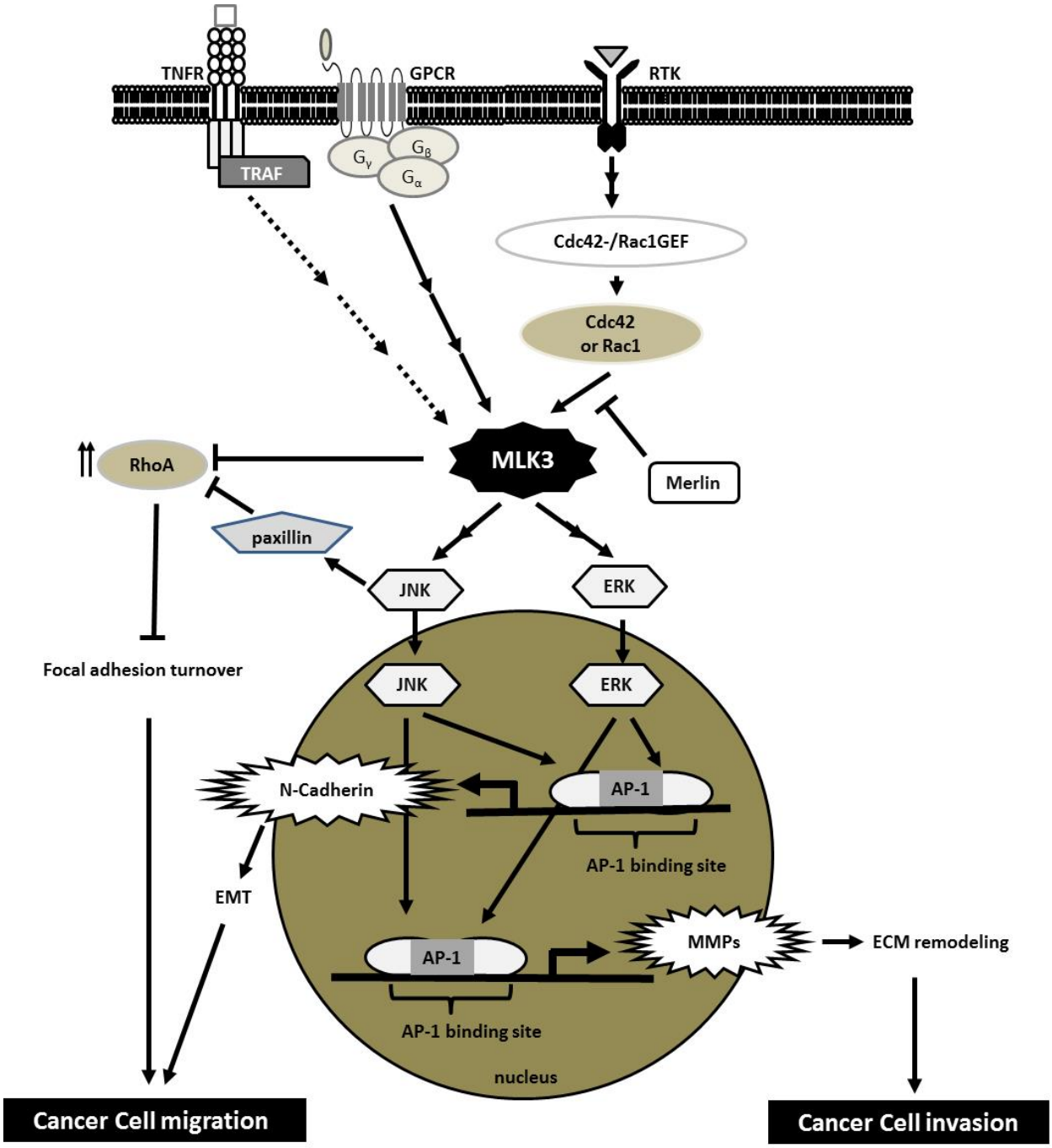

Figure 1.8 MLK3 signaling in cancer cell migration and invasion. MLK3 transduces signal from multiple cell surface receptors to various downstream signaling effectors to control cancer cell migration and invasion. See text for details (Rattanasinchai and Gallo, 2016).

6.1 Receptor tyrosine kinases (RTKs)

The RTK family of cell surface receptors has intrinsic tyrosine kinase activity. Upon ligand binding through the extracellular domain, these RTKs undergo dimerization, followed by trans-autophosphorylation within the cytoplasmic domain. Many of these phosphorylated regions serve as docking sites for intracellular proteins ultimately resulting in transduction of extracellular signals to several important downstream cascades including the MAPK pathways. Dysregulation of RTKs, such as members of epidermal growth factor receptor (EGFR) family, or overexpression of their ligands is frequently found in breast cancer (Masuda et al., 2012; Park et al., 2014), glioblastoma (GBM) (Furnari et al., 2007; Taylor et al., 2012) and non-small cell lung carcinoma (NSCLC) (Gazdar, 2010; Zhang et al., 2010), and results in sustained pro-proliferative, pro-invasive and pro-survival signaling (Chong and Janne, 2013; Citri and Yarden, 2006; Furnari et al., 2007; Gajria and Chandarlapaty, 2011; Gazdar, 2010; Larsen et al., 2013; Luo and Lam, 2013; Marmor et al., 2004; Masuda et al., 2012; Park et al., 2014; Taylor et al., 2012; Tebbutt et al., 2013; Yan et al., 2011; Yewale et al., 2013; Zhang et al., 2010).

In various cancer cell lines, MLK3 has been shown to signal through multiple receptors including EGFR (Chen and Gallo, 2012), hepatocyte growth factor (HGF) receptor, more commonly known as c-MET (Chadee and Kyriakis, 2004a), and Discoidin domain receptor 1 (DDR1) (Shintani et al., 2008). Depending upon the context, downstream JNK, ERK, or P38 signaling is subsequently activated. Both EGF and HGF can induce several Rac1 or Cdc42 specific Guanine nucleotide exchange factors (GEFs) (Feng et al., 2014; Feng et al., 2015; Innocenti et al., 2002; Tamás et al., 2003; Zhu et al., 2015); however, it is still unclear which RacGEF(s) are responsible for MLK3 activation. On the other hand, collagen I-induced DDR1 activation coupling with integrin α2β1 is thought to increase MLK3 activity through a small

GTPase Rhoptry associated protein 1 (Rap1) dependent mechanism (Shintani et al., 2008); however there is no evidence for a direct interaction between MLK3 and Rap1. Depending upon cell types, ligands, and RTKs, MLK3 relays RTK signaling to multiple intracellular targets, resulting in biological responses including cell proliferation, cell migration and invasion, and cell survival.

6.2 Chemokine receptors

Chemokine receptors, a subgroup of cytokine receptors, are a family of seven-transmembrane, G-protein-coupled receptors (GPCRs). GPCR signaling is mediated through association of an intracellular heterotrimeric G-protein with the intracellular portion of the inactive receptor. Upon chemokine activation, GPCRs undergo a conformational change that triggers dissociation of G_α from $G_\beta G_\gamma$ subunits. These subunits then interact with and transduce signals to multiple pathways including MAPKs (Kakinuma and Hwang, 2006). C-X-C chemokine receptor 4 (CXCR4) is a GPCR that is overexpressed in numerous types of malignant cancers, including breast (Liu et al., 2009), gastric (Iwasa et al., 2009), ovarian (Guo et al., 2011), cervical (Huang et al., 2013), and colorectal cancers (Song et al., 2014). CXCR4 and its specific ligand, stromal-derived factor 1 (SDF-1), also referred to as C-X-C ligand 12 (CXCL12), together play a crucial role in cancer survival and metastasis. Notably, CXCL12/CXCR4 appears to be important for site-specific metastasis of several cancers; CXCR4-positive cancer cells invade along CXCL12 gradients thus promoting metastasis to CXCL12-expressing organs such as lymph nodes, lung, livers and bone (Furusato et al., 2010; Teicher and Fricker, 2010).

CXCR4 has been shown to be critical for metastasis of triple negative breast cancer (TNBC). In an experimental model of TNBC, MLK3 has been shown to mediate

CXCR4/CXCL12-induced TNBC cell invasion, and knockdown of MLK3 is sufficient to block TNBC metastasis in a xenograft model (Chen and Gallo, 2012). However, the mechanism through which CXCR4 activates MLK3 in this context is still unclear. In addition, carbachol, a chemical ligand for the G-protein coupled acetylcholine receptor, can activate MLK3 in lung cancer cells (Swenson-Fields et al., 2008).

6.3 Tumor necrosis factor receptors (TNFRs)

TNFRs, another subgroup of cytokine receptors, are membrane-bound receptors that bind TNF ligands and play key roles in immune homeostasis to promote survival or apoptosis of cancer cells depending on the specific conditions (Bremer, 2013; van Horssen et al., 2006). Upon ligand activation, TNFR recruits an adaptor protein, TNFR-associated factor (TRAF) followed by other intracellular signaling proteins. The consequence of TNFR signaling in cancer is still controversial; depending upon the specific circumstances it can either promote or suppress tumor progression. How TNFR signals to MLK3 in cancer cells is also not known. However it is worth mentioning that TNF-α was the first ligand identified to activate MLK3 (Sathyanarayana et al., 2002). TNF-α stimulates the interaction between MLK3 and TRAF2 and this is crucial for MLK3-JNK signaling (Korchnak et al., 2009; Sondarva et al., 2010). Of note, the mechanism of TRAF2-dependent MLK3 activation is still unclear as TRAF2 lacks a kinase domain and, thus, is unable to phosphorylate MLK3. However, two possibilities have been considered: 1) TRAF2 may recruit a protein kinase to phosphorylate and activate MLK3, or 2) the interaction of MLK3 with TRAF2 causes MLK3 to undergo conformational change leading to an increase in its kinase activity (Rana et al., 2013). In addition, TRAF6 can promote MLK3 activation (Korchnak et al., 2009) .

7. Implication of MLK3 in cancer invasion

7.1 Cancer cell migration, focal adhesion dynamics and epithelial-to-mesenchymal transition (EMT)

Cell migration is a required step in the process of metastasis, allowing malignant cancer cells to move from a primary tumor to the metastatic sites. The first data to support the role of MLKs in cell migration came from a study in Drosophila within which Rac-JNK signaling was shown to be critical for dorsal closure, a process of epithelial cell sheet movement, during Drosophila embryogenesis. Of the six Drosophila MAP3Ks: MLK, LZK, TGFβ-activated kinase (TAK), apoptosis signal-regulating kinase (ASK), MEK kinase (MEKK) and Tumor progression locus (TPL) homologs, only the MLK homolog, *Slipper*, is critical for JNK-induced dorsal closure in this model (Stronach and Perrimon, 2002). In breast cancer, the requirement of a MLK3-JNK signaling axis in cell migration has also been investigated. Our lab has shown that ectopic expression of MLK3 is sufficient to induce cell migration in both immortalized breast epithelial cells and poorly invasive breast cancer cells, while inhibition of either the MLK3 or JNK pathways, or silencing of MLK3, can block migration of highly migratory, TNBC cells (Chen et al., 2010).

Cytoskeletal rearrangement and focal adhesion (FA) dynamics are critical for migrating cells and are spatially and temporally regulated by the activities of Cdc42, Rac1 and RhoA GTPases (Mitra et al., 2005). Cdc42 and Rac1 promote membrane protrusion and regulate the formation of nascent FAs while active RhoA induces the formation of actin stress fibers, promotes maturation of FAs, and stimulates actomyosin contraction, pushing the cell to move forward. In addition, spatial suppression of RhoA activity is necessary for FA turnover (Nobes and Hall, 1995; Vicente-Manzanares et al., 2005). Hence the cycling between these RhoGTPases

is critical for efficient cell migration. It has been shown that both catalytic activity and scaffolding function of MLK3 are critical for limiting excess RhoA activity and allowing dynamic cell migration. In breast cancer, MLK3 signals through paxillin, which has been shown, in other settings, to activate p190RhoGAP (Huang et al., 2004). Depletion of MLK3 or inhibition of its activity results in elevated RhoA activity, excessive FA and stress fiber formation, and decreased cell migration in highly invasive breast cancer cells (Chen and Gallo, 2012). In addition, MLK3-mediated suppression of RhoA activity is also required for lung cancer migration (Swenson-Fields et al., 2008). Unlike in breast cancer, the proposed mechanism for suppression of RhoA is through MLK3 binding and sequestration of p63RhoGEF (Swenson-Fields et al., 2008). Of note, antagonism between Rac1 and RhoA activity is well-documented; under conditions of high Rac1, RhoA activity is suppressed and *vice versa*. One possible mediator of Rac1-driven Rho inactivation is through p190RhoGAP (Wildenberg et al., 2006). Since Rac1 is an upstream regulator of MLK3, it is possible that MLK3 could be involved in establishing this Rac1-RhoA antagonism by transducing signals from active Rac1 to suppress RhoA via regulation of the activity of 190RhoGAP in JNK-paxillin dependent manner.

Most human cancers are derived from epithelial tissue, the polarized cells that form tight cell-cell contacts and line the cavities and the surface of organs (Royer and Lu, 2011). In cancer cells, the loss of cell polarity and cell-cell adhesion results in EMT, which strongly enhances cancer cell motility and cancer cell invasion. In prostate cancer, MLK3 facilitates the collagen type I-induced EMT switch. In this model, MLK3 transduces signaling from two collagen receptors, α2β1 integrin and discoidin domain receptor 1 (DDR1), leading to a MKK7-JNK-mediated increased expression of EMT marker, N-cadherin. Interestingly, among 12 human MAP3Ks (MEK kinase 1-4, TAK1, ASK 1/2, MLK1-3, DLK and LZK) that were investigated

for involvement in promoting EMT phenotype, only MLK3 knockdown was found to be sufficient to block JNK activity, collagen I-induced cell scattering and N-cadherin expression (Shintani et al., 2008).

7.2 Cancer cell invasion and metastasis

The ability of cancer cells to promote extracellular matrix (ECM) remodeling and invade through the layer of ECM is a key for their invasiveness and metastatic capability. ECM, a complex network of macromolecules secreted by cells, not only functions as a structural element in tissue but also serves as a stimulus for adjacent cells, leading to several biological responses such as cell migration (Bonnans et al., 2014). Of note, the most abundant type of ECM protein in the human body is collagen (Ricard-Blum, 2011). More than 90% of the collagen in vertebrates is fibrillar collagen type I (Exposito et al., 2010), while collagen type IV is a major component of basement membrane, which functions as a barrier separating epithelium from surrounding stroma (Tanjore and Kalluri, 2006). The importance of MLK3 in cancer cell invasion has been demonstrated in several types of cancer including breast cancer (Chen and Gallo, 2012), ovarian cancer (Abi Saab et al., 2012; Blessing et al., 2014; Zhan et al., 2012a), melanoma (Zhang et al., 2014) and non-small cell lung cancer (Chien et al., 2011). Though the mechanism of MLK3 in cell invasion is still unclear, inhibition of MLK3 is sufficient to block *in vitro* invasion of cancer cells through Matrigel, which resembles the process of cancer cells breaching through the basement membrane during early steps of cancer metastasis. Blocking ECM invasion could, in theory, result in failure of cancer cells to form distant metastases. Indeed, two research groups have reported that stable knockdown of MLK3 in TNBC cells is sufficient to block formation of pulmonary micrometastases and lymph node metastases (Chen and Gallo, 2012; Cronan et al., 2012).

One mechanism through which MLK3 may regulate cancer cell invasion is by controlling of expression of matrix metalloproteinases (MMPs). Specific MMPs are required for ECM remodeling that occurs during tissue development and cancer invasion (Kessenbrock et al., 2010). For example, MMP-1, -2, -7, -8, -13 and MT1-MMP were found to be responsible for type I collagen degradation while collagen type IV, a component of basement membrane, is a substrate of MMP-2, -3, -7, -9, -10, and -13 (Woessner, 1991). Of note, several invasive cancer cells also express high level of MMPs (Egeblad and Werb, 2002; Kessenbrock et al., 2010; Woessner, 1991). In ovarian cancer, the MLK3-ERK-AP1 axis is responsible for production of MMP2 and 9, suggesting that MLK3 may facilitate cancer invasion, in part, through upregulation of MMPs (Blessing et al., 2014; Zhan et al., 2012b).

8. Other functions of MLK3

8.1 Cancer cell proliferation and viability

Sustained proliferative signaling is one of the hallmarks of cancer (Hanahan and Weinberg, 2011a) and one key axis that can control such signaling is the Ras/Raf/MEK/ERK (Roberts and Der, 2007; Talotta et al., 2010). In this context, B-Raf, a serine/threonine kinase, acts as a MAP3K relaying Ras signaling to the ERK MAPK pathway (Roberts and Der, 2007; Talotta et al., 2010). In certain cancer models, MLK3 is required for EGF-induced B-Raf activation by providing scaffolding for Raf-1/B-Raf complex; thus this scaffolding function of MLK3 allows Ras-dependent, activated Raf-1 to phosphorylate and activate B-Raf, resulting in increased ERK activity (Chadee and Kyriakis, 2004a; Chadee et al., 2006). Interestingly, a gain-of-function mutation in B-Raf is frequently found in cancers including melanoma (Ascierto et al., 2012a) and papillary thyroid carcinoma (Kebebew et al., 2007). Approximately 90% of B-Raf mutations found in melanomas and papillary thyroid carcinomas are point mutations

resulting in substitution of a valine with glutamic acid at amino acid 600 (B-Raf V600E) (Ascierto et al., 2012a). This phosphomimetic B-Raf V600E mutation results in constitutively active B-Raf and sustained ERK signaling. Although the B-Raf inhibitor, vemurafinib, has proved efficacious and increases survival in patients with this B Raf mutation, most patients relapse and become resistant to this treatment (Fisher and Larkin, 2012). Many mechanisms of vemurafenib resistance exist. A recent study has shown that MLK1, -2, -3, and -4 can contribute to acquired vemurafinib resistance in B-Raf V600E tumor model. RNA sequencing reveals upregulation of MLKs in drug-resistant tumor specimens and biochemical assays have also shown that catalytic activity of MLK1, -2, -3, and/or -4 is required for direct phosphorylation of MEK, in a Raf-independent manner, leading to reactivation of ERK signaling and decreased sensitivity to the B-Raf inhibitor (Marusiak et al., 2014).

9. Summary

Cancer invasion is one of the key steps during the metastatic process. Several signalosomes have been shown to govern the invasiveness of the malignant cells. In this chapter, we have focused on a protein kinase MLK3 and its role in cancer invasion and metastasis. MLK3 functions as a signaling hub mediating multiple extracellular signals to the intracellular environment. In normal cells, MLK3 activity and localization is tightly controlled by both intrinsic and extrinsic mechanisms including autoinhibition, dimerization, and interaction with other signaling proteins and protein scaffolds. Dysregulation of MLK3 activity and/or increased expression of MLK3 is found in numerous types of malignant cancer and substantial studies provide the evidence for the role of MLK3 in cancer invasion. Thus MLK3 may serve as a potential target for the development of therapeutics against the metastatic disease.

Despite MLK3 being recognized for its role in cancer migration, invasion and

metastasis, its upstream regulators or downstream signaling cascades, and how they contribute to cancer metastasis, have not been fully defined. MLK3 can signal to both JNK and ERK. It is noteworthy that both JNK and ERK are major contributors to the activation of the dimeric AP-1 transcription factors. Several invasion genes are controlled by the AP-1 (Ozanne et al., 2007). Thus identifying MLK3-dependent AP-1 target genes that are critical for cancer progression will not only provide a better understanding of how MLK3 signaling controls cancer invasion and metastasis, but also potentially lead to the development of predictive biomarkers which could be used to select cancer patients most likely to respond to treatments that block MLK3 function.

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CHAPTER II

MLK3 IS A KEY REGULATOR OF THE FRA-1-MMP-1 AXIS AND DRIVES TRIPLE-NEGATIVE BREAST CANCER INVASION AND TRANSENDOTHELIAL MIGRATION.

This chapter has been accepted for publication in an open access manuscript for *Oncogenesis 2017*.

1. Abstract

Mixed-lineage kinase 3 (MLK3), a mitogen-activated kinase kinase kinase (MAP3K), plays critical roles in metastasis of triple-negative breast cancer (TNBC), in part by regulating paxillin phosphorylation and focal adhesion turnover. However the mechanisms and the distinct step(s) of the metastatic processes through which MLK3 exerts its influence are not fully understood. Here we report that in non-metastatic, estrogen receptor positive (ER+) breast cancer cells, induced MLK3 expression robustly upregulates the oncogenic transcription factor, FOS-related antigen-1 (FRA-1), which is accompanied by elevation of matrix metalloproteinases (MMPs), MMP-1 and MMP-9. MLK3-induced MMP-1 upregulation is abrogated by FRA-1 silencing, demonstrating that MLK3 signals through FRA-1 to control MMP-1. Conversely, in metastatic TNBC models, high FRA-1 levels are significantly reduced upon depletion of MLK3 by either gene silencing or by the CRISPR/Cas9n editing approach. Furthermore, ablation of MLK3 or MLK inhibitor treatment decreases expression of both MMP-1 and MMP-9. Consistent with the established role of tumor cell-derived MMP-1 in endothelial permeability and transendothelial migration, both of these are reduced in MLK3-depleted TNBC cells. In addition, MLK inhibitor treatment or MLK3 depletion renders TNBC cells defective in Matrigel invasion. Furthermore, circulating tumor cells (CTCs) derived from a TNBC-bearing mouse display increased levels of FRA-1 and MMP-1 compared with parental cells, supporting a role for the MLK3-FRA-1-MMP-1 signaling axis in tumor cell invasion and vascular intravasation. Consistent with the role of MMP-1 in multiple steps of metastasis, high levels of MMP-1 in breast cancer patient tumor samples are associated with increased distant metastases and poorer overall survival. Our results demonstrating the requirement for MLK3 in controlling the FRA-1/MMP-1 axis suggest that MLK3 is a potentially an effective therapeutic target for TNBC.

2. Introduction

Metastatic breast cancer is responsible for nearly half a million deaths worldwide (Ferlay et al., 2015) and 40,000 deaths in the US (Siegel et al., 2015), annually. A major contributor is a lack of efficacy of the current standard treatments in preventing and treating metastatic disease. Breast cancer metastasis is a multistep process initiated by cancer cells within the primary tumor that gain invasive capacity. These cancer cells must breach the basement membrane, invade through extracellular matrix (ECM) and intravasate into blood vessels. The cells that infiltrate into the bloodstream, called circulating tumor cells (CTCs), must survive in the circulation and extravasate to a distant site, such as bone, lung, brain, or liver, and colonize to form metastatic lesions (Fidler, 2003). Of the three major clinical breast cancer subtypes, triple-negative breast cancer (TNBC) is considered the most aggressive and has the highest rate of metastasis and early recurrence (Colleoni et al., 2016). Given the relative dearth of targeted therapy for treating TNBC, standard treatment relies on surgical removal, adjuvant radiotherapy, and toxic chemotherapy.

Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAP3K) that transduces signals from multiple cell surface receptors to activate several MAPK cascades in a context-dependent manner (Gallo and Johnson, 2002; Rattanasinchai and Gallo, 2016). Upon activation, MAPKs can directly phosphorylate cytosolic substrates or undergo nuclear translocation to regulate transcription factors, including activating protein-1 (AP-1) (Gallo and Johnson, 2002; Rattanasinchai and Gallo, 2016). MLK3 has been demonstrated to be critical for TNBC metastasis (Chen and Gallo, 2012; Cronan et al., 2012). Mechanistically, we showed that MLK3 functions through JNK-dependent paxillin phosphorylation to facilitate focal adhesion turnover and cell migration (Chen and Gallo, 2012). In addition, MLK3 signaling leads

to JNK-mediated c-JUN phosphorylation (Chen et al., 2010). which can activate AP-1 to drive gene expression.

AP-1 transcription factors are comprised of various, usually heterodimeric, combinations of JUN and FOS family members which include c-JUN, JUN-B, JUN-D, c-FOS, FOS-B, FRA-1, and FRA-2 (Hess et al., 2004). Increased AP-1 activity, as well as overexpression of certain AP-1 family members, results in transcriptional regulation of a subset of genes that promote cancer progression (Ozanne et al., 2007; Zhao et al., 2014). Among the AP-1 members, high levels of FRA-1 are associated with poor prognosis in TNBC (Bakiri et al., 2015; Zhao et al., 2014). FRA-1 is elevated in TNBC lines compared with the other breast cancer subtypes (Belguise et al., 2005; Belguise et al., 2012); and is required for proliferation (Belguise et al., 2005), epithelial-to-mesenchymal transition (EMT) (Bakiri et al., 2015; Liu et al., 2015), invasion (Adiseshaiah et al., 2008; Henckels and Prywes, 2013), and metastasis (Desmet et al., 2013). Many invasion genes are controlled by FRA-1, including matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases involved in matrix degradation and extracellular matrix (ECM) remodeling (Kessenbrock et al., 2010). Elevated levels of several MMPs are found in various types of cancer; these MMPs play crucial roles in multiple steps of tumor progression including tumor growth, angiogenesis, invasion and metastasis (Gialeli et al., 2011).

In this study, we demonstrate that MLK3 is a key regulator of FRA-1 expression in both ER+ BC and TNBC models. Furthermore, we show that the MLK3-FRA-1 axis controls levels of MMP-1 and MMP-9. Consistent with the roles of these MMPs, loss of MLK3 blocks Matrigel invasion as well as transendothelial migration of highly aggressive 4T1 cells. Importantly, an MLK inhibitor diminishes FRA-1 and its target genes, MMP-1 and MMP-9, in TNBC cells suggesting that targeting MLK3 may interfere with metastatic progression.

3. Materials and methods

3.1 Chemicals and antibodies

Chemicals: 5(6)-Carboxyfluorescein (6-FAM), Bovine Serum Albumin, gelatin, and 4', 6-Diamidino-2-Phenylindole (DAPI) were obtained from Sigma-Aldrich (St Louis, MO, USA). SP600125, U0126, and SB203580 were from Calbiochem (San Diego, CA, USA). CEP-1347 and CEP-11004 were generously provided by Cephalon, Inc., a wholly owned subsidiary of Teva Pharmaceuticals, Ltd (North Wales, PA, USA). AP21967 was generously provided by Ariad Pharmaceuticals (Cambridge, MA, USA). Calcein AM and Simple Blue SafeStain were purchased from Invitrogen (Carlsbad, CA, USA).

Antibodies: anti-MLK3 (A-20) (for detection of murine MLK3), anti-FRA-1 (R-20), anti-JNK1/3 (C-17), anti-ERK1 (K-23), anti-P38 (C-20), anti-actin (C-2), anti-p-c-JUN (S63)(KM-1), and anti-c-JUN (H-79) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit monoclonal anti-MLK3 (C-terminal) (for detecting human MLK3) was obtained from Epitomics (Cambridge, MA, USA), anti-p-MLK3, anti-p-ERK 1/2 (T202/Y204)(E-10), anti-p-JNK1/2 (T183/Y185)(81E11), anti-p-P38 (T180/Y182) were obtained from Cell Signaling (Danvers, MA, USA), and anti-MMP-1 was obtained from R&D systems (Minneapolis, MN, USA), anti-p-paxillin S178 was obtained from Bethyl Laboratory (Montgomery, TX, USA) IRDye 800CW goat anti-mouse IgG, IRDye 680 goat anti-rabbit IgG, and IRDye 800 CW donkey anti-goat IgG, were obtained from Li-COR Biosciences (Lincoln, NE, USA) for immunoblotting. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 and 546 were purchased from Invitrogen (Carlsbad, CA, USA) and used for immunofluorescence staining.

3.2 Cell lines

MCF7 and ZR-75-1 cells obtained from ATCC (Manassas, VA, USA) were maintained in DMEM supplemented with 10% FBS and RPMI-1640 supplemented with10%FBS, respectively. MCF7iMLK3 cells engineered to inducibly express MLK3 (MCF7iMLK3) were previously described (Chen et al., 2010; Zhang et al., 2004). 4T1-luc2 cells obtained from Perkin Elmer (Massachusetts, USA) were maintained in RPMI 1640 (Gibco, Life Technology, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). SUM-159-GFP cells (a gift from Dr. Chengfeng Yang (University of Kentucky)) were maintained in Ham's F-12 (Gibco) supplemented with 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and containing penicillin/streptomycin.

3.3 RNA interference and plasmid transfection

For siRNAs, Mission®siRNA Universal Negative control #1, siRNA duplexes targeting human MLK3 (5'-CUGACUGCCACUCAUGGUG-3'and its antisense) (Chadee and Kyriakis, 2004; Chen et al., 2010) and human FRA-1(5'-GGGCAGUGACGUCUGGAG-3' and its antisense) (Belguise et al., 2005) were obtained from Sigma-Aldrich (St Louis, MO, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as a transfection reagent according to the manufacturer's protocol.

For plasmid transfection, pRK-MLK3 or pRK-MLK3 K144M were previously described (Schachter et al., 2006). Lipofectamine 2000 and Lipofectamine 3000 were used as transfection reagents for MCF7 and ZR-75-1 cells, respectively. In a recovery assay of MLK3-knockout 4T1 cells, pCMV-EGFP-MLK3 (Xu et al., 2005) were reverse-transfected into 4T1KO-1 cells using Lipofectamine 3000.

3.4 CRISPR-Cas9n constructs

The MLK3 CRISPR construct was generated based on a previously described protocol (Ran et al., 2013b). Briefly, two pairs of guide RNAs for CRISPR-Cas9n construct were designed (crispr.mit.edu) to target exon 1 of murine MLK3: Pair 1 - guide A 5'-CATTGGGCTCATAGTCGAAC-3', guide B 5'-CCTGAGGAAGGGCGACCGTG-3', Pair 2 – guide A 5'-ATTGGGCTCATAGTCGAACA-3', guide B 5'-GGCAGGACGAACTAGCCCTG-3', and were cloned into pSpCas9n(BB)-2A-GFP (PX461) (Ran et al., 2013b) (a gift from Feng Zhang (Addgene plasmid #48140). After reverse transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), GFP positive clones were screened for MLK3 deletion by immunoblotting. Genomic DNAs from selected clones were collected and amplified using forward primer 5'- ATGGAGCCCTTGAAGAACCT-3' and reverse primer 5'–ACGGTAGACCTTGCCGAAG-3'. Purified PCR products were subjected to TOPO TA cloning (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. At least 5 clones were subjected to nucleotide sequencing to identify the genomic alterations.

3.5 Immunoblot analysis

Cellular lysates were prepared in 1% NP-40 lysis buffer (1% NP-40, 150 mM sodium chloride, and 50 mM Tris pH 8.0) and immunoblotting was performed as previously described (Chen and Gallo, 2012; Chen et al., 2010).

3.6 Immunofluorescence (IF) analysis

IF staining was performed as previously described (Chen and Gallo, 2012). Images were acquired using an Olympus fluorescence microscope (MetaMorph software).

3.7 Gelatin zymography

4T1 cells or their derivatives (2.5 x 10^5 cells) were seeded to 35-mm culture dishes. The following day, at about 70% confluence, cells were incubated in serum-free medium for 24 h, in the presence of an inhibitor if indicated. Conditioned media was concentrated using a Centricon-10 and amounts corresponding to equal cellular equivalents were loaded onto 10% polyacrylamide gels containing 1 mg/mL gelatin. The gels were then incubated for 1 h in 2.5% Triton X-100, then developed for 24 h at 37 ^{o}C in a developing buffer (5 mM $CaCl_2$, 200 mM NaCl, and Tris-HCl, pH 7.6), and stained for 16 h with Simple Blue SafeStain (Invitrogen). After 30 min de-staining with water, gels were scanned using a scanner and the images were processed using Image J software.

3.8 Quantitative Real Time PCR

Total RNAs were extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) and cDNA synthesis was performed using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Real-time qPCR was performed using PerfeCTa SyBR green superMix (Quanta, Gaithersburg, MD, USA) or SYBR® Green Master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Specific primer sequences were designed using PrimerBank (Wang et al., 2012) and are listed in **table 2.1**.

Table 2.1 Lists of primers

Genes	Forward primer	Tm	Reverse Primer	Tm
hMLK3	GCAGCCCATTGAGAGTGAC	60.5	CACTGCCCTTAGAGAAGGTGG	61.8
hFRA-1	CAGGCGGAGACTGACAAACTG	62.9	TCCTTCCGGGATTTTGCAGAT	61.5
hMMP-1	GAGCAAACACATCTGAGGTACAGGA	58.5	TTGTCCCGATGATCTCCCCTGACA	61.5
hMMP-2	CCCACTGCGGTTTTCTCGAAT	62.9	CAAAGGGGTATCCATCGCCAT	62
hMMP-9	AGACCTGGGCAGATTCCAAAC	62	CGGCAAGTCTTCCGAGTAGT	61.3
hGAPDH	GGCTGAGAACGGGAAGCTTGTCAT	61.4	AGCCTTCTCCATGGTGGTGAAGA	60.8
mFRA-1	ATGTACCGAGACTACGGGGAA	61.8	CTGCTGCTGTCGATGCTTG	61.5
mMMP1a	AACTACATTTAGGGGAGAGGTGT	60.2	GCAGCGTCAAGTTTAACTGGAA	61.3
mMMP-2	CAAGTTCCCCGGCGATGTC	63	TTCTGGTCAAGGTCACCTGTC	61.3
mMMP-9	GGACCCGAAGCGGACATTG	63	CGTCGTCGAAATGGGCATCT	62.6
mGAPDH	TGTGTCCGTCGTGGATCTGA	58.1	CCTGCTTCACCACCTTCTTGA	57.2

3.9 *In vitro* Matrigel invasion assay

In vitro Matrigel invasion assay was performed as previously described (Chen and Gallo, 2012; Chen et al., 2010). Briefly, MCF7iMLK3 cells were pre-treated with either 50 nM Control siRNA or 50 nM MLK3siRNA as described above. 16 h after transfection, cells were serum-deprived for approximately 8 h in the presence of 25 nM AP21957. In the following day, $1x10^5$ cells were then seeded into the upper chamber and allowed to invade in the presence of vehicle control or 25 nM AP21967 for 24 h. For 4T1 cells and their derivatives, cells were serum-deprived overnight. 4T1 cells ($2x10^4$) were then introduced into the upper chamber and allowed to invade for 24 h toward 10% FBS in the presence of indicated inhibitors. Mitomycin C (2 μg/mL) was added to exclude the effect of cell proliferation. All invasion experiments were performed in duplicate and were repeated at least three times.

3.10 Transendothelial migration and endothelial permeability

Transendothelial migration was performed essentially as described (Juncker-Jensen et al., 2013; Kim et al., 2011). Briefly, $1x10^5$ EA.hy926 cells were grown as a confluent monolayer on 5-micron pore Transwell inserts for 1-2 days and 1 x 10^5 4T1 cells, or their derivatives, that had

been deprived of serum for 18 h were introduced to the upper chamber. 4T1 cells were allowed to migrate toward 10% FBS for 24 h. To assess permeability of the endothelial layer, 5(6)-Carboxyfluorescein (6-FAM)-conjugated albumin (10 μM final concentration) was added to the upper chamber and culture medium was collected from the bottom chamber after 30 min at 37 ^{o}C. Permeability was assessed by measuring the fluorescence due to leakage of 6-FAM-conjugated albumin (excitation = 495 nm, emission = 520 nm) into the bottom chamber. To assess transendothelial migration, the cells inside the Transwell inserts were wiped out with the cotton swabs, and the extent of migration through the Transwell membranes was then determined by relative bioluminescence activity using IVIS imaging.

3.11 Circulating tumor cell (CTC) isolation from the 4T1 tumor-bearing mice

This experiment was carried out in accordance with standard protocols approved by All University Committee on Animal Use and Care at Michigan State University. Briefly, puromycin-resistant 4T1-luc2 cells ($7.5x10^5$ cells) were injected into the 4th mammary gland of the 8 week old female athymic nu/nu mice (N=2). After 24 days, the mice were euthanized and CTC isolation was performed as described (Wang et al., 2009). Briefly, 200-uL blood collected by cardiac puncture was cultured in RPMI-1640 supplemented with 20% FBS, 2 μg/mL puromycin, and penicillin/streptomycin for 10 days. Approximately 40 colonies per sample were obtained, pooled, and propagated as a population, named 4T1-CTC#1 and 4T1-CTC#2. The 4T1-CTC lines were maintained in RPMI-1640 supplemented with 10% FBS containing penicillin/streptomycin.

3.12 Statistical analysis

Data are expressed as mean ± standard deviation (SD). An unpaired, 2-tailed Student's t test was used to calculate P values.

4. Results

4.1 MLK3 is required for FRA-1 expression in breast cancer cells.

High FRA-1 levels are found in aggressive TNBC, while estrogen receptor positive (ER+) breast cancer cell lines have low FRA-1 levels and are typically poorly invasive (Belguise et al., 2005; Belguise et al., 2012). To investigate whether MLK3 regulates FRA-1 expression in ER+ breast cancer, we transiently expressed either a wild type MLK3 or its kinase dead mutant (MLK3 K144M) (Schachter et al., 2006) into MCF7 cells. As shown in **Figure 2.1**, ectopic expression of wildtype MLK3 increases both JNK and ERK activities, and is sufficient to drive FRA-1 protein expression. Conversely, ectopic expression of equivalent protein levels of the kinase dead mutant MLK3-K144M does not significantly activate JNK or ERK, and fails to upregulate FRA-1 (Figure 2.1). These data demonstrate that the kinase activity of MLK3 is required for FRA-1 expression in MCF7 breast cancer cells.

To further investigate this phenomenon, we utilized MCF7 cells engineered to overexpress MLK3 upon a treatment of transcriptional inducer AP21967 (MCF7iMLK3) (Chen et al., 2010; Schachter et al., 2006; Zhang et al., 2004). As shown in **Figure 2.2**, Induced MLK3 expression with 50 nM AP29167 increases FRA-1 protein expression along with JNK and ERK pathway activation. Of note, induced expression of MLK3 in MCF7iMLK3 is also sufficient to drive cell migration in both Transwell (Chen et al., 2010) and wound healing assays (Data not shown). MLK3 overexpression also drives FRA-1 expression in another ER+ breast cancer cell line, ZR-75-1 (**Figure 2.3**), suggesting MLK3 regulation of FRA-1 is a general phenomenon in breast cancer cells. FRA-1 is an AP-1 driven gene; and MLK3 is a known activator of AP-1, in part through JNK-mediated c-Jun phosphorylation. Accordingly, qRT-PCR analysis in **Figure 2.4** demonstrates that overexpression of MLK3 robustly increases the FRA-1 transcript level in

MCF7iMLK3, suggesting that the regulation is, in part, at the level of transcription.

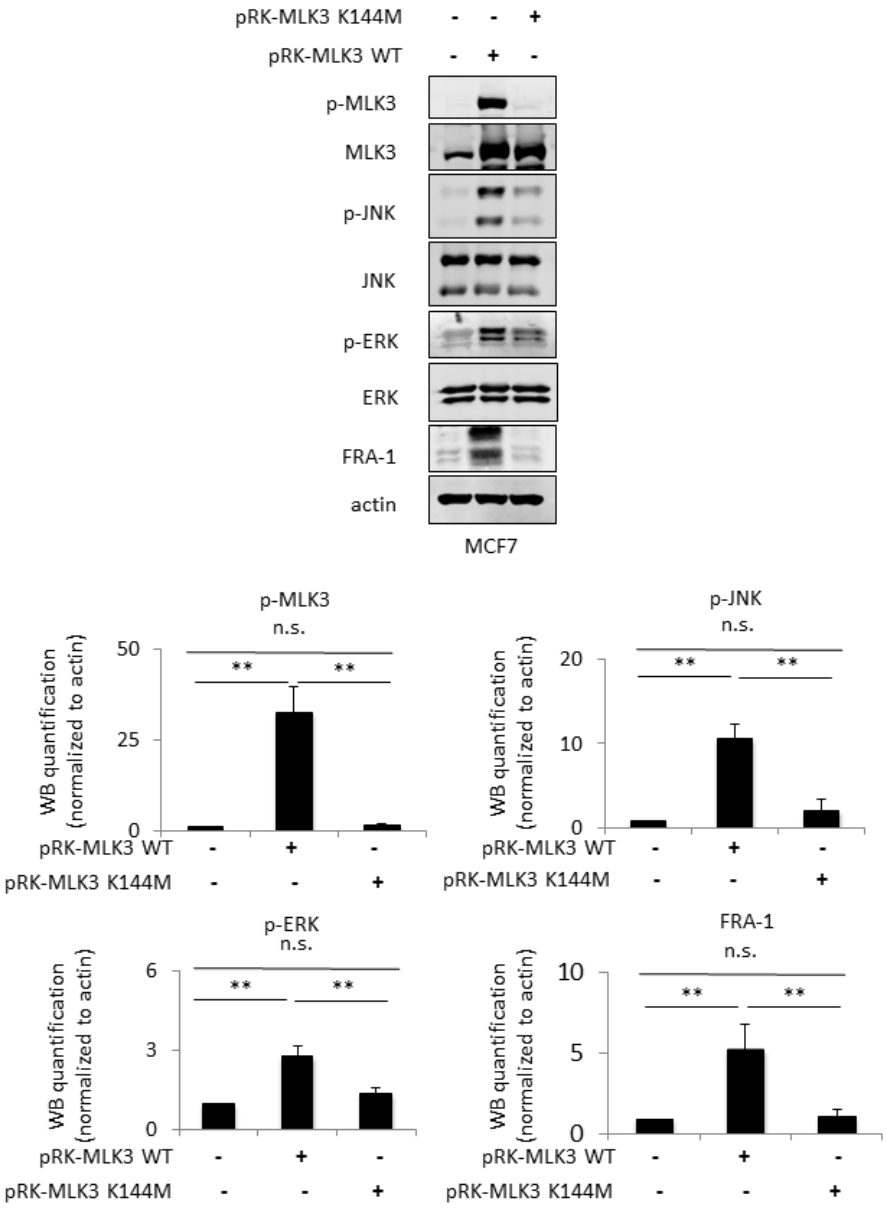


Figure 2.1 Catalytic activity of MLK3 is required to drive FRA-1 expression ER+ breast cancer MCF7. MCF7 cells were transiently transfected with an expression plasmid encoding wild type MLK3 (pRK-MLK3) or a kinase inactive MLK3 (pRK-MLK3-K144M) for 24 h. Cellular lysates were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; n.s. – not statistically significant; ** $p < 0.01$.

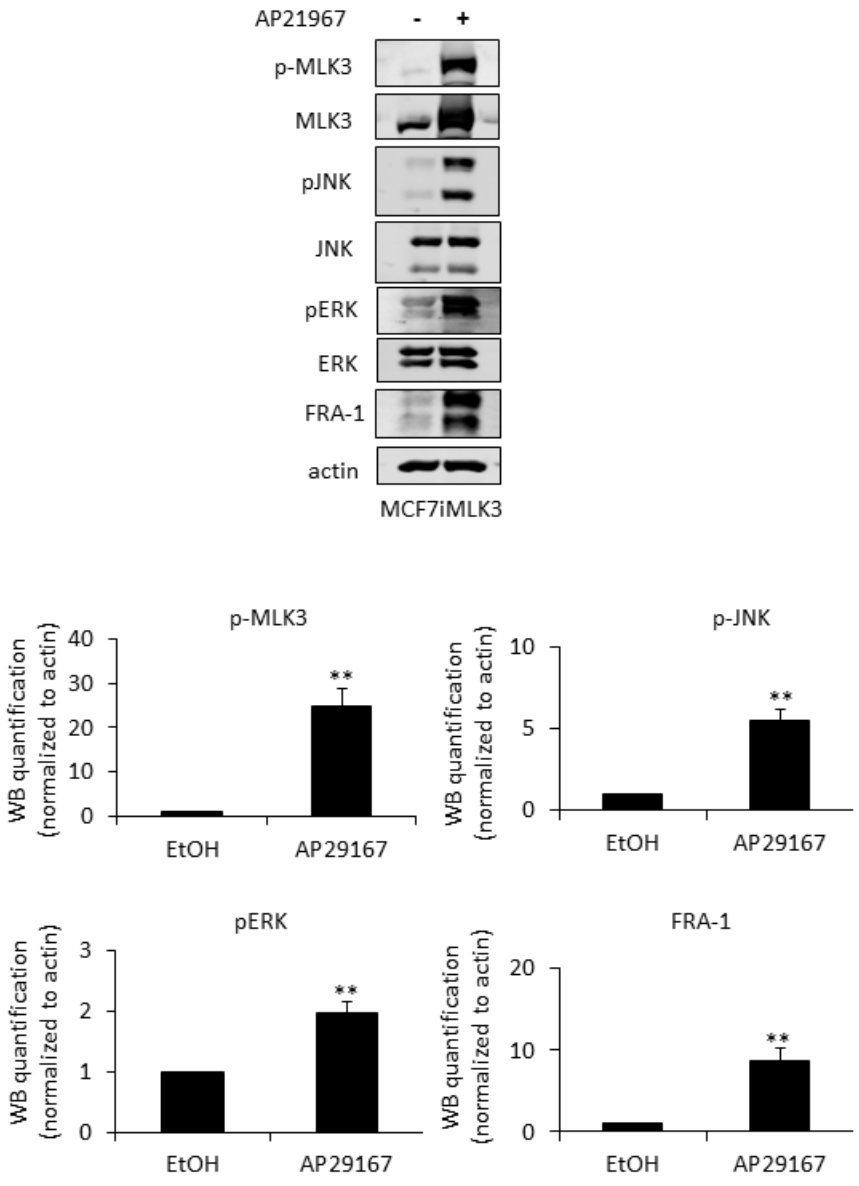


Figure 2.2 Induced expression of MLK3 increases FRA-1 levels in MCF7iMLK3 cells. MCF7iMLK3 cells were treated ± 50 nM AP21967 for 24 h to induce MLK3 expression. Cellular lysates were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments;** $p < 0.01$.

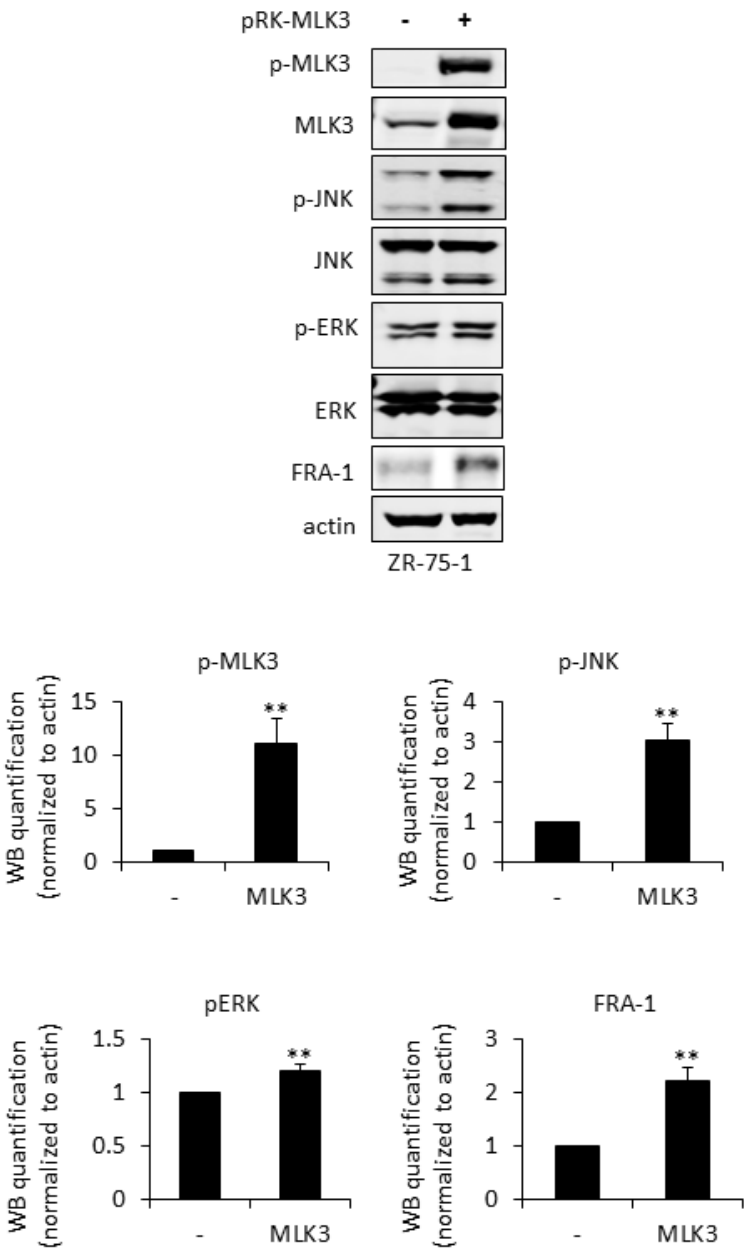


Figure 2.3 Ectopic expression of MLK3 drives FRA-1 expression in ER+ breast cancer ZR-75-1. ZR-75-1 cells were transiently transfected with an expression vector encoding wild type MLK3 (pRK-MLK3) or a kinase dead MLK3 (pRK-MLK3-K144M) for 24 h. Cellular lysates were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; ** $p < 0.01$.

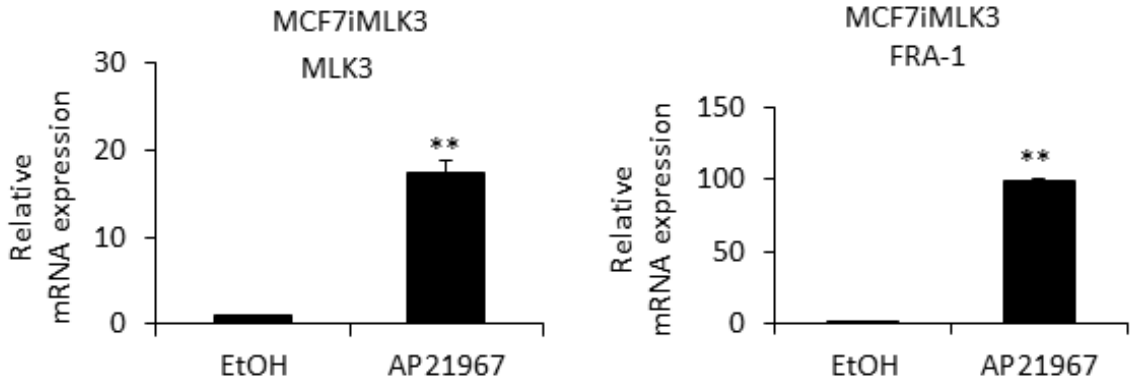


Figure 2.4 Induced expression of MLK3 increases FRA-1 transcript levels in breast cancer cells. MCF7iMLK3 cells were treated ± 50 nM AP21967 for 24 h to induce MLK3 expression. The mRNAs were subjected to qRT-PCR with indicated primers. Relative mRNA expression levels are displayed as mean ± SD from at least three independent experiments performed in triplicate; ** $p < 0.01$.

In complementary experiments, we investigated the requirement for MLK3 in metastatic TNBC cell lines, which are known to possess high levels of FRA-1 (Belguise et al., 2005). When short interfering RNA (siRNA) targeting MLK3 was used to knockdown MLK3 in highly invasive TNBC SUM-159 cells (Chen and Gallo, 2012; Chen et al., 2010), immunoblot analysis revealed efficient MLK3 knockdown and a corresponding 60% reduction in FRA-1 protein levels, compared with SUM-159 cells transfected with control siRNA (**Figure 2.5A**). As in the MCF7 inducible system, MLK3-mediated regulation of FRA-1 transcription is supported by qRT-PCR, which shows 80% and 50% reduction in MLK3 and FRA-1 mRNA levels in MLK3 siRNA-transfected SUM159 cells, respectively, compared with control siRNA-transfected SUM159 cells (**Figure 2.5**).

The murine 4T1 line is a highly metastatic TNBC model. In order to evaluate the function of MLK3 in this model, we first generated MLK3 gene knockout 4T1-luc2 cells using the

CRISPR/Cas9n (nickase) system (Ran et al., 2013a). Two pairs of guide RNAs, M1 and M4 (**Figure 2.6A**), were designed against the exon 1 region of murine *MAP3K11* genomic DNA (crispr.mit.edu), cloned into pSpCas9n (BB)-2A-GFP plasmid (PX461, addgene.org), and transfected into 4T1 cells. Clones were screened for MLK3 expression by immunoblotting. Three MLK3 knockout (MLK3 KO) clones named 4T1KO-1, 4T1KO-2, and 4T1KO-3, and a wild type (WT) clone that maintained MLK3 expression, were furthered analyzed by sequencing to confirm the genomic alteration on *MAP3K11* gDNA (**Figure 2.6B**).

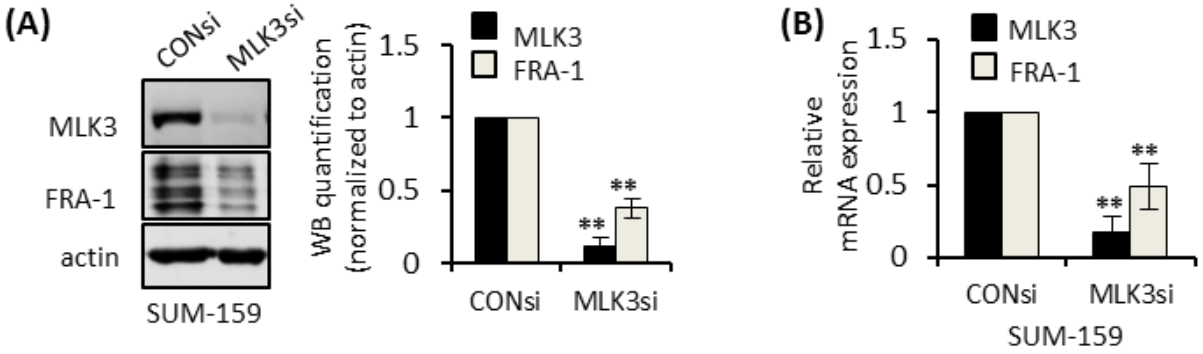


Figure 2.5 MLK3 is required for FRA-1 expression in TNBC SUM-159 cells. SUM-159 cells were incubated with 50 nM control or 50 nM MLK3 siRNA for 24 h. **(A)** Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments. **(B)** Relative mRNA expression levels are displayed as mean ± SD from at least three independent experiments performed in triplicate; ** $p < 0.01$.

(A)

	Guide A	Guide B
M1	CATTGGGCTCATAGTCGAACAGG	CCTGAGGAAGGGCGACCGTGTGG
M4	ATTGGGCTCATAGTCGAACAGGG	GGCAGGACGAACTAGCCCTGAGG

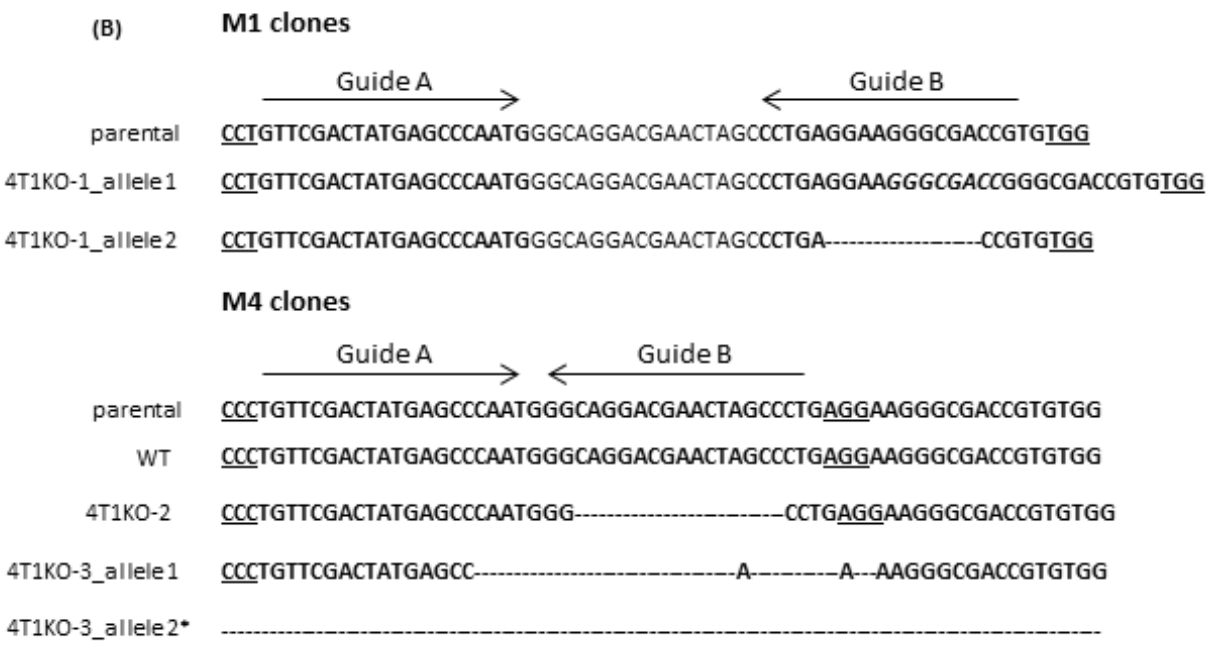


Figure 2.6 Genome-edited sequencing of 4T1 CRISPR clones. (A) Two pairs (M1 and M4) of gRNAs targeting exon 1 region of murine MAP3K11 genomic DNA were designed using the tools at crispr.mit.edu. **(B)** Genomic DNAs from parental 4T1 cells, a wildtype clone and three 4T1 MLK3 knockout (KO) clones: 4T1KO-1, 4T1KO-2, and 4T1KO-3 were isolated and subjected to sequencing to identify genomic alteration. The protospacer adjacent motif (PAM) sequences are underlined.

As shown in **Figure 2.7A**, all three 4T1 CRISPR clones lacked MLK3 expression, in contrast to parental 4T1 cells and the WT clone. These 4T1 CRISPR MLK3 KO clones also exhibited decreased FRA-1 protein expression. Based on qRT-PCR analysis FRA-1 mRNA expression is reduced in MLK3 KO 4T1 cells compared with parental cells (**Figure 2.7B**), consistent with the idea that MLK3 controls FRA-1 at the transcriptional level. Despite a shift in FRA-1 mRNA and protein levels when MLK3 is either overexpressed or silenced in breast cancer cells, there is no change observed in the level of c-FOS protein levels in the same samples, suggesting that MLK3 specifically controls expression of FRA-1 but not another AP-1 member c-FOS (**Figure 2.8**).

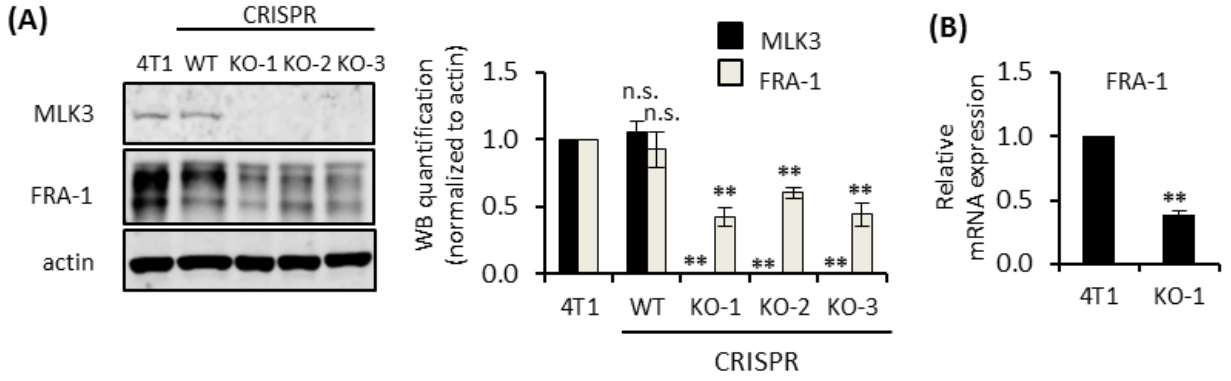


Figure 2.7 MLK3 knockout 4T1 cells display decreased FRA-1 expression at both the protein and transcript levels. (A) Cellular lysates from parental 4T1, WT clone, and 4T1 CRISPR knockout clones (KO-1, KO-2 and KO-3) were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments. **(B)** The mRNAs of parental 4T1 or 4T1KO-1 were subjected to qRT-PCR with indicated primers. Relative mRNA expression levels are displayed as mean ± SD from at least three independent experiments performed in triplicate; n.s. – not statistically significant; ** $p < 0.01$.

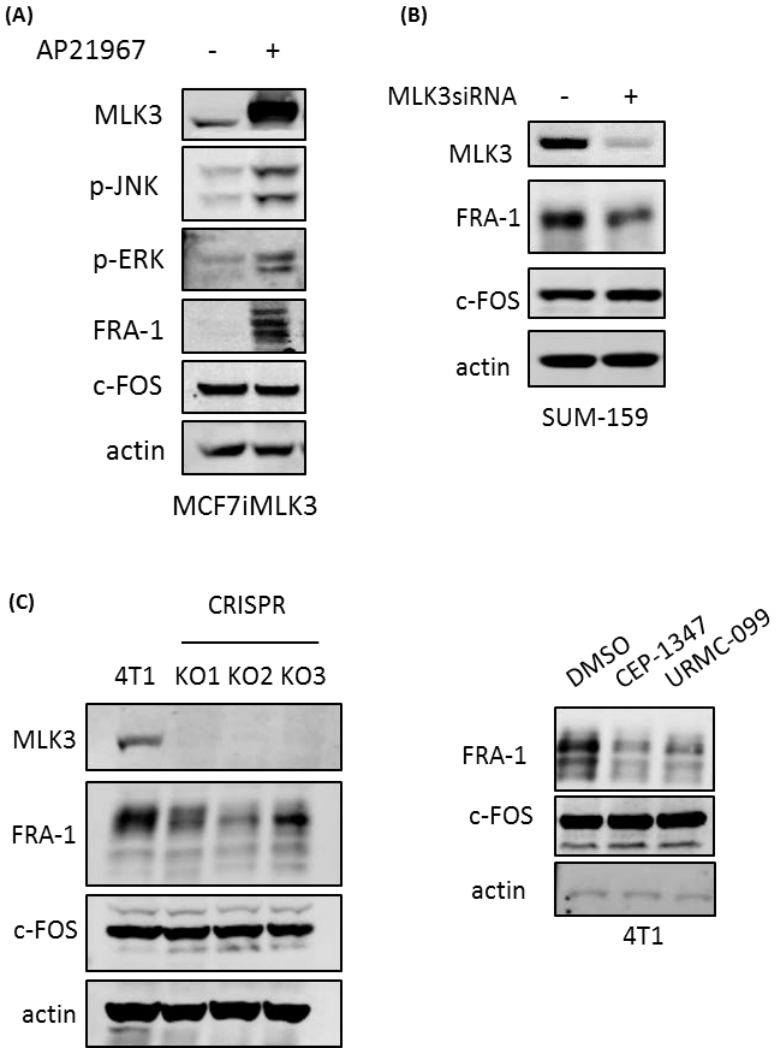


Figure 2.8 MLK3 regulates FRA-1 but not c-FOS expression in breast cancer cells. (A) MCF7iMLK3 cells were treated ± 25 nM AP21967 for 24 h to induce MLK3 expression, **(B)** SUM-159 were treated with 50 nM control siRNA or MLK3siRNA for 24 h, **(C)** 4T1 cells and their derivatives and, **(D)** 4T1 cells were treated with vehicle, 400 nM CEP-1347, or 400 nM URMC-099) for 24 h. Cellular lysates were subjected to immunoblotting with indicated antibodies.

To validate the specificity of CRISPR MLK3 KO, a rescue experiment was performed using the 4T1KO-1 clone. As shown in **Figure 2.9**, 4T1 cells exhibit predominantly nuclear expression of FRA-1 as judged by a co-staining of FRA-1 with 4', 6-Diamidino-2-

Phenylindole (DAPI). FRA-1 expression was nearly absent in the 4T1KO-1 cells. Upon a transient transfection of the bi-cistronic pCMS-EGFP-MLK3 vector, more than 98% of the EGFP-positive 4T1KO-1 cells, which also express ectopic MLK3, regained nuclear FRA-1 staining (**Figure 2.8**). Taken together, these data indicate that MLK3 is required for FRA-1 expression in these TNBC lines.

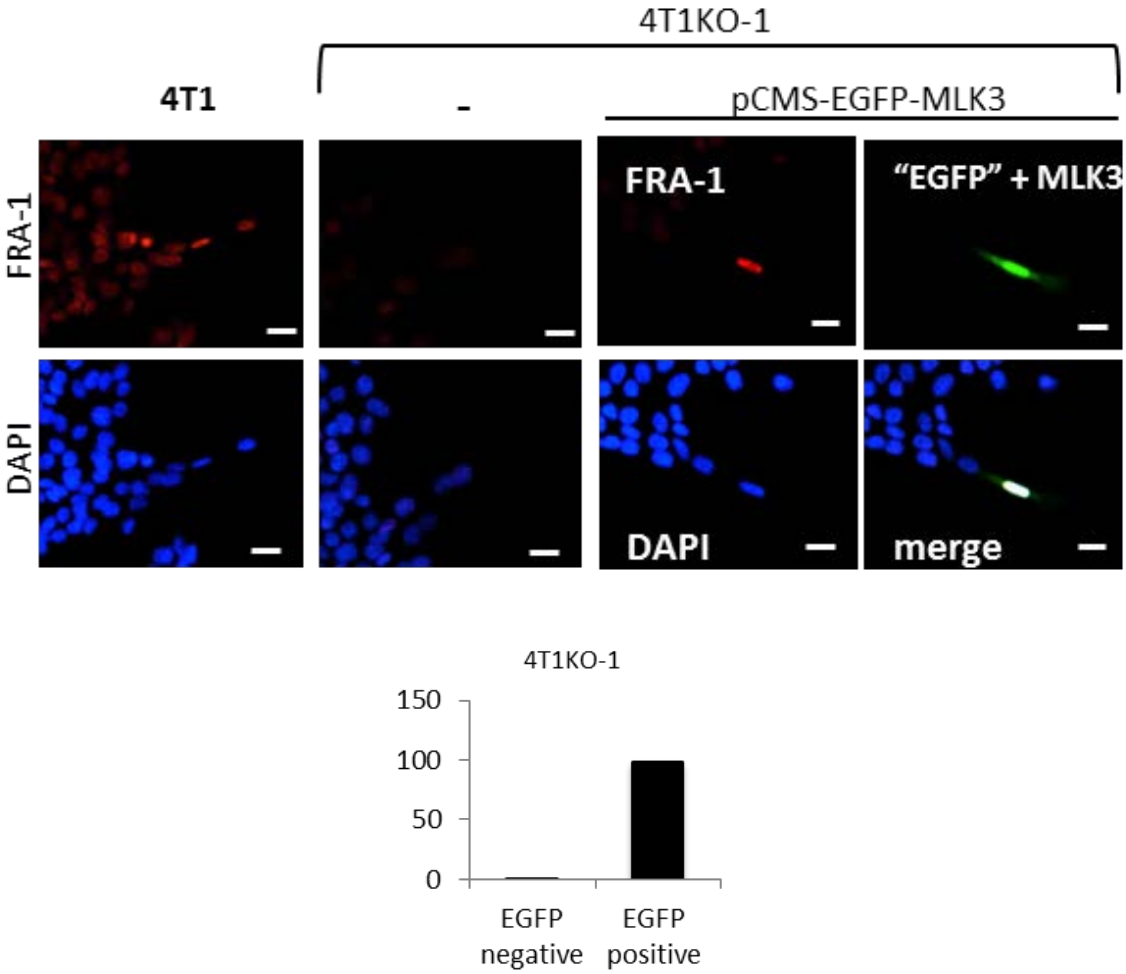


Figure 2.9 FRA-1 expression is rescued by ectopic expression of human MLK3 in MLK3-knockout 4T1 cells re-express FRA-1 protein in a rescue experiment. 4T1 cells or 4T1KO-1 ± pCMS-EGFP-MLK3 for 24 h were subjected to immunofluorescence staining against FRA-1 (red); nuclei were counterstained with DAPI (blue); GFP shown in green; Scale bar = 25 μm.

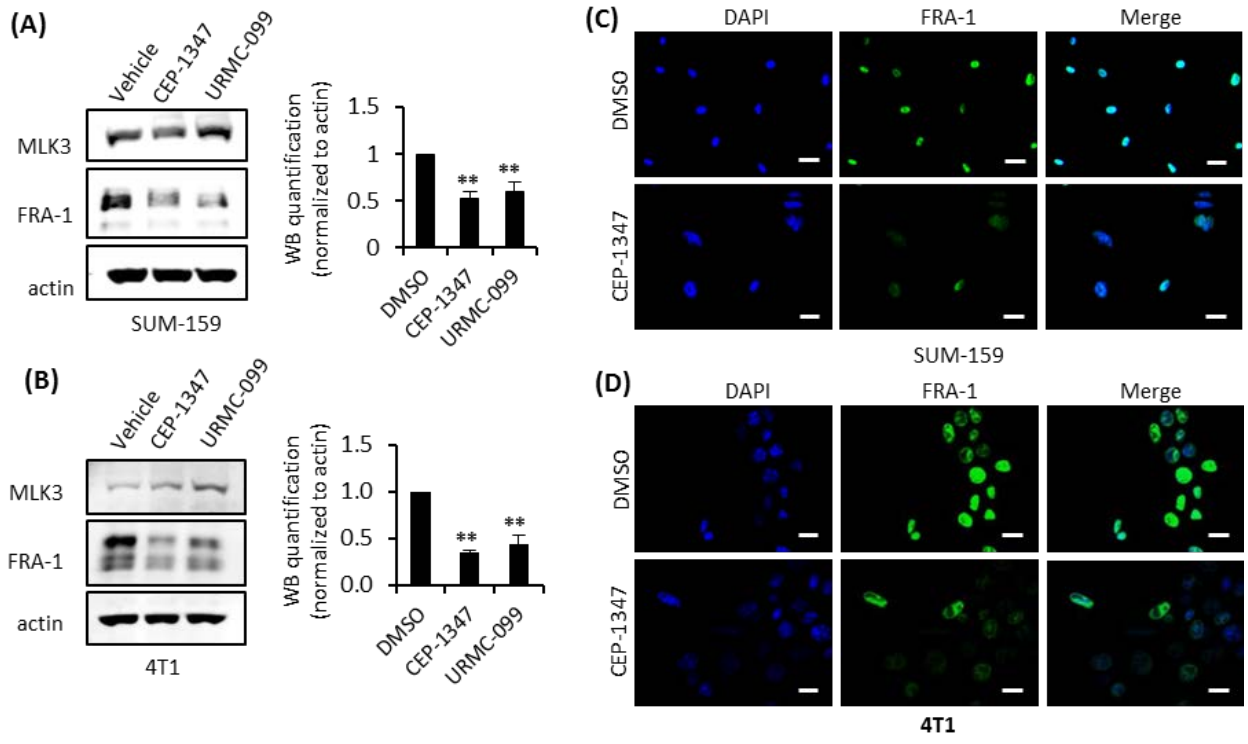


Figure 2.10 Pharmacological inhibition of MLKs reduces FRA-1 in TNBC cells. Cellular lysates from **(A)** SUM-159 or, **(B)** 4T1 cells treated for 24 h with DMSO vehicle or 400 nM CEP-1347 or 400 nM URMC-099) were subjected to immunoblotting with indicated antibodies. Western blot quantification of FRA-1 normalized to actin is expressed as mean ± SD from at least three independent experiments; ** $p < 0.01$. **(C)** SUM-159 cells or **(D)** 4T1 cells were grown on glass-coverslips and treated with DMSO vehicle or 400 nM CEP-1347 for 24 h, and subjected to immunofluorescence staining against FRA-1 (green); nuclei were counterstained with DAPI (blue); scale bar = 50 μm in **2C** and 25 μm in **2D**.

To assess if blockade of MLK activity is sufficient to reduce FRA-1 levels in highly invasive breast cancer cells, SUM-159 and 4T1 cells were treated with CEP-1347 (400 nM) and URMC-099, an MLK3 selective inhibitor (Rhoo et al., 2014), with a chemical scaffold distinct from that of CEP-1347, for 24 h and subjected to immunoblot analysis. As shown in **Figure 2.10A and 2.10B**, both CEP-1347 and URMC-099 treatment downregulates FRA-1 expression in SUM-159 and 4T1 cells. Immunofluorescence staining of SUM-159 and 4T1 cells treated with CEP-1347 revealed loss of FRA-1 staining, similar to the phenotype of the 4T1KO-1 cells

(**Figure 2.10C and 2.10D).** These findings strongly demonstrate that MLK3 activity is required for FRA-1 expression in these highly invasive breast cancer cell lines.

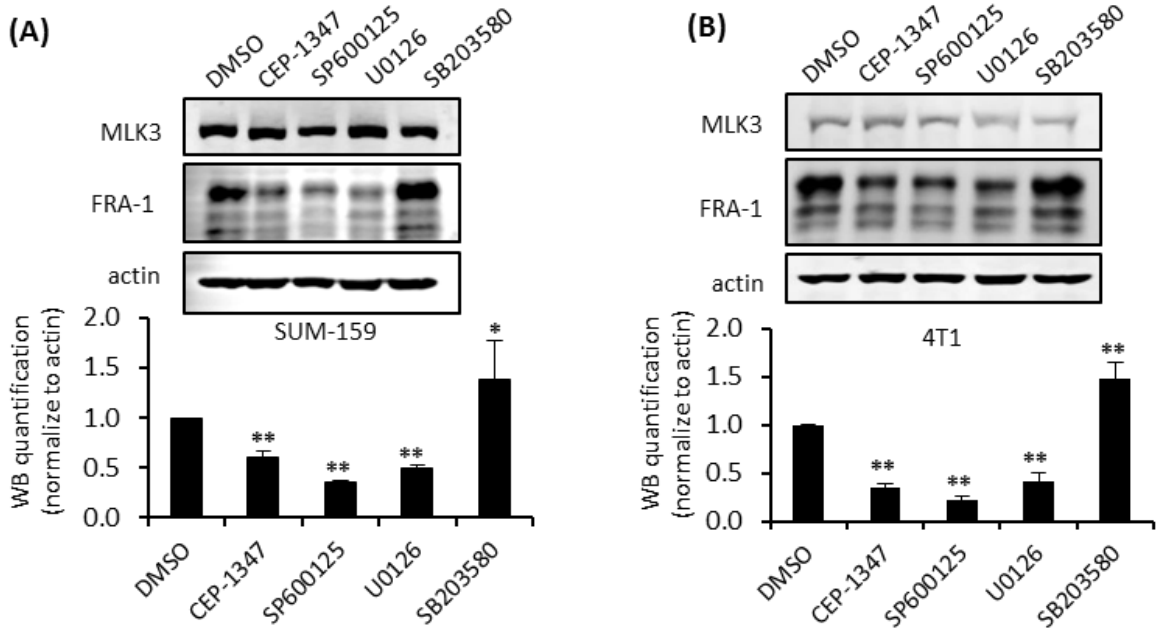


Figure 2.11 Inhibition of MLK, JNK, ERK but not P38 pathways decreases FRA-1 protein expression. (A) SUM-159 cells or **(B)** 4T1 cells with DMSO vehicle, 400 nM CEP-1347, 15 μM SP600125, 10 μM U0126, or 10 μM SB203580 for 24 h. Cellular lysates were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; ** $p < 0.01$.

4.2 MLK3-dependent FRA-1 regulation is mediated through JNK and ERK pathways.

Our data show that MLK3 activity is critical for expression of FRA-1. We then addressed which downstream MLK3-mediated pathways contribute to FRA-1 expression. To test the requirement for MAPK signaling on basal levels of endogenous FRA-1 in TNBC cells, MAPK pathways were blocked by treatment with small molecule inhibitors SP600125 (JNK), U0126 (MEK/ERK), or SB203580 (P38). The treatments with MLK, JNK, or ERK inhibitors significantly decreased basal, endogenous FRA-1 levels in SUM-159 (**Figure 2.11A**) as well as

4T1 cells (**Figure 2.11B**). The treatment with CEP-1347 or the previous generation of this inhibitor, CEP-11004, for 1 h is sufficient to decrease both JNK and ERK phosphorylation (**Figure 2.12**). Treatment of SP600125 and U0126 for 1 h also effectively blocks the activation of JNK, as judged by phopho-c-JUN levels, and MEK/ERK signaling, as judged by phosphor-ERK levels, respectively. Time course experiments performed on 4T1 cells demonstrated that, by 16 h, the treatments with JNK and ERK pathway inhibitors significantly decrease FRA-1 protein levels. While SP600125 treatment had no impact on ERK signaling throughout the 24 h time course experiment, we observed that U0126 treatment for 16 and 24 h reduced phospho-c-JUN. **(Figure 2.13**). Interestingly, the treatment with the P38 MAPK inhibitor SB203580 failed to reduce FRA-1, and instead increased FRA-1 expression in these two invasive breast cancer cell lines. This may result from established antagonistic activity between the JNK and p38 pathways (Wada et al., 2008).

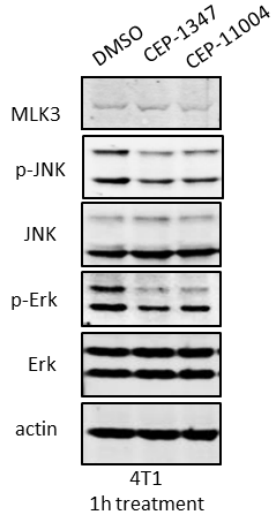


Figure 2.12 MLK inhibition downregulates ERK and JNK phosphorylation. 4T1 cells were treated with an MLK inhibitor (400 nM CEP-1347 or 400 nM CEP-11004) for 1 h. Cellular lysates were subjected to immunoblotting with indicated antibodies.

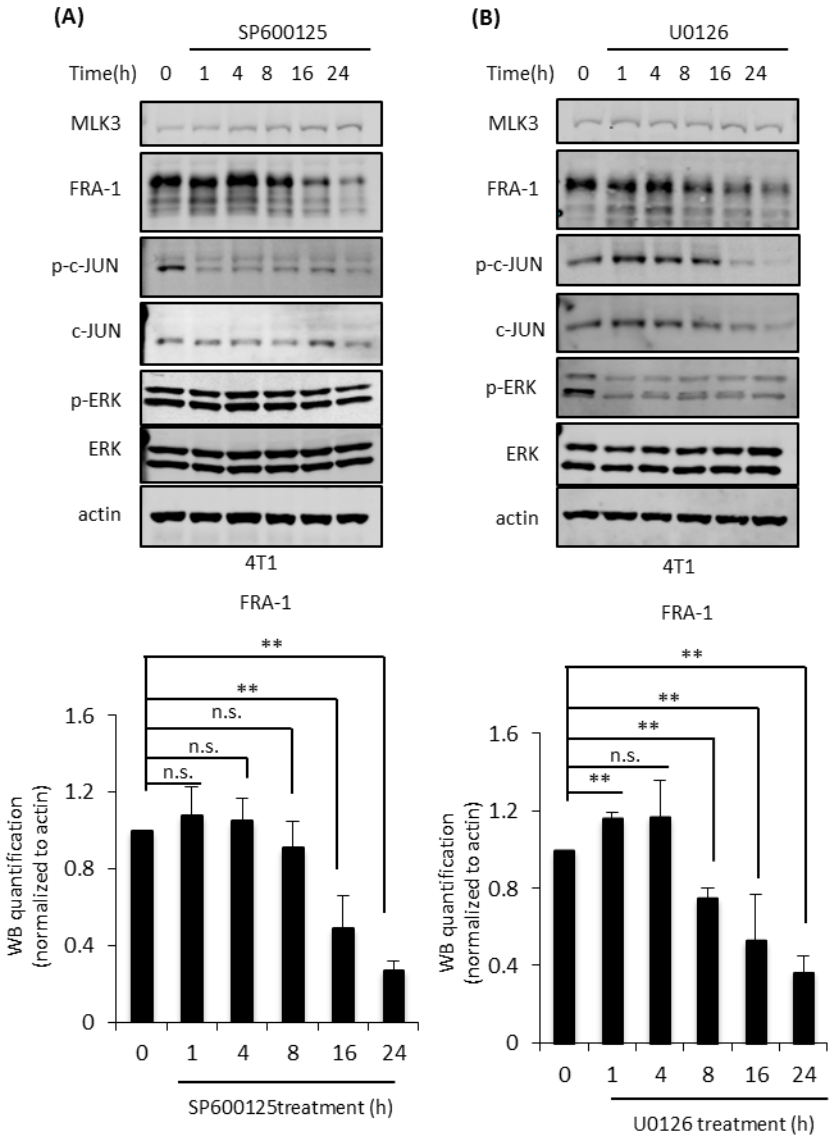


Figure 2.13 Time course analyses for JNK and ERK pathway inhibition on FRA-1 expression. 4T1 cells treated with (**A**) 15 μM SP600125 and (**B**) 10 μM U0126 for 1, 4, 8, 16, and 24 h. At the indicated times, cellular lysates were harvested and were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; n.s. – not statistically significant; ** $p < 0.01$.

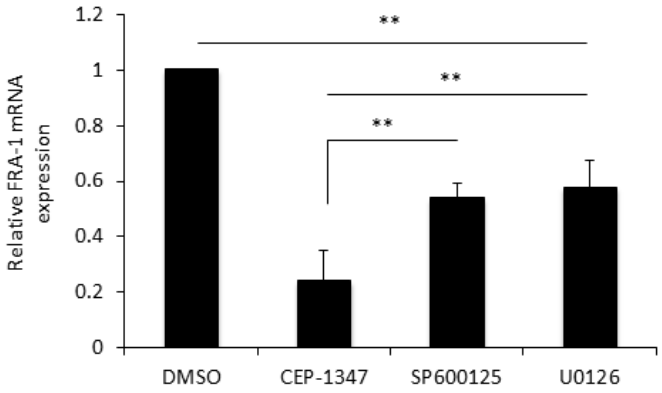


Figure 2.14 Inhibition of MLK, JNK and ERK pathways decrease FRA-1 transcript levels. The mRNAs from 4T1 cells treated with DMSO vehicle, 400 nM CEP-1347, 15 μM SP600125, or 10 μM U0126 for 24 h were subjected to qRT-PCR with FRA-1 primers. Relative mRNA expressions are displayed as mean ± SD from at least three independent experiments performed in triplicate; n.s. – not statistically significant; ** $p < 0.01$.

FRA-1 transcript levels were determined by qRT-PCR of mRNA isolated from 4T1 cells treated with either CEP-1347, SP600125, or U0126, to block activation of MLKs, JNK, or ERK pathway respectively, for 24 h were analyzed for FRA-1 transcript levels. As shown in **Figure 2.14**, all three inhibitors decrease FRA-1 transcript levels, suggesting that both JNK and ERK pathways contribute to FRA-1 regulation at the transcriptional level.

4.3 MLK3 increases MMP-1 and MMP-9 through FRA-1.

FRA-1 is an oncogenic member of the AP-1 transcription factor family (Young and Colburn, 2006) which is known to regulate expression of genes involved in cancer progression, including multiple matrix metalloproteinases (MMPs). Based on our data, we hypothesized that MLK3 functions to control the expression of FRA-1-dependent MMPs, including MMP-1(Belguise et al., 2005; Henckels and Prywes, 2013; Kimura et al., 2011), MMP-2(Bergman et al., 2003; Singh et al., 2010), and MMP-9 (Henckels and Prywes, 2013). Following MLK3 induction in MCF7iMLK3 cells, the mRNA levels of selected MMPs were determined by qRT-PCR.

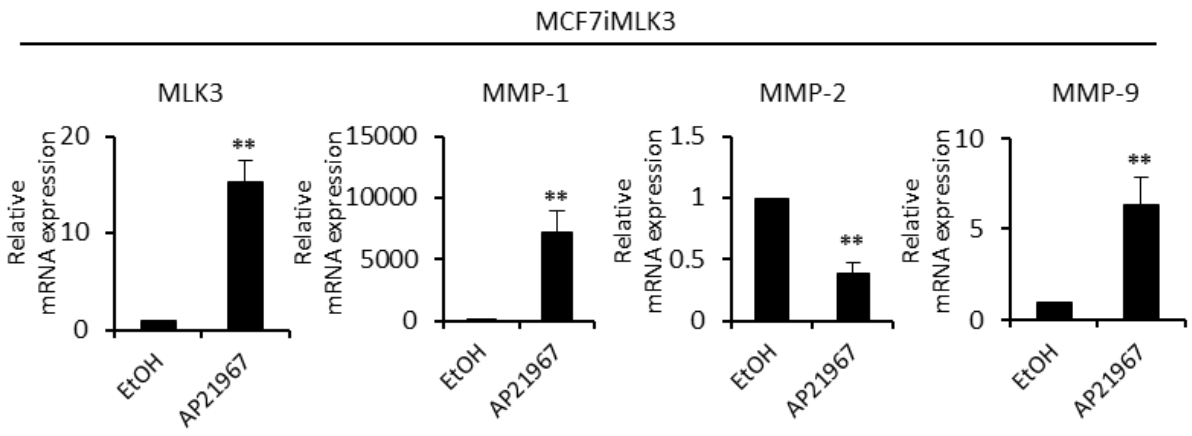


Figure 2.15 Induced expression of MLK3 increases MMP-1 and MMP-9 transcript levels in MCF7iMLK3 cells. The mRNAs isolated from MCF7iMLK3 cells treated with vehicle or 50 nM AP21967 for 24 h were subjected to qRT-PCR with indicated primers. Relative mRNA levels are displayed as mean ± SD from at least three independent experiments performed in triplicate; **$p < 0.01$.

As shown in **Figure 2.15**, induced expression of MLK3 upregulates MMP-1 mRNA expression by 5,000-fold and MMP-9 mRNA expression by 6-fold. Overexpression of MLK3 reduced, rather than increased, MMP-2 mRNA expression. Elevation of MMP-1 protein upon MLK3 induction in MCF7iMLK3 cells was assessed by immunoblotting. Uninduced MCF7 cells exhibit very low cytosolic and secreted MMP-1 protein as judged by the immunoblotting of cellular lysates and concentrated conditioned media, respectively (**Figure 2.16A and 2.16B**). Upon MLK3 overexpression, MCF7iMLK3 cells display elevated expression of cytosolic and secreted MMP-1 protein. Importantly, the increase in MMP-1 protein levels is abrogated by treatment with CEP-1347 (**Figure 2.16A**), or by FRA-1 gene silencing (**Figure 2.16B**), suggesting that MLK3 functions through FRA-1 to induce MMP-1 expression in these breast cancer cells.

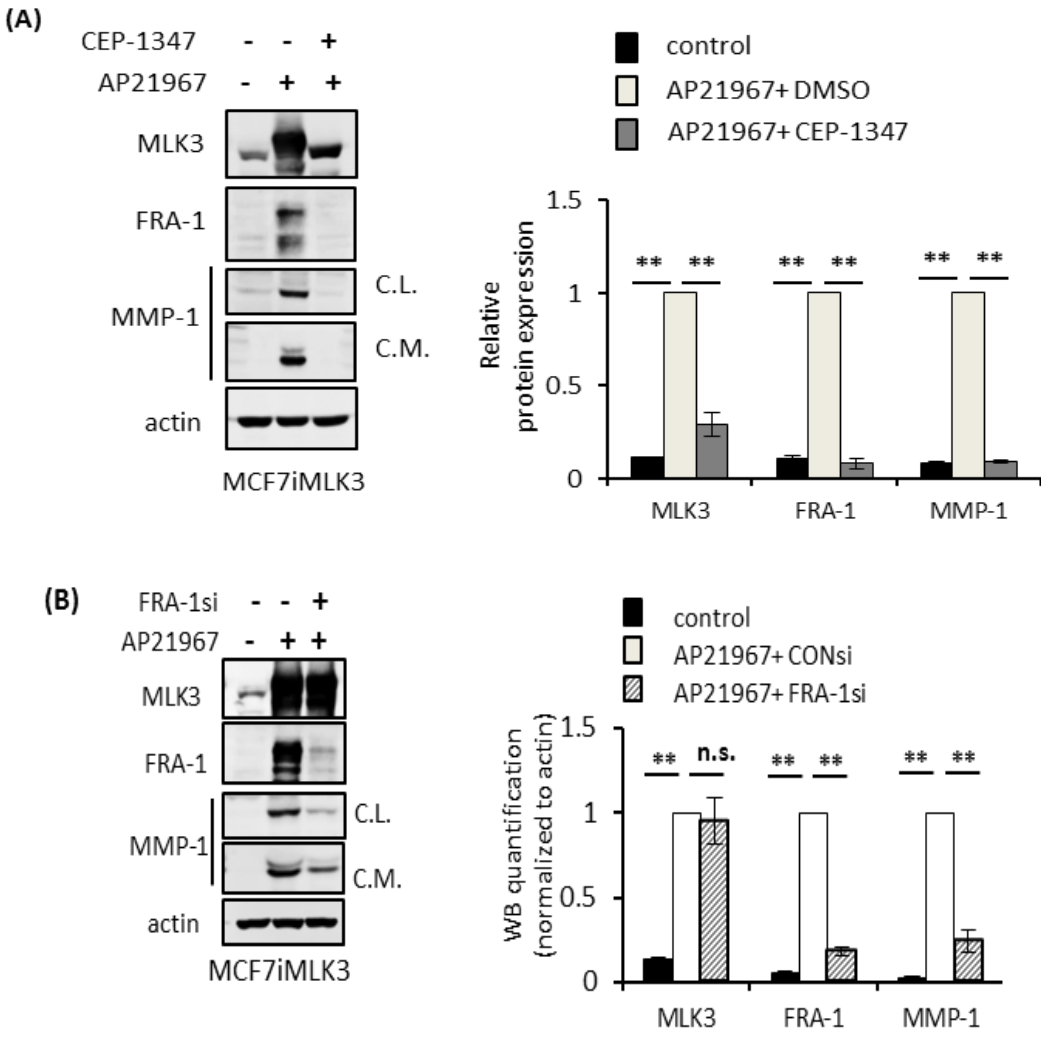


Figure 2.16 MLK3 functions through FRA-1 to drive MMP-1 expression in MCF7iMLK3 cells. Cellular lysates from MCF7iMLK3 cells treated with vehicle or 50 nM AP21967 in the presence or absence of **(A)** DMSO or 400 nM CEP-1347 and **(B)** 50 nM control siRNA or 50 nM FRA-1 siRNA for 24 h were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; n.s. – not statistically significant; **p < 0.01; C.L. – Cellular lysate; C.M. – concentrated conditioned medium.

We then evaluated mRNA levels of the same MMPs in the metastatic 4T1 cell line compared with its MLK3 KO clones: 4T1KO-1 and 4T1KO-2. The qRT-PCR analysis shows reduction of MMP-1a, the functional ortholog of human MMP-1 (Foley and Kuliopulos, 2014), in 4T1KO-1 and 4T1KO-2 by 99 and 95%, respectively, when compared with control parental

4T1 cells (**Figure 2.17A**). Consistent with the finding in the MCF7iMLK3 cells, loss of MLK3 decreased MMP-9 mRNA by 60 and 50% in 4T1KO-1 and 4T1KO-2 cells, respectively, and slightly increased MMP-2 expression in 4T1KO-1 cells compared with control cells. Moreover, similar effects on MMP mRNA levels were found upon treatment of 4T1 cells with CEP-1347 (**Figure 2.17B**). Decrease of MMP-1 mRNA upon MLK3 gene silencing or MLK3 inhibitor treatment was also confirmed in SUM-159 cells (**Figure 2.17C and 2.17D**).

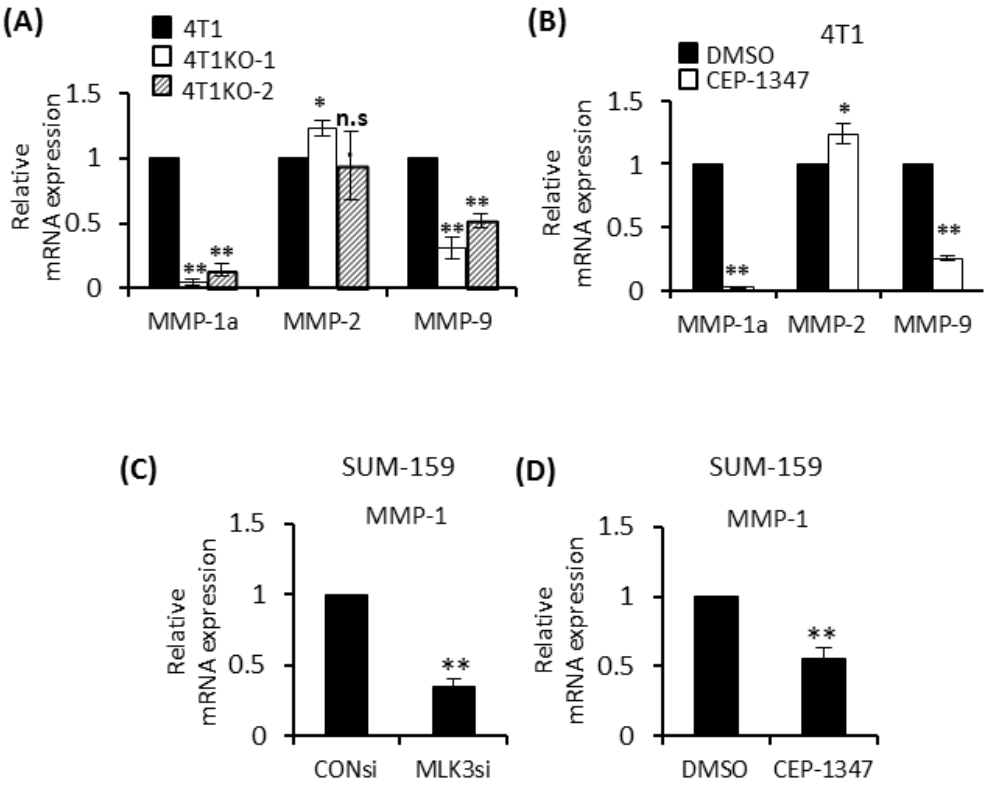


Figure 2.17 Induced expression of MLK3 increases MMP-1 and MMP-9 transcript levels in MCF7iMLK3 cells. The mRNAs from MCF7iMLK3 cells ± 50 nM AP21967 for 24 h were subjected to qRT-PCR with indicated primers. Relative mRNA expression levels are displayed as mean ± SD from at least three independent experiments performed in triplicate; **$p < 0.01$.

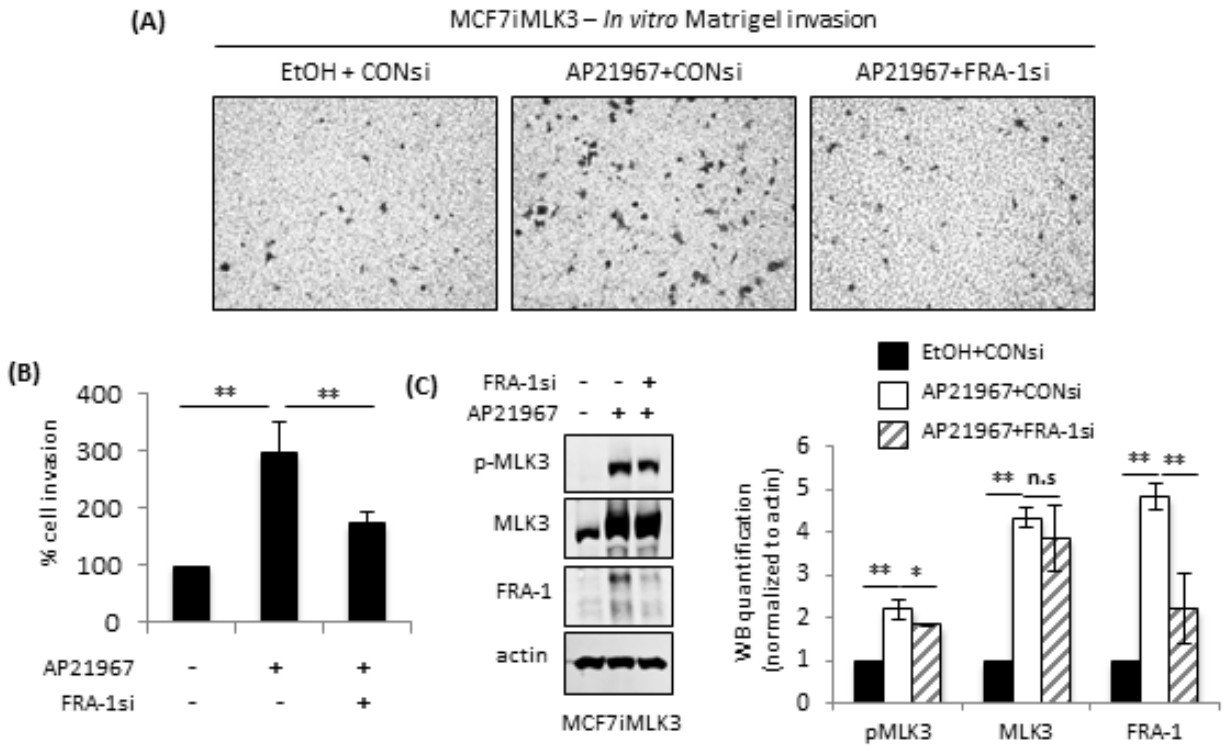


Figure 2.18 MLK3 drives cancer cell invasion through FRA-1 axis. (A) MCF7iMLK3 cells were treated with ± 25 nM AP21967 and ± 50 nM FRA-1 siRNA were subjected to an *in vitro* Matrigel invasion assay for 24 h**. (B)** Relative cell invasion is expressed as mean ± SD from three independent experiments. **(C)** Cellular lysates from the remaining MCF7iMLK3 receiving the indicated treatments were collected at the end point of an invasion assay and were subjected to immunoblotting with indicated antibodies. Western blot quantification of the indicated protein normalized to actin is expressed as mean ± SD from at least three independent experiments; n.s. – not statistically significant; *p < 0.05; **p < 0.01.

4.4 MLK3 regulates cancer cell invasion and transendothelial migration

To assess whether MLK3 drives cancer cell invasion through FRA-1, we determined the impact of FRA-1 silencing on Matrigel Transwell invasion of MCF7iMLK3 cells. As shown in **Figure 2.18A-C**, induced expression of MLK3 in MCF7iMLK3 cells upregulates FRA-1 expression and enhances invasion which is reduced by silencing of FRA-1, indicating FRA-1 functions downstream of MLK3 in cancer cell invasion. Of note, MLK3 is also known to control breast cancer cell migration through JNK-paxillin pathway in TNBC cells (Chen and Gallo,

2012). However, while induced expression of MLK3 in MCF7 cells no change in phosphorylation of paxillin was observed in these FRA-1-silencing MCF7iMLK3 cells (**Figure 2.19**) indicating that MLK3 regulation of paxillin is independent of FRA-1, consistent with the idea that MLK3 controls multiple pathways in cancer cell migration and invasion.

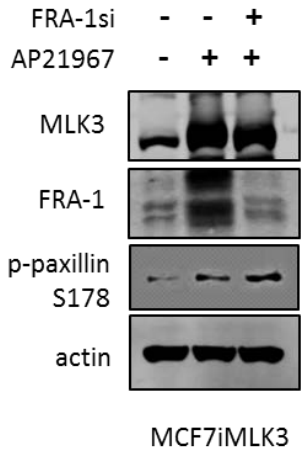


Figure 2.19 Loss of FRA-1 does not affect MLK3-induced phosphorylation of paxillin. MCF7iMLK3 cells ± 25 nM AP21967 ± 50 nM FRA-1siRNA for 48 h from the Transwell invasion assay were collected and analyzed by immunoblotting with indicated antibodies.

In TNBC models, tumor cell-derived MMP-9 is required for Matrigel invasion and formation of pulmonary metastases (Lee et al., 2012; Mehner et al., 2014; Zeng et al., 1999). Recently, MMP-1 has been shown to play a critical role in transendothelial migration and vascular intravasation (Juncker-Jensen et al., 2013). Since MLK3 deletion decreases both MMP-1 and MMP-9 levels, we tested whether deletion of MLK3 impacts Matrigel invasion and transendothelial migration in these TNBC cells. Indeed the three independent 4T1 MLK3 KO clones show impaired invasion through Matrigel compared with parental 4T1 cells. Furthermore, treatment with CEP-1347 similarly inhibits Matrigel invasion (**Figure 2.20A**).

Gelatin zymography was performed to evaluate secreted MMP-9 protein from these samples. Consistent with the invasion experiment, conditioned media from 4T1 MLK3 KO clones and 4T1 treated with CEP-1347 showed the reduction of bands at approximately 90 kDa, corresponding to the molecular weight of MMP-9, when compared to the control (**Figure 2.20B**).

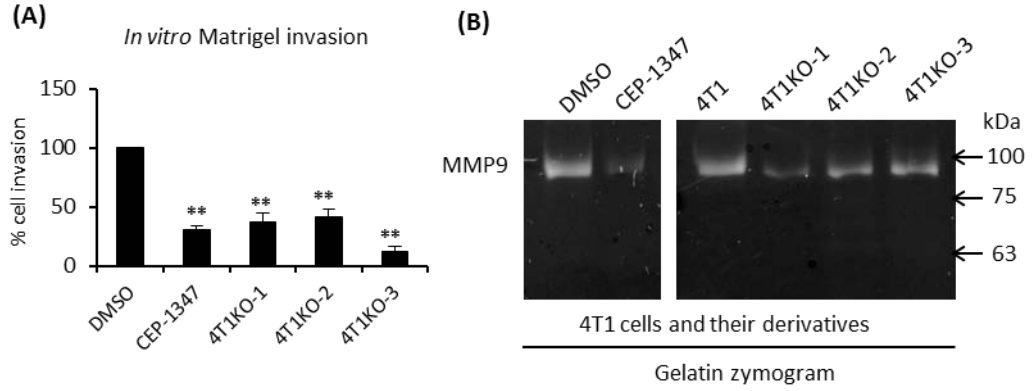


Figure 2.20 Loss of MLK3 or inhibition of its catalytic activity impairs cancer cell invasion. (A) Parental 4T1 cells were treated ± 400 nM CEP-1347 and MLK3-KO 4T1 clones (KO-1, KO-2, and KO-3) were subjected to an *in vitro* Matrigel invasion assay for 24 h. Relative cell invasion is expressed as mean ± SD from three independent experiments; ** $p < 0.01$. **(B)** Conditioned media from 4T1 cells ± 400 nM CEP-1347 and MLK3-KO 4T1 clones (KO-1, KO-2, and KO-3) were harvested and subjected for a gelatin zymography. The images are the representative from three independent experiments.

Transendothelial migration assays were performed to assess the ability of 4T1 or 4T1KO-1 cells to disrupt and invade through a confluent endothelial cell monolayer. The 4T1KO-1 clone was chosen for these experiments because its bioluminescence activity is comparable to that of parental 4T1 cells (**Figure 2.21**).

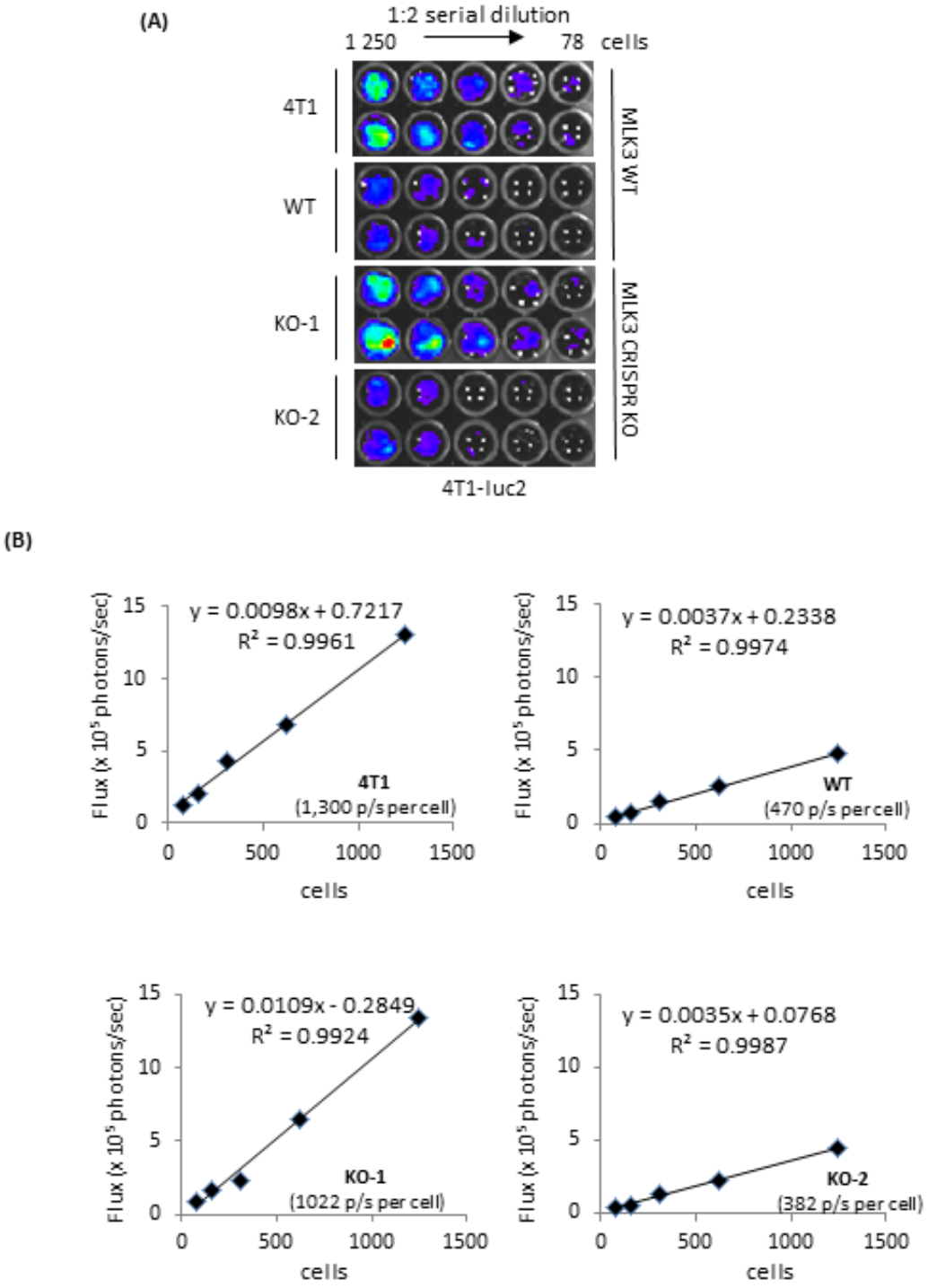


Figure 2.21 Bioluminescence of 4T1, WT, and 4T1 MLK3 KO clones. (A) Parental 4T1-luc2 cells, a WT clone and two different 4T1 MLK3 KO clones (4T1KO-1 and 4T1KO-2) were seeded in a black, 96-well plate overnight. Cells were then treated with 2 μg/mL D-luciferin for 3 min and the bioluminescence was measured using the IVIS system. **(B)** The bioluminescence activity of each line was plotted against cell number to determine the average activity per cell.

While parental 4T1 cells increase permeability of the endothelial cell layer as judged by leakage of fluorescent albumin, 4T1KO-1 cells show markedly reduced endothelial permeability (**Figure 2.22A**). In addition, 4T1KO-1 cells show a 4-fold reduction in transendothelial migration compared with parental 4T1 cells (**Figure 2.22B**).

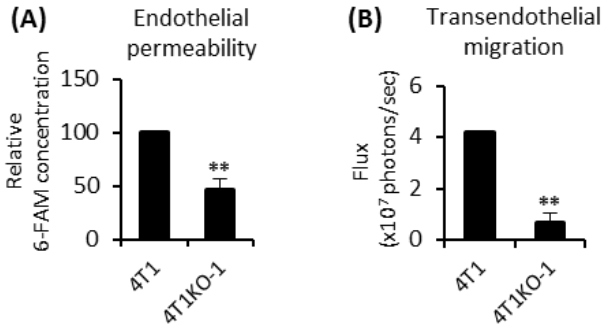


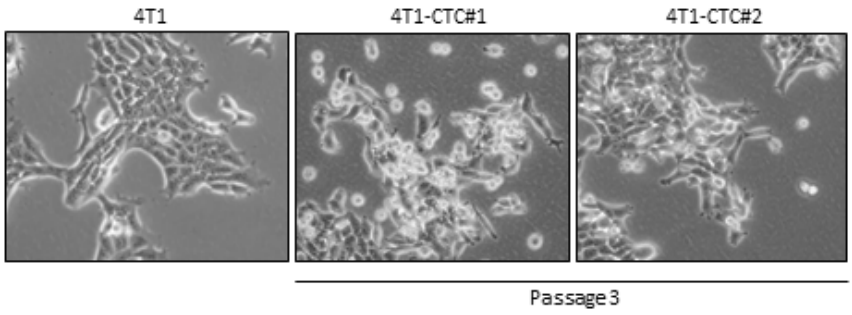
Figure 2.22 Loss of MLK3 impairs both the ability of cancer cells to disrupt the endothelial barrier and trans-endothelial migration. Parental 4T1 and 4T1KO-1 were subjected to transendothelial migration toward 10 % FBS for 24 h. **(A)** 5(6)-Carboxyfluorescein (6-FAM)-conjugated albumin (**10** μM final concentration) was added into the upper chamber and the endothelial permeability was assessed by measuring the leakage 6-FAM-conjugated albumin into the lower chamber, and **(B)** the cells in the upper chamber were removed and the extent of transendothelial migration was assessed using bioluminescence imaging after a treatment with luciferin (150 μg/mL final concentration) for 3 min. Relative 6-FAM conjugated albumin concentration and bioluminescence activity are expressed mean ± SD from three independent experiments performed in triplicates. ** $p < 0.01$.

4.5 Upregulation of FRA-1 and MMP-1a is found in circulating tumor cells (CTCs) derived from 4T1 tumors.

The established role of MMP-1 in cancer intravasation, coupled with our findings that MLK3 controls MMP-1 levels, prompted us to assess the components of the MLK3-FRA-1-MMP-1 signaling axis in circulating tumor cells, which have undergone intravasation. A clonogenic assay was used to isolate circulating tumor cells (CTCs) from the blood of a mouse bearing a 4T1-luc2 mammary tumor and associated metastases. By phase contrast imaging, the

morphology of the isolated CTCs (4T1-CTC) shows distinct morphology compared with the morphology of the parental 4T1 cells. Many of these cells are able to detach and re-attach to tissue culture plates (**Figure 2.23A**). Bioluminescence imaging demonstrated that the isolated CTCs retain luciferase activity, confirming that they originated from the 4T1-luc2 tumor (**Figure 2.23C**).

(A)



(B)

Samples	Number of CTC colonies* at Day 9
CTCs from mouse#1 + puro	42
CTCs from mouse#2+ puro	30

*From 200 ul blood collected from cardiac puncture.

(C)

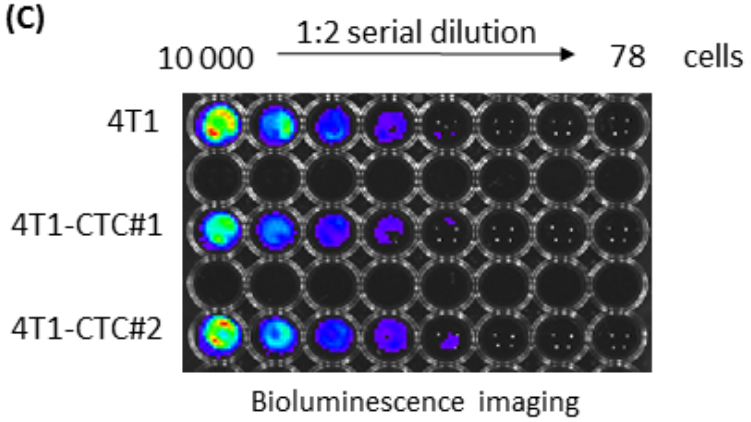


Figure 2.23 Characterization of 4T1-CTC cells derived from 4T1-luc2 cells. (A) Representative images of parental 4T1 cells and 4T1-CTC lines which were isolated from the blood of 4T1 (containing luc2 construct) tumor-bearing mice using a colony formation assay. **(B)** Number of colonies from blood cultures in the presence of puromycin (2 μg/mL) was counted at day 9. Colonies from each plate were pooled and named 4T1-CTC#1 and 4T1-CTC#2. **(C)** Parental 4T1-luc2**,** 4T1-CTC#1 and 4T1-CTC#2 cells were seeded in a black, 96-well plate overnight. Cells were then treated with 2 μg/mL D-luciferin for 3 min and bioluminescence was measured using the IVIS system.

Interestingly, qRT-PCR analysis of two 4T1-CTC lines demonstrated that these cells possess increased FRA-1 and MMP-1a, expression, compared with parental 4T1 cells. Furthermore in the 4T1-CTC lines, FRA-1 and MMP-1a levels are dependent upon MLK activity, since their levels are decreased by CEP-1347 treatment (**Figure 2.24**). The fact that the components of the MLK3-FRA-1-MMP1 signaling axis are upregulated in CTCs compared with parental 4T1 cells, and that CTCs retain sensitivity to CEP-1347, is consistent with the idea that enhanced MLK3 signaling promotes invasion, intravasation, and, possibly, CTC survival.

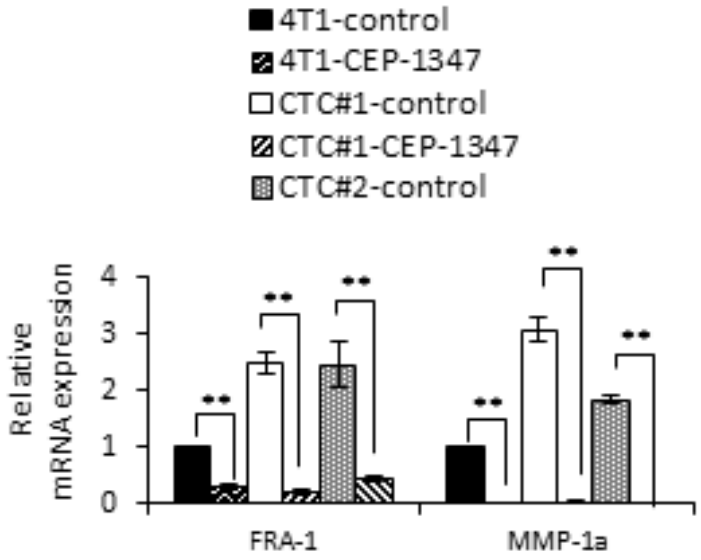


Figure 2.24 4T1-derived circulating tumor cells (CTCs) upregulate FRA-1 and MMP-1a. The mRNAs from 4T1, 4T1-CTC#1 and 4T1-CTC#2 treated with vehicle or 400 nM CEP-1347 for 24 h were subjected to qRT-PCR analysis using FRA-1 and MMP-1a primers. Relative mRNA expression are displayed as mean ± SD from at least three independent experiments performed in triplicate; ** $p < 0.01$.

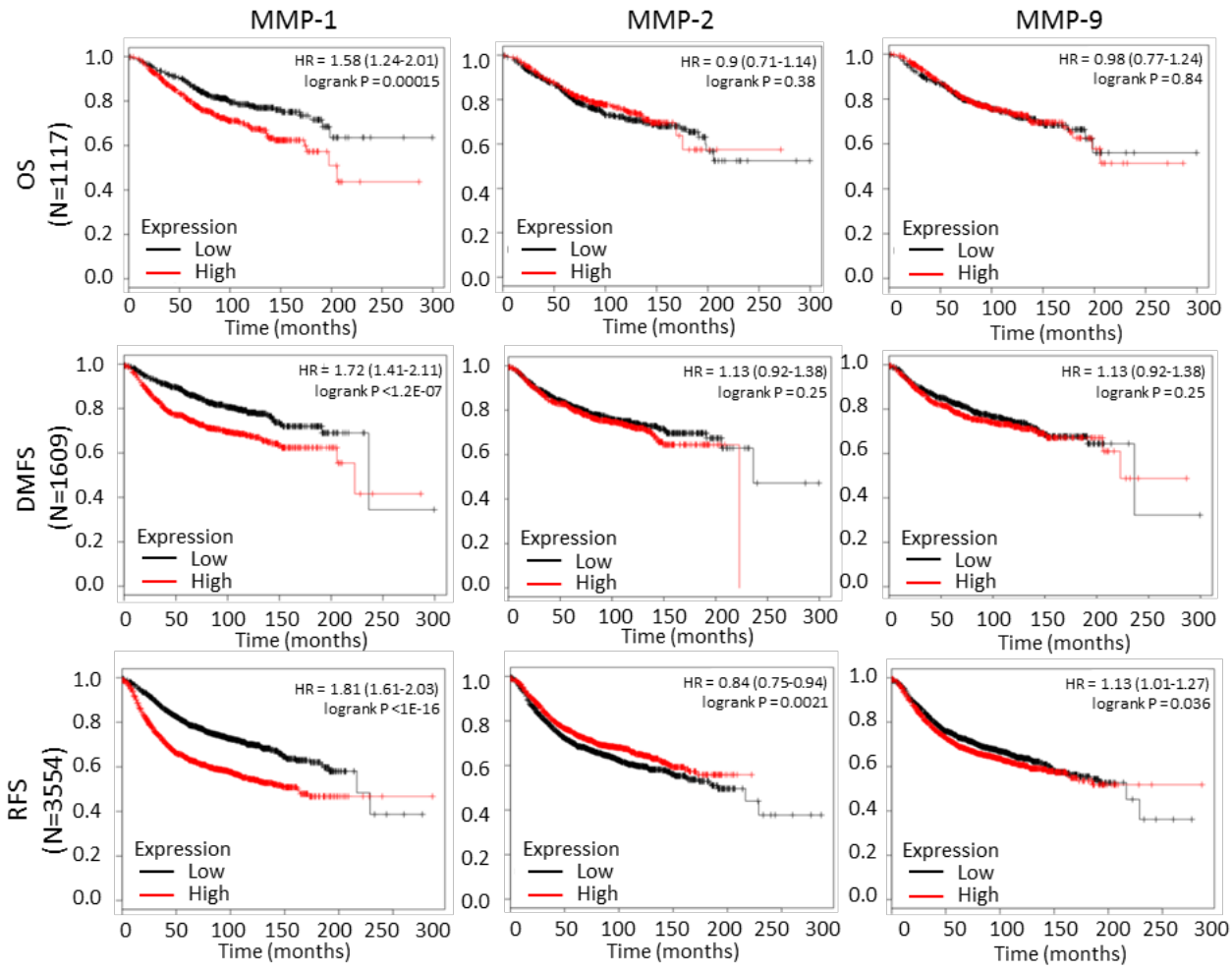


Figure 2.25 Correlation of MMP-1, -2, -9 expression with Overall Survival (OS), Distant Metastasis Free Survival (DMFS), and Relapse Free Survival (RFS) of breast cancer patients. Kaplan-Meier survival analyses of MMP-1, MMP-2 and MMP-9 in OS, RFS, and DMFS of breast cancer patients were generated from a database of 1117, 3554, and 1610 breast cancer patients, respectively, split by median expression among all breast tumor samples without stratification by breast cancer subtype (kmplot.com).

4.6 Expression of MMP-1 negatively correlates with Overall Survival (OS), Relapse Free Survival (RFS), and Distant Metastasis Free Survival (DMFS).

High levels of MMPs have been associated with poor outcome in breast cancer patients. We analyzed clinical data sets (*http://kmplot.com)*(Gyorffy et al., 2010) to examine the relationship between MMP-1, MMP-2, and MMP-9 levels and overall survival (OS) (N=1117),

relapse-free survival (RFS) (N=3354), and distant metastasis free survival (DMFS) (N=1609) in breast cancer patients, regardless of subtype. Elevated expression of MMP-1 is predictive of decreased OS (hazard ratio (HR)=1.58; P=0.00015), RFS (HR=1.81; $P<1x10^{-16}$), and DMFS (HR=1.72; $P<1.2x10^{-7}$). Analogous comparisons show that high levels of MMP-2 do not significantly decrease OS, RFS, or DMFS; whereas high MMP-9 levels are associated with a small, but statistically significant, decrease in RFS (HR=1.13; P=0.036), but do not affect OS or DMFS (**Figure 2.25**).

5. Discussion

Metastasis is overwhelmingly the cause of breast cancer-related death yet the complexity of the metastatic process makes it therapeutically challenging to treat this fatal disease (Sethi and Kang, 2011). Previous studies in our lab and others have demonstrated that the protein kinase MLK3 is crucial for TNBC metastasis in a xenograft model (Chen and Gallo, 2012; Cronan et al., 2012). Herein, we utilized the CRISPR/Cas9 approach to deplete MLK3 in the highly aggressive 4T1 mammary cancer model in order to elucidate the role(s) of MLK3 in discrete steps of metastasis and to identify the key signaling pathways through which MLK3 regulates these events. Mechanistically, we have deciphered a novel function for MLK3 in controlling the FRA-1/MMP-1 axis in breast cancer cells. In this context, both JNK and ERK signal downstream of MLK3 (**Figure 2.11-2.14**) to enhance FRA-1 expression. JNK is well-known to phosphorylate c-JUN (Johnson and Nakamura, 2007), the AP-1 member which is required for transcriptional control of FRA-1(Adiseshaiah et al., 2005). ERK, in turn, phosphorylates FRA-1 which enhances the stability of the FRA-1/c-JUN heterodimer (Basbous et al., 2007; Belguise et al., 2012). Thus MLK3 signaling is well-poised to control FRA-1/c-JUN-mediated transcription.

FRA-1 has emerged as a key driver of metastatic progression in multiple cancer types

including breast cancer (Belguise et al., 2005; Belguise et al., 2012; Henckels and Prywes, 2013; Zhao et al., 2014), lung cancer (Adiseshaiah et al., 2008), colorectal cancer(Diesch et al., 2014a; Iskit et al., 2015b; Liu et al., 2015), and glioblastoma (Debinski and Gibo, 2005). FRA-1 not only controls expression of genes involved in cell motility, epithelial-to-mesenchymal transition (EMT) (Bakiri et al., 2015; Cheng et al., 2016; Diesch et al., 2014b; Lemieux et al., 2009), and cell invasion (Belguise et al., 2005; Henckels and Prywes, 2013; Kimura et al., 2011; Moquet-Torcy et al., 2014), but it also controls proliferation (Albeck et al., 2013; Belguise et al., 2005; Burch et al., 2004), metastatic outgrowth (Iskit et al., 2015a), and the stem cell phenotype of cancer cells (Tam et al., 2013). Several FRA-1 regulated genes (Belguise et al., 2005; Henckels and Prywes, 2013; Kimura et al., 2011; Moquet-Torcy et al., 2014) have been demonstrated to facilitate cancer cell invasion including MMP-1, MMP-2, and MMP-9 (Adiseshaiah et al., 2008; Henckels and Prywes, 2013). Our studies show that MLK3 deletion decreases both MMP-1 and MMP-9 expression (**Figure 2.17**) but does not significantly affect MMP-2 levels. Functionally, MMP-9, a type IV collagenase, facilitates extracellular matrix remodeling and basement membrane degradation; and, like MLK3, is critical for TNBC metastasis (Mehner et al., 2014). Likewise, MMP-9 silencing in multiple TNBC lines has been shown to block Matrigel invasion (Mehner et al., 2014), analogous to our findings that MLK3 deletion or CEP-1347 treatment inhibits Matrigel invasion (**Figure 2.20**).

In TNBC, hematogenous metastasis, which requires transendothelial migration (Reymond et al., 2013a) is more common than lymphatic spread (Mohammed et al., 2011). During vascular intravasation and extravasation, cancer cells must disrupt endothelial barrier integrity and transmigrate through the endothelial layer. MMP-1 is described as an interstitial collagenase-I required for fibrillar collagen remodeling (Klein and Bischoff, 2011; Manka et al.,

2012; Reymond et al., 2013b). However non-collagenolytic mechanisms of MMP-1 are implicated in transendothelial migration of tumor cells (Juncker-Jensen et al., 2013). In epidermoid cancer, for instance, tumor cell-derived MMP-1 increases endothelial barrier permeability by proteolytically activating the endothelial thrombin receptor PAR-1, facilitating transendothelial migration (Juncker-Jensen et al., 2013). In our TNBC model, tumor cells are able to induce endothelial barrier permeability and to transmigrate through an endothelial barrier, whereas, the ability of MLK3-deleted tumor cells to induce permeability and to undergo transendothelial migration is impaired (**Figure 2.22**). These findings suggest that MLK3 in TNBC cells could facilitate vascular intravasation and/or extravasation.

Cancer cells that have intravasated and remain circulating in the bloodstream are known as circulating tumor cells (CTCs) (Cristofanilli et al., 2004). Over the past decade CTCs have risen to prominence as potential prognostic and predictive biomarkers for metastatic burden, metastatic recurrence and therapeutic response (Bidard et al., 2008; Budd et al., 2006; Cierna et al., 2014; Cristofanilli et al., 2007; Cristofanilli et al., 2004; Cristofanilli et al., 2005; Giuliano et al., 2011; Hayes et al., 2006; Liu et al., 2009; Riethdorf et al., 2007; Tewes et al., 2009; Yu et al., 2013a). Our isolated 4T1-CTCs display a distinct morphology to the parental 4T1 cells. An appearance of low cell-cell/cell-matrix adhesion, resembling the cells undergoing epithelial-to-mesenchymal transition (EMT), observed in these cells is also analogous to what has been observed in a CTC line derived from colorectal cancer (Cayrefourcq et al., 2015). Expression of EMT markers identifies more aggressive and chemoresistant CTCs (Aceto et al., 2015; Barriere et al., 2014; Raimondi et al., 2011; Wu et al., 2015; Yu et al., 2013b) thus, making this “CTC-EMT” subpopulation an attractive target for therapeutic development. It would be interesting to further elucidate whether these cells possess epithelial- EMT phenotypes and whether MLK3 is

required in this process. Nevertheless, our current data point to a link between MLK3 signaling and TNBC CTCs.

We demonstrate that these 4T1-CTCs have elevated mRNA levels of FRA-1 and MMP-1a compared with parental 4T1 cells (**Figure 2.24)**. Several lines of evidence support a key role for MMP-1 in CTCs and metastatic progression. For instance, patients with CTCs expressing EMT markers have significantly higher MMP-1 expression in their corresponding primary tumors compared with patients whose CTCs lack EMT marker expression (Cierna et al., 2014). Local recurrence is posited to arise from CTCs that are capable of "self-seeding", which is the process of infiltrating and colonizing the tumor of origin (Comen et al., 2011); MMP-1 was identified as a key upregulated gene in infiltration of self-seeding CTCs (Kim et al., 2009). Elevated MMP-1 was also observed in TNBC MDA-MB-231 subclones selected for their ability to metastasize to lung (Minn et al., 2005) and brain (Bos et al., 2009). Recently, single cell gene expression analysis studies utilizing TNBC patient-derived xenografts showed that MMP-1, as well as components of a proliferative gene signature, significantly increases in late stage, high burden metastatic cells compared with early stage, low burden metastatic cells (Lawson et al., 2015). These data, along with the role of MMP-1 in vascular intravasation, suggest that MMP-1 is not only required for early stages of metastatic process, but may also contribute to colonization.

TNBC is considered the most aggressive subtype of breast cancer; however, therapeutic options are limited. A major challenge is to identify important targetable signaling pathway essential for this cancer type. MMP-1 expression is significantly elevated in aggressive breast tumors and correlates with both tumor size and grade (McGowan and Duffy, 2008). These findings point to MMP-1 as a promising therapeutic target. Indeed, MMPs, including MMP-1,

have a long history as targets for cancer therapeutics yet early clinical trials using MMP inhibitors were unsuccessful due, in part, to inadequate preclinical and clinical design, lack of drug specificity and high toxicity (Cathcart et al., 2015; Vandenbroucke and Libert, 2014). Recently FRA-1, an upstream regulator of MMP-1, has also emerged as a key driver of cancer progression; however, generally, transcription factors are not readily druggable. One approach has been to use an existing inhibitor of the FRA-1 regulated gene, adenosine receptor A_{2B}, ADORA2B, which was shown to block formation of lung metastases in a TNBC experimental metastasis xenograft model (Desmet et al., 2013). However, it is unclear whether targeting a single FRA-1-regulated gene will always be sufficient to inhibit breast cancer progression as FRA-1 controls a suite of genes involved in cancer invasion and metastasis (Diesch et al., 2014b). Based on our findings, we propose that an alternative strategy would be to target MLK3, an upstream regulator of FRA-1.

Multiple MLK inhibitors exist, including CEP-1347 (Maroney et al., 2001) and URMC-099 (Marker et al., 2013). Our data show that both of these MLK inhibitors, built upon different chemical scaffolds, reduce FRA-1 levels in 4T1 cells. In addition, either CEP-1347 treatment or MLK3 deletion reduces FRA-1, MMP-1, and MMP-9 expression to similar levels in multiple TNBC cells, indicating that MLK3, specifically, controls FRA-1 and MMP-1 expression. CEP-1347 progressed through Phase II/III clinical trials for Parkinson's disease, and although it failed to delay progression, no significant toxicity was observed (Parkinson Study Group, 2007), suggesting that it could potentially be repurposed for breast cancer treatment.

In summary, we provide evidence that MLK3 signaling is a crucial regulator of FRA-1 and its target genes, MMP-1 and MMP-9 in models of TNBC. As a consequence, depletion or inhibition of MLK3 in TNBC cells impairs both Matrigel invasion and transendothelial

migration. Consistent with these findings, FRA-1 and MMP-1 are upregulated in our isolated CTC line. Importantly these CTCs retain sensitivity to the MLK inhibitor, CEP-1347. Taken together, our data reveal important roles of MLK3 during basement membrane degradation and transendothelial migration and suggest that MLK3 inhibitors may be a useful addition to the limited armament for combating TNBC.

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CHAPTER III

CONCLUSIONS AND PERSPECTIVES

Metastatic breast cancer is incurable and remains the major cause of death in breast cancer patients. A major issue is the lack of effective therapeutics against this highly complex disease. This dissertation aims to decipher molecular mechanisms underlying the process of metastasis in breast cancer. Specifically, this work focuses on the roles of mixed lineage kinase 3 (MLK3), a protein kinase previously demonstrated by our lab and others to be critical for triple negative breast cancer (TNBC) metastasis. The goal of this dissertation is to dissect the function(s) of MLK3 in discrete steps of the metastatic cascade in the hopes of identifying susceptible windows for therapeutic strategies using an MLK inhibitor.

This investigation has identified the oncogenic transcription factor FOS-related antigen-1 (FRA-1) as a novel downstream effector of MLK3 in breast cancer. In this context, evidence shows that MLK3 functions as a mitogen-activated protein kinase kinase kinase (MAP3K) to activate the MAPKs: c-JUN N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which consequently promote FRA-1 expression at the RNA and protein levels. FRA-1 is a member and component of the activator protein-1 (AP-1) transcription factor family, which controls a large number of genes required for multiple biological processes, including tumor invasion. This work pinpoints two FRA-1 target genes, matrix metalloproteinase-1 (MMP-1) and MMP-9, as MLK3-dependent in a TNBC model. MMP-9 is well-recognized as a protease facilitating basement membrane degradation during local tumor invasion while MMP-1 has recently been described as a protease critical for vascular intravasation. Taken advantage of well-controlled *in vitro* experimental methods for studying cancer invasion and vascular intravasation, experiments described in this thesis demonstrate the requirement of MLK3 in both invasion and transendothelial migration. In addition, these findings are supported by the finding that circulating tumor cells (CTCs) derived from mouse TNBC tumors displayed upregulation of

FRA-1 and MMP-1 transcript levels when compared with parental tumor cells. Furthermore, high levels of MMP-1 mRNA in primary tumors correlate with a greater risk of developing distant metastases and/or metastatic recurrence and poorer overall survival of breast cancer patients. Taken together, these findings point to the importance of MLK3/FRA-1/MMP-1 axis in breast cancer metastasis.

Gene expression reprogramming caused by genetic and/or epigenetic alterations is thought to initiate and facilitate the metastatic cascade. Our data provide strong evidence that MLK3 regulates expression of FRA-1, an oncogenic member of activator protein-1 (AP-1) transcription factor, which has a vast number of target genes. In this study, only two FRA-1 regulated genes: MMP-1 and MMP-9 were examined and shown to depend upon MLK3. MLK3-regulated global gene expression in TNBC is still unknown and could further be explored using a gene profiling approaches such as microarray or RNA-seq. Results from such analyses would provide the opportunity to identify novel downstream effectors of MLK3 signaling and would improve our understanding of the roles of MLK3 in cancer progression.

In this dissertation, the evidence is provided for a requirement for MLK3 in both local invasion and intravasation using well-established *in vitro* experimental approaches. The next critical step is to confirm these findings using an appropriate animal model. However, as most breast cancers arise from the luminal epithelial cells lining the mammary ducts, it is nearly impossible to assess the involvement of MLK3 during basement membrane degradation using a tumor transplant model in which the tumor cells are introduced into mammary fat pad rather than within the mammary ducts. Transgenic animal models that spontaneously develop mammary tumors in the mammary ducts could be considered. Nevertheless, an orthotopic tumor transplant model would be applicable for assessing local invasion of cancer cells into the surrounding

stroma or tissues as well as evaluating tumor intravasation. In a study using control and MLK3-deleted tumor cells, the degree of local invasion could be assessed using immunohistochemistry (IHC) or immunofluorescence (IF) staining on samples that contain the primary tumors as well as parts of adjacent normal tissues. IF staining would also provide the opportunity to analyze the expression and localization of protein markers such as. MLK3, FRA-1, MMP-1, and MMP-9 within control and MLK3-deleted tumor tissue. The extent of tumor vascular intravasation could be evaluated by comparing the difference between the number of CTCs from control tumor-bearing animals and from MLK3-deleted tumor-bearing animals. Notably, as tumor intravasation predominantly occurs near or inside primary tumors where neovasculaturization is present, it is possible that this event could also be captured using IF staining of primary tumor samples.

The metastatic cascade is comprised of multiple highly complex, sequential events including - but not limited to - local invasion, intravasation, survival in the circulation/anoikis, extravasation, and colonization. The role of MLK3 in late stages of the metastatic cascade can also be tested. The experimental metastasis model in which tumor cells are introduced directly into the circulation is the most suitable approach currently-available to study the involvement of MLK3 during the process of extravasation and, to some extent, colonization. Although intravasation and extravasation processes are mechanistically different, these two share common characteristics including the requirement of cancer cells to induce disruption of the endothelial barrier integrity. Cleavage and activation of the endothelial protease-activated receptor-1 (PAR-1) by MMP-1 is critical for vascular intravasation. It is possible that MLK3 could also be required for tumor extravasation. Conceivably MLK3 may signal to MMP-1 to cleave and activate endothelial protease-activated receptor-1 (PAR-1), in a similar fashion as for vascular intravasation, or MLK3 may control other as yet unidentified molecules to facilitate this process.

Nevertheless, the extent of tumor extravasation could, in principle, be assessed by comparing the number of cancer cells that have extravasated out of blood vessels into the surrounding tissue of the target organs, *e.g.* lungs and bones. Note that the interpretation of this result could be limited by the fact that most cancer cells are destroyed by immune cells or other host tissue defenses in the new microenvironment of the metastatic niche; thus the appropriate timing of this experiment would be a crucial factor for performing such an experiment. If MLK3 is not required for extravasation, no difference between tumor extravasation would be expected between control and MLK3-deleted cells. For these studies, the extent of colonization could, in principle, be assessed using *in vivo* imaging techniques, *e.g.* bioluminescence imaging and fluorescence imaging; however, sensitivity of these techniques would likely present a problem. IHC and IF analyses could be performed to analyze tumor cells in lung or other metastatic tissues. If MLK3 is necessary for vascular extravasation, fewer MLK3-deleted tumor cells are expected in the metastasis-targeted tissues when compared to the corresponding tissues from experiments using the control tumor cells. A better approach to study the extent of colonization may be to use an inducible MLK3 knockdown system in which cancer cells are allowed to infiltrate in the target organs prior to induction of MLK3 silencing.

This thesis describes the generation and characterization of the CRISPR-mediated MLK3 knockout 4T1 model. The 4T1 model is a syngeneic mouse tumor model renowned for its highly metastatic features that resemble stage IV human breast cancer. Another advantage of using the syngeneic 4T1 allograft model rather than a human tumor xenograft model is the fact that the allograft model is immune competent. It is well established that cancer cells can be targeted and destroyed through immune surveillance thus suppressing tumor formation and metastasis. However, in some contexts, interactions between tumor cells and immune cells can facilitate

tumor progression. The MLK3 knockout 4T1 model described in this thesis may provide an opportunity to investigate the importance of MLK3 signaling in immune escape mechanisms of cancer cells as well as to assess the involvement of MLK3 signaling in tumor-macrophage interactions.

The MLK inhibitor CEP-1347 has previously been tested in phase II/III clinical trial for Parkinson's disease. Despite the lack of efficacy for this indication, no major toxicity was observed in the patients, making drug repurposing feasible for targeting MLKs in cancer. In this dissertation, the evidence that CEP-1347 effectively blocks the activation of MLK3 downstream targets is demonstrated in multiple *in vitro* models. Given that our data predict the requirement of MLK3 during the early steps of the metastatic cascade, it would be expected that this drug could prevent the metastatic formation in an orthotopic tumor transplant model if administered at the early time point. However, there is no sufficient data available at the moment to predict whether this inhibitor would remain effective in an experimental metastasis model in which local invasion and vascular intravasation are bypassed. In addition, both MLK3 deletion and MLK inhibitor treatment effectively reduce expression of MMP-1, a protease associated with the more aggressive, highly metastatic breast tumors. Thus an MLK inhibitor could be a useful add-on therapeutic option for breast cancer patients, especially those with high levels of MMP-1 who are likely to develop the metastatic disease or the metastatic recurrence, to prevent or reduce the formation of distant metastases.

In conclusion, the work presented in this dissertation thesis demonstrates a novel mechanism through which MLK3 regulates cancer invasion and intravasation of TNBC cells. This new findings provide a mechanistic framework for understanding the contribution and possible targeting of MLK3 in breast cancer metastasis.