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MLK3 regulates paxillin phosphorylation in chemokine-mediated breast cancer cell migration and invasion to drive metastasis

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Precis: Important mechanistic findings identify an MLK3-JNK-paxillin signaling axis as an important potential therapeutic target in metastatic breast cancer.

Keywords: MLK3, JNK, migration, paxillin, FAK, breast cancer, metastasis
Abstract
MLK3 kinase activates multiple MAPKs and plays a critical role in cancer cell migration and invasion. In the tumor microenvironment, pro-metastatic factors drive breast cancer invasion and metastasis, but their associated signaling pathways are not well-known. Here, we provide evidence that MLK3 is required for chemokine (CXCL12)-induced invasion of basal breast cancer cells. We found that MLK3 induced robust phosphorylation of the focal adhesion scaffold paxillin on Ser 178 and Tyr 118, which was blocked by silencing or inhibition of MLK3-JNK. Silencing or inhibition of MLK3, inhibition of JNK, or expression of paxillin S178A all led to enhanced Rho activity, indicating that the MLK3-JNK-paxillin axis limits Rho activity to promote focal adhesion turnover and migration. Consistent with this, MLK3 silencing increased focal adhesions and stress fibers in breast cancer cells. MLK3 silencing also decreased the formation of breast cancer lung metastases in vivo, and breast cancer cells derived from mouse lung metastases showed enhanced Ser 178 paxillin phosphorylation. Taken together, our findings suggest that the MLK3-JNK-paxillin signaling axis may represent a potential therapeutic target and/or prognostic marker in breast cancer metastasis.

Introduction
Recent decreases in breast cancer mortality are primarily due to improved diagnosis and treatment. However, approximately 40,000 deaths annually in the US are due to breast cancer(1), primarily from metastasis to distant organs. Metastasis is a multistep process requiring tumor cell migration, intravasation,
survival in circulation, extravasation and colonization to a secondary site. Interrupting the metastatic process is key to reducing breast cancer mortality.

Chemokines and growth factors drive breast cancer migration, invasion and metastasis. The chemokine, CXCL12/SDF1-α, binds its G-protein-coupled receptor, CXCR4, to promote cytoskeletal remodeling and migration in human breast cancer cells (2); and CXCL12-CXCR4 signaling is critical for breast cancer metastasis in mouse xenograft models (2, 3). High levels of CXCR4 are found in breast tumor cells isolated from pleural effusions (4), and correlate with lymph node metastases (5) and poor overall survival in patients (5, 6). Hepatocyte growth factor/scatter factor (HGF), through binding to its receptor, c-met, promotes cell motility and invasion (7). Aberrant c-met signaling and the MET oncogene are associated with basal breast cancer (8); and c-met overexpression predicts poor outcome in breast cancer patients (9, 10).

MAPK signaling contributes to breast cancer cell migration, invasion, and metastasis. MLK3 is a MAPKKK that regulates the three major MAPK pathways (11). MLK3 contains an N-terminal SH3 domain, followed sequentially by a serine/threonine kinase domain, leucine zippers, a Cdc42/Rac interactive binding (CRIB) motif, and a C-terminal Pro-rich region. MLK3 is autoinhibited through its SH3 domain. Binding of GTP-bound Rac or Cdc42 through MLK3’s CRIB motif disrupts SH3-mediated autoinhibition and promotes zipper-mediated homodimerization, resulting in transautophosphorylation within the kinase domain, yielding the active kinase (12-14). MLK3 regulates cancer cell migration and invasion (15-19). We recently demonstrated that MLK3 signaling through
JNK to the transcription factor AP-1 is required for migration and invasion in human mammary basal epithelial and breast cancer cells. Activation of the MLK3-JNK-AP1 signaling axis induces expression of several breast cancer invasion genes (16).

The mechanics of cancer cell migration involve cytoskeletal remodeling and focal adhesion dynamics (20). Paxillin is a multi-domain adaptor protein which localizes to focal adhesions, the multiprotein complexes that bridge the extracellular matrix and cytoskeleton. The dynamics of focal adhesion assembly and disassembly are controlled by protein interactions and phosphorylation events within the paxillin signaling hub. Paxillin, itself, is phosphorylated at numerous sites (21). Recruitment of FAK to focal adhesions and subsequent tyrosine phosphorylation of paxillin leads to focal adhesion disassembly (21-23). Phosphorylation of paxillin on Ser 178 by JNK is required for focal adhesion disassembly and migration in a basal breast cancer cell line (24).

Small Rho family GTPases include Rac, Cdc42 and Rho (25). Temporal and spatial activation of Rho GTPases is tightly controlled in migrating cells (20). Disrupting the activity cycle of Rho family GTPases results in inefficient cell migration (26). Cdc42 regulates formation of filopodia, while Rac controls formation of lamellipodia. Rho triggers formation of stress fibers and regulates focal adhesion (25). Phosphorylation of paxillin on Tyr 31 and Tyr 118 leads to enhanced Rac activity and decreased RhoA activity (27, 28).

Herein, we demonstrate that MLK3 is required for migration and invasion in response to CXCL12 and both CXCL12 and HGF signal to JNK through MLK3.
We provide evidence that MLK3-activated JNK phosphorylates paxillin on Ser178 which, in turn, recruits FAK to paxillin, resulting in tyrosine phosphorylation. Disruption of the MLK3-JNK-paxillin signaling pathway increases Rho activity, focal adhesions, and stress fibers in basal breast cancer cells. MLK3 silencing decreases formation of breast cancer lung metastases in a mouse xenograft model. In addition, breast cancer cells derived from mouse lung metastases show enhanced Ser 178 paxillin phosphorylation, which can be blocked by an MLK inhibitor. Based on these findings, we propose that targeting MLK3-JNK-paxillin signaling axis may be a useful strategy to combat breast cancer metastasis.

**Materials and Methods**

**Cell lines, Chemicals, Antibodies, DNA constructs and siRNAs**

Human mammary epithelial and breast cancer cell lines were from ATCC. MDA-MB-231-luc2-tdTomato cells were from Caliper Life Sciences. Cell line authentication was performed using STR and amelogenin profiling. The p-paxillin (S178) antibody was from Bethyl Laboratories. All other phospho-antibodies were from Cell Signaling Biotechnology. Other antibody suppliers were Santa Cruz Biotechnology (ERK, JNK and FAK); Sigma (Anti-Flag M2, HA, vinculin and actin), Millipore (paxillin), Abcam (CD44), and Clontech (GFP). MLK3 antibody was homemade or from Epitomics. Recombinant human CXCL12 was from R&D systems. Collagen I and Matrigel were from Becton Dickinson. Pharmacological inhibitors SP600125, U0126 and SB203580 were from...
Calbiochem. CEP-1347 was kindly provided by Cephalon. Flag-MLK3 or MLK3 K144R constructs were described (14). GFP-FAK was a gift from Dr. Jun-Lin Guan (University of Michigan). HA–PaxS178A construct was generated from wild type HA-Pax construct (a gift from Dr. Ravi Salgia, University of Chicago) using site-directed mutagenesis (Stratagene) following manufacturer’s instructions. #1 Mlk3 siRNA (5’-GGGCAGUGACGUCUGGAGUUU-3’) and #2 Mlk3 siRNA (5’-CUGGAGGACUCAAGCAAUG-3’) were from Dharmacon (11, 15). JNK1/2 siRNA (5’-AAAGAAUGUCCUACCUUCU-3’) was from Qiagen (29). AP21967 was provided by Ariad Pharmaceuticals.

**Stable cell populations and transfections**

MCF10A-MLK3 cells (16) were treated with +/- 50 nM AP21967 to induce MLK3 expression. MDA-MB-231 cells expressing pSuper or Mlk3 shRNA have been described (16). Transfection of DNA constructs was performed using Lipofectamine 2000 (Invitrogen). Transfection of siRNA (30-100 nM) was performed using INTERFERin (Polyplus-transfection). 48 h posttransfection, cells were subjected to migration, invasion assays, immunofluorescence or immunoblotting.

**Immunoblotting, co-immunoprecipitations, and Rho GTPase assays**

Preparation of cellular lysates and immunoblotting was as previously described (16). Western blots were developed by chemiluminescence or by fluorescence using LI-COR Odyssey infrared imaging (LI-COR). Co-
immunoprecipitation experiments were performed as described (13). Rho-GTP was measured using the Rhotekin-RBD pulldown assay (Cytoskeleton). Briefly, cells were lysed in ice-cold Triton X-100 lysis buffer and cleared cellular extracts (500 μg) were incubated with Rhotekin-RBD agarose beads (10 μg). Beads were pelleted, washed and resuspended in 1.5x SDS sample buffer. GTP-bound Rho was detected by immunoblotting.

**Migration and invasion assays**

Chemotactic migration was quantified using a Boyden chamber transwell assay as described (16). The chemoattractant was 100 ng/ml CXCL12. For invasion assays, chambers were coated with Matrigel (1:5 dilution in DMEM/F12).

**Immunofluorescence**

Formaldehyde-fixed cells were permeabilized with 0.5% Triton X-100, blocked in 4% BSA, and stained with anti-vinculin antibody (1:200 dilution), followed by Alexa Fluor-488-conjugated anti-mouse IgG (1:200 dilution). To visualize stress fibers, cells were stained with Alexa Fluor-546-conjugated phalloidin (1:50, Invitrogen); and nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml). Images were acquired using an Olympus FV1000 confocal laser scanning microscope. Focal adhesions were quantified using Image J software.

**Spontaneous metastasis model**
All experiments involving animals were performed in accordance with standard protocols approved by All University Committee on Animal Use and Care at MSU. Female athymic nu/nu mice (6 week old, Harlan Laboratory) were maintained in microisolation cages under specific pathogen-free conditions. MDA-MB-231 cells-pSuper or -shMlk3 (2x10^6 cells/site) were resuspended in PBS and Matrigel (1:1 v/v) and surgically inoculated bilaterally into No. 4 mammary glands. Tumors were measured using a caliper twice weekly. Mice were euthanized after 7 weeks, primary tumors were excised and lysed using RIPA buffer. Mouse lungs were fixed in formalin overnight and paraffin sections were analyzed using anti-CD44 specific human antibody and Vectastain Elite ABC kits and DAB Substrate (Vector laboratory). Metastatic nodules were quantified in 10 lung sections per mouse and statistical analysis was performed using GraphPad Prism 5.

**Experimental metastasis model**

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

**Results**
MLK3 is required for migration and invasion of basal breast cancer cells towards CXCL12

CXCL12 is critical in breast cancer cell migration and metastasis (3, 30). The highly invasive, basal-like breast cancer cell lines, MDA-MB-231 and BT549, both express high levels of CXCR4 (30). In a transwell migration assay, CXCL12 increased migration of MDA-MB-231 cells expressing control vector by approximately 3-fold. MLK3 expression is efficiently ablated in a stable population of MDA-MB-231 cells (16). CXCL12-induced migration was completely blocked in MDA-MB-231 cells stably expressing shMlk3 or treated with CEP-1347, a selective MLK inhibitor (Figure 1A). As shown in Figure 1A, CXCL12 activated JNK. To confirm the efficacy of CEP-1347, we used phospho-JNK (p-JNK) as a readout for active MLK signaling. Immunoblotting using a p-JNK antibody showed that CEP-1347 blocked CXCL12-induced JNK activation (Figure 1A). In addition, transient silencing of MLK3 in BT549 cells reduced CXCL12-induced migration (Figure 1B).

In a Matrigel invasion assay using CXCL12 as a chemoattractant, CEP-1347 reduced invasion of MDA-MB-231 cells by approximately 5-fold (Figure 1C). In addition, CEP-1347 blocked invasion of BT549 cells (Figure 1D). Silencing of MLK3 in BT549 cells also largely inhibited invasion (Figure 1E). Mlk3 silencing had negligible effect on proliferation of BT549 cells (Supplementary figure S1). Taken together, these results support the idea that MLK3 signaling is required for migration and invasion of invasive basal breast cancer cells in response to CXCL12.
Active MLK3 promotes phosphorylation of paxillin through JNK

We recently showed that induced expression of MLK3 promotes migration and invasion of MCF10A mammary epithelial cells, which requires JNK-AP1 (16). We hypothesized that, in addition to its impact on gene expression, MLK3-JNK signaling might act upon cytoskeleton or focal adhesions to regulate cell migration and invasion. Phosphorylation of Ser 178 of paxillin by JNK is necessary for focal adhesion turnover and cell migration (24).

To investigate whether MLK3 can promote phosphorylation of paxillin, MCF10A cells engineered to inducibly express MLK3 were used (16). Upon MLK3 induction, JNK was activated and robust Ser 178 phosphorylation of paxillin was observed. Inhibition of MLKs with K252a, or of JNK with SP600125, blocked phosphorylation of paxillin at Ser 178. In contrast, inhibition of ERK signaling with U0126, or p38 with SB203580, had no effect on MLK3-induced paxillin phosphorylation (Figure 2A). Like K252a, its derivative CEP-1347 blocked MLK3-induced paxillin phosphorylation (Supplementary figure S2). In MDA-MB-231 cells, transient expression of wildtype MLK3, but not kinase inactive, MLK3 K144R, induced JNK activation and Ser 178 phosphorylation of paxillin (Figure 2B). Furthermore, silencing of JNK1/2 in MDA-MB-231 cells decreased Ser 178 phosphorylation of paxillin (Figure 2C), confirming the requirement for JNK in paxillin phosphorylation. Thus the MLK3-JNK signaling axis promotes paxillin phosphorylation at Ser 178.
Chemokine and growth factor induce paxillin phosphorylation in an MLK3-dependent manner

To determine whether MLK3 is required for phosphorylation of paxillin at Ser 178, serum-deprived MDA-MB-231 cells stably expressing control or shMlk3 vector were treated with 10% serum and paxillin phosphorylation was assessed. Serum treatment led to JNK activation and maximal phosphorylation of paxillin at Ser 178 at 30 min, both of which were largely abrogated in cells expressing shMlk3 (Figure 3A), suggesting MLK3 is a major mediator of JNK signaling to paxillin. Since phosphorylation of paxillin on Ser 178 is associated with breast cancer migration, we investigated the impact of CXCL12 and HGF on paxillin phosphorylation. Both factors induced JNK activation and phosphorylation of paxillin at Ser 178 in MDA-MB-231 cells, which was reduced by CEP-1347 (Figure 3B).

Likewise, in BT549 cells, based on 4 independent experiments, both CXCL12 and HGF induced JNK activation (3 and 3.4-fold, respectively) and paxillin phosphorylation at Ser 178 (3.2 and 4.4-fold, respectively), which was attenuated by silencing with Mlk3 siRNA (Figure 3C). MLK inhibition with CEP-1347 also reduced both JNK activation and Ser 178 paxillin phosphorylation (Figure 3D). The requirement of MLK3 in paxillin phosphorylation was confirmed using a different siRNA sequence (Supplementary figure S3 A and B). In BT549 cells, HGF potently activated ERK, whereas only a small increase in ERK activation was observed in response to CXCL12. ERK activation was refractory to the MLK inhibitor, consistent with the proposed scaffolding role of MLK3 in
ERK activation (11). Since MDA-MB-231 cells harbor activating mutations in both Ras and Raf (31), resulting in constitutive ERK activation, it is not too surprising that CXCL12 and HGF have relatively little effect on ERK activity in these cells. These data demonstrate a requirement for active MLK3 in JNK activation and Ser 178 paxillin phosphorylation, triggered by either a prometastatic chemokine or growth factor in basal breast cancer cells.

**MLK3 controls Tyr 118 phosphorylation of paxillin and its association with focal adhesion kinase**

Cell migration requires efficient assembly and disassembly of focal adhesion complexes. Paxillin undergoes phosphorylation at multiple sites to modulate protein-protein interactions in focal adhesions (21). The role of Ser 178 phosphorylation of paxillin is not completely clear. In corneal epithelial cells, Ser 178 phosphorylation of paxillin recruits FAK to mediate tyrosine phosphorylation of paxillin(32).

Since MLK3 controls Ser 178 phosphorylation of paxillin, we tested whether MLK3 indirectly modulates tyrosine phosphorylation of paxillin and regulates interactions among focal adhesion proteins. Ectopic expression of MLK3 in BT549 cells promoted both Ser 178 and Tyr 118 paxillin phosphorylation, demonstrating that active MLK3 drives Tyr 118 phosphorylation of paxillin (Figure 4A). In response to serum stimulation in MDA-MB-231 cells, CEP-1347 reduced Tyr 118 phosphorylation of paxillin by 2-fold at 5 min and 3.5-fold at 20 min, paralleling the effects of JNK inhibition (Figure 4B). Consistently, HGF-induced
Tyr 118 phosphorylation of paxillin was dramatically reduced in MDA-MB-231 cells expressing shMlk3 (Figure 4C). From these data we conclude that MLK3 is critical for Tyr 118 phosphorylation of paxillin.

To test whether MLK3 influences association of paxillin with FAK, we took advantage of 293T cells as an efficient cotransfection system. Ectopically expressed GFP-FAK and HA-paxillin showed weak association in coimmunoprecipitations from serum-deprived 293T cells (Figure 4D). EGF has previously been shown to facilitate the interaction between FAK and paxillin in 293T cells (32). Our data confirm that the association between GFP-FAK and HA-paxillin is enhanced by EGF (Figure 4D). However, pre-treatment with CEP-1347 abrogated the EGF-induced association of GFP-FAK and HA-paxillin (Figure 4D). We were able to detect endogenous paxillin in a FAK immunoprecipitate from MDA-MB-231 cells in growth medium, which was reduced by CEP-1347. In the immunoprecipitated FAK complex, levels of Ser 178 phosphorylated paxillin and total paxillin correlate directly, consistent with the idea that Ser 178 phosphorylation drives association of FAK with paxillin (Figure 4E). Conversely, forced expression of active Flag-MLK3 in MDA-MB-231 cells increased interaction of endogenous paxillin and FAK as well as phosphorylation of paxillin at both Ser 178 and Tyr 118 (Figure 4F). These data provide strong evidence that MLK3 regulates both paxillin phosphorylation and FAK-paxillin interactions.

**MLK3 silencing increases the number of focal adhesions**
MLK3 modulates phosphorylation of Ser 178 and Tyr 118 of paxillin, which is required for focal adhesion disassembly (23, 24). Consistent with this, silencing of MLK3 in BT549 cells increased focal adhesions, which were quantified as vinculin-staining focal adhesions, particularly at the cell periphery (Figure 5 A and B). Similar effects were observed using two different Mlk3 siRNA sequences. Silencing of MLK3 had no effect on total vinculin protein levels (Figure 5 C). These data suggest that MLK3 is important for focal adhesion turnover.

**MLK3-JNK-paxillin signaling negatively regulates Rho activity**

Tyr 118 phosphorylation of paxillin leads to decreased Rho activity, enhancing focal adhesion turnover and cell migration (28). Ectopic expression of MLK3 and wildtype paxillin in 293T cells resulted in robust Ser 178 paxillin phosphorylation. As expected, no phospho-Ser 178-paxillin signal was detected upon coexpression of the phosphorylation-defective mutant, paxillin S178A with MLK3 (Supplementary figure S4A). In 293T cells, expressing control vector or wildtype paxillin, Tyr 118 phosphorylation of paxillin was observed. However, the paxillin mutant S178A was refractory to serum-induced Tyr 118 phosphorylation, suggesting that Ser178 phosphorylation of paxillin is a prerequisite to Tyr 118 phosphorylation (Supplementary figure S4B). Likewise, in BT549 cells, ectopically expressed wildtype paxillin, but not paxillin S178A, was phosphorylated on Tyr 118 (Figure 6A).
Increased cellular stress fibers are observed in keratinocytes upon expression of the paxillin S178A mutant (24). As shown in Figure 6B, a similar phenotype was observed upon silencing of MLK3 in BT549 breast cancer cells. Since Rho promotes stress fiber formation, we assessed whether interruption of MLK3 and its signaling to JNK affects Rho activity in breast cancer cells. In both MDA-MB-231 and BT549 cells, Rho activity was increased upon silencing of MLK3 (Figure 6C). Furthermore, inhibition of MLK3, using CEP-1347, as well as inhibition of downstream signaling to JNK with SP600125, increased Rho activity in MDA-MB-231 cells (Figure 6D, E).

If MLK3-JNK suppresses Rho activity through Ser 178 phosphorylation of paxillin, then expression of the paxillin S178A mutant should enhance Rho activity. As shown in Figure 6F, expression of paxillin S178A, which fails to undergo Tyr 118 phosphorylation, resulted in a marked increase of Rho activity and decreased migration of MDA-MB-231 cells (Figure 6G). These data reveal the MLK3-JNK-paxillin signaling axis as a negative modulator of Rho activity in basal breast cancer cells.

**MLK3 silencing decreases formation of lung metastases of human breast cancer cells**

To determine whether silencing of MLK3 is sufficient to prevent metastases, MDA-MB-231 cells stably expressing control vector or Mlk3 shRNA were introduced into the mammary gland of athymic nude mice. Both MDA-MB-231-control and -shMlk3 formed primary tumors. MDA-MB-231-control tumors
were slightly larger than MDA-MB-231-shMlk3 tumors, but this did not reach statistical significance (Figure 7A). Seven weeks post-inoculation, primary tumors were excised. MLK3 silencing was maintained in primary tumors over the course of the experiment as shown in immunoblots of tumor lysates (Supplementary figure S5).

Lung micrometastases were detected by immunohistochemistry of lung sections using a human-specific CD44 antibody. Numerous micrometastases were observed in lung sections of mice inoculated with MDA-MB-231-control cells, whereas few were found in those arising from MDA-MB-231-shMlk3 cells (Figure 7B). Lung and liver tissue lysates from nude mice showed no human CD44 immunoreactivity, confirming species specificity of the antibody. Furthermore, silencing of MLK3 in MDA-MB-231 cells had no impact on CD44 protein levels (Supplementary figure S6). These data provide evidence for a critical role of MLK3 in breast cancer metastasis.

**Ser 178 phosphorylation of paxillin is associated with the metastatic phenotype**

In this study we have shown that prometastatic factors signal through MLK3-JNK to promote Ser 178 phosphorylation of paxillin (Figure 7C) and migration/invasion. Using an experimental metastasis model, MDA-MB-231 cells expressing luciferase (luc2) were injected into the tail vein of nude mice. Bioluminescence imaging revealed overt metastases 12 weeks post injection. Tumor cells isolated from lung metastases (Lu) show higher paxillin
phosphorylation on Ser 178 compared with parental MDA-MB-231 cells (Pa), both basally as well as in response to either CXCL12 or HGF (Figure 7D). Furthermore, CEP-1347 inhibits both CXCL12- and HGF-induced Ser 178 phosphorylation of paxillin in Lu cells (Figure 7E), indicating these cells are still sensitive to an MLK inhibitor.

Screening of a panel of human mammary epithelial and breast cancer cell lines revealed a correlation between p-Ser 178 paxillin and metastatic potential (Supplementary figure S7). These data, taken together, suggest that phosphorylation of paxillin on Ser 178 may be a predictor of lung metastatic potential.

Discussion

Deciphering key signaling pathways underlying breast cancer cell migration and invasion may reveal novel therapeutic targets for effectively treating or preventing metastatic breast cancer. We previously demonstrated MLK3-JNK signaling upregulates multiple AP-1-driven invasion genes and promotes a malignant phenotype in mammary epithelial cells (16). JNK is important in breast cancer cell migration and invasion, and breast cancer progression (24, 33, 34). Yet, how MLK3-JNK signaling regulates cell migration machinery remains largely unknown.

In this study, we report, for the first time, that in response to CXCL12 and HGF, MLK3 signals to JNK to control phosphorylation of paxillin on both Ser 178 and Tyr 118 (Figures 2-4), phosphorylation events that are essential in cell
migration (24, 35). MLK3 modulates interactions between two key focal adhesion proteins, paxillin and FAK (Figure 4). MLK3-JNK-paxillin signaling negatively regulates Rho activity to promote focal adhesion turnover in cell migration (Figures 5, 6). Finally, MLK3 is critical for formation of breast cancer lung micrometastases in a mouse xenograft model (Figure 7). The importance of paxillin phosphorylation is highlighted by the finding that cells derived from MDA-MB-231 lung metastases show higher phosphorylation of paxillin at Ser 178, compared with parental MDA-MB-231 cells, implicating this phosphorylation site in breast cancer metastasis. Taken altogether, our data reveal a novel MLK3-JNK-paxillin signaling pathway that regulates breast cancer cell migration and invasion.

Paxillin undergoes dynamic phosphorylation during cell migration (21). Ser 178 phosphorylation of paxillin is essential for cell migration (24). We show both CXCL12 and HGF signal through MLK3 to paxillin, consistent with our data showing that MLK3 is required for CXCL12-induced breast cancer cell migration (Figures 1-3). Thus, MLK3 emerges as an important signal node that relays extracellular cues to JNK to control paxillin phosphorylation. While it is possible that other MAPKKKs contribute to paxillin phosphorylation through JNK, MLK3 appears to play a dominant role, at least in basal breast cancer cells. Because CXCL12 and HGF are consistently linked with invasion and metastasis, our findings provide a strong rationale for targeting MLK3 in the context of breast cancer metastasis. In agreement with our findings, localized JNK activation and Ser 178 phosphorylation of paxillin is observed during migration of rat kidney
epithelial cells, which involves the aPKC-Exocyst complex (36). Interestingly, PKC is important for activation of MLK3 in response to free fatty acids (37). Whether PKC plays a role in CXCL12- or HGF- induced MLK3 activation remains to be determined.

Rapid assembly and disassembly of focal adhesions is a well-described property of many migrating cancer cells. Experimental disruption of focal adhesion turnover typically results in migratory defects in cancer cells (38). Phosphorylation of paxillin at Ser 178 by JNK (24) and Tyr 118 by FAK/Src (39, 40) is critical for focal adhesion turnover and cell migration (24, 35, 41, 42). For instance, a tyrosine phosphomimetic mutant of paxillin enhances focal adhesion turnover, whereas a non-phosphorylatable mutant shows defective focal adhesion turnover and migration (22, 23). Furthermore, phosphorylation of Tyr 118 on paxillin is implicated in cancer invasion and metastasis (43). Our results support a model in which MLK3 is required for focal adhesion turnover in cell migration through controlling Tyr 118 phosphorylation of paxillin (Figure 4). Indeed, MLK3 silencing increases the number of focal adhesions in breast cancer cells (Figure 5). Furthermore, experiments using a nocodazole-based assay (44), in which nocodazole washout promotes microtubule formation and focal adhesion turnover, reveal a defect in focal adhesion disassembly upon MLK3 silencing in MDA-MB-231 cells (data not shown), supporting the necessity of MLK3 in focal adhesion turnover.

Elevated levels and activity of FAK are found in high grade human cancers, including breast cancer, and correlate with invasive phenotypes,
metastatic disease and poor prognosis (45). FAK inhibitors are currently in clinical trials for treating human solid tumors (46). Activated FAK recruits Src to form an active FAK/Src complex. Our data showing that MLK3 promotes interaction of FAK with paxillin may explain how MLK3 controls Tyr 118 phosphorylation of paxillin and promotes focal adhesion turnover, since association of FAK with paxillin promotes tyrosine phosphorylation of paxillin and is correlated with less stable focal adhesions (47). Ablation of the Ser 178 phosphorylation site on paxillin decreased phosphorylation of Tyr 118 (Figure 6), suggesting that, at least in this experimental context, Ser 178 phosphorylation is a prerequisite for Tyr 118 phosphorylation. This is in agreement with the finding that paxillin S178A has decreased affinity for FAK (32). In our working model, MLK3-JNK-Ser 178 paxillin phosphorylation regulates association of FAK with paxillin and indirectly controls subsequent tyrosine phosphorylation of paxillin (Figure 7C).

Focal adhesion dynamics are tightly controlled by Rho GTPases (20, 25). Active Rho increases stress fibers and focal adhesion maturation and decreases focal adhesion turnover (48). While Rho activity is required for cell migration, aberrantly high Rho activity also impairs cell migration (26). FAK promotes focal adhesion turnover, in part, through suppression of Rho activity (48). In particular, FAK/Src-mediated Tyr 118 phosphorylation of paxillin has been proposed to release p190 Rho-GAP from its sequestration with Ras-GAP, leading to downregulation of Rho activity (28). Our experimental evidence supports a model in which the MLK3-JNK-paxillin Ser 178 signaling axis negatively regulates Rho
activity, through FAK-mediated tyrosine phosphorylation of paxillin. Indeed, MLK3 silencing in breast cancer cells resulted in increased focal adhesions (Figure 5) and stress fibers (Figure 6), two Rho-associated phenotypes. In A549 lung carcinoma cells, a role for MLK3 in limiting Rho activity through an interaction with p63 RhoGEF has also been described (17). In summary, we have identified in basal breast cancer cells, a distinct pathway involving an active MLK3-JNK-paxillin axis that functions to negatively control Rho activity.

In a xenograft model in which MDA-MB-231 cells were introduced into mammary fat pad of nude mice (49), MLK3 silencing inhibited spontaneous lung micrometastases (Figure 7), in agreement with findings that MLK3 knockdown reduced lymph node metastases of MDA-MB-231 cells (50). Although MLK3 has been shown to promote cell survival (16, 50), which might contribute to formation of metastases, we propose that an important mechanism through which MLK3 promotes metastasis is through facilitating cancer cell migration and invasion.

Our study demonstrates that MLK3 controls Ser 178 phosphorylation of paxillin, which is required for cell migration. Interestingly, phospho-Ser 178 paxillin correlates with metastatic potential of breast cancer cells, suggesting that phospho-Ser 178 paxillin might be a predictive biomarker for metastasis. Our novel findings demonstrate that prometastatic factors found in the tumor microenvironment converge on MLK3 to promote breast cancer cell migration and invasion. Since the MLK inhibitor, CEP-1347, efficaciously blocks invasion in response to such factors, we are currently testing the effect of this compound in a preclinical study using a mouse xenograft model. Our findings indicate that
MLK3 regulates phosphorylation of paxillin and its interaction with FAK. We also provide evidence that the MLK3-JNK-paxillin axis negatively regulates Rho activity and focal adhesion turnover. Finally, we demonstrate a critical role of MLK3 in breast cancer metastasis. Thus, targeting MLK3 could be a promising therapeutic strategy for treatment or prevention of metastatic disease in breast cancer.

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**Figure Legends**

Figure 1. Silencing or inhibition of MLK3 blocks CXCL12-induced migration and invasion. (A) Serum-deprived MDA-MB-231-pSuper or –pSuper-shMLk3 were treated
with CXCL12 (100 ng/ml) +/- CEP-1347 (400 nM) for 30 min. Total cellular lysates were analyzed by immunoblotting using indicated antibodies (upper panel). MDA-MB-231-pSuper or -pSuper-shMlk3 were allowed to migrate towards CXCL12 (100 ng/ml) for 24 h. Migrated cells were quantified as described (16). Column, mean of three experiments. Bar, SE. (B) BT549 cells were treated with control or Mlk3 siRNA for 48 h, serum-deprived and allowed to migrate towards CXCL12 (100 ng/ml) for 48 h. (C) MDA-MB-231 cells, pretreated +/-400 nM CEP-1347 for 6 h in serum-free medium, were allowed to invade towards CXCL12 (100 ng/ml) for 24 h. (D) BT549 cells were pretreated +/- 400 nM CEP-1347 and CXCL12-induced transwell invasion was determined as in (B) . (E) BT549 cells were treated as in (B) and subjected to a transwell invasion assay. Column, mean of four experiments. Bar, SE.

Figure 2. MLK3-JNK signaling promotes phosphorylation of paxillin at Ser 178. (A) MCF10A-MLK3 cells were treated +/-50 nM AP21967 for 20 h with indicated inhibitors: 15 μM SP600125, 10 μM U0126, 10 μM SB203580 or 400 nM K252a, for an additional 24 h. Cellular lysates were analyzed by western-blotting. (B) MDA-MB-231 cells were transfected wildtype or MLK3 K144R vector for 24 h. Cellular lysates were analyzed by western-blotting. (C) MDA-MB-231 cells were treated with control or JNK1,2 siRNA for 48 h. Cellular lysates were analyzed immunoblotting. Quantitation of blots normalized to actin was performed using LI-COR Odyssey software V3.0.

Figure 3. MLK3 silencing or an MLK inhibitor impairs paxillin phosphorylation at Ser 178. (A) Serum-deprived MDA-MB-231-pSuper or pSuper-shMlk3 were treated with 10% serum for indicated times. Total cellular lysates were analyzed by immunoblotting. Quantitation of p-JNK/JNK determined by LI-COR Odyssey software V3.0 is shown. (B) Serum-deprived MDA-MB-231 cells treated +/-CEP-1347 (400 nM) for 6 h, followed by CXCL12 (100 ng/ml) or HGF (100 ng/ml) for 30 min. (C) BT549 cells were transfected with control or Mlk3 siRNA and treated for 30 min with 100 ng/ml CXCL12 or HGF. (D) Serum-deprived BT549 cells were treated +/- CEP-1347 (400 nM) for 6 h, followed by 100 ng/ml CXCL12 or HGF for 30 min.

Figure 4. MLK3 promotes Tyr 118 phosphorylation of paxillin and interaction of FAK with paxillin. (A) Immunoblots of cellular lysates from BT549 cells transfected with control or Flag-Mlk3 vector are shown. (B) Serum-deprived MDA-MB-231 cells were treated +/-400 nM CEP-1347 for 6 h followed by 10% serum for indicated times. Immunoblots are shown. (C) Serum-deprived MDA-MB-231-pSuper or pSuper-shMlk3 were treated with 100 ng/ml HGF. (D) 293T cells were cotransfected with indicated constructs, serum deprived, and treated +/- CEP-1347 followed by 100ng/ml EGF (100 ng/ml) for 30 min. Lysates were immunoprecipitated using HA antibody and subjected to western-blotting. (E) After overnight treatment of MDA-MB-231 cells +/-CEP-1347, lysates were immunoprecipitated using control IgG or FAK antibody, followed by western-blotting. Ratios of relative intensities of FAK to paxillin with control (=1) are shown. Column, mean of three experiments. Bar, SE. (F) Immunoprecipitation and immunoblots from MDA-MB-231 cells transfected with control or Flag-MLK3 vector. FAK-paxillin association was quantified as in (E).
Figure 5. MLK3 knockdown increases vinculin-containing focal adhesions. (A) BT549 cells were transfected with control or two different Mlk3 siRNAs for 48 h. Fixed cells were stained with vinculin antibody and DAPI. Images were taken using Olympus FluoView confocal microscope. Bar, 50 μm. (B) Vinculin-positive focal adhesions were quantified from over 20 cells per group using Image J software. Column, mean of two experiments. Bar, SE. (C) Immunoblots corresponding to (A).

Figure 6. MLK3-JNK-mediated Ser 178 phosphorylation of paxillin is necessary for Tyr 118 phosphorylation of paxillin and inhibits Rho activity. (A) Immunoblots from BT549 cells transiently expressing HA-paxillin wt or HA-paxillin S178A mutant after serum-deprivation and treatment with 10% serum for 30 min. (B) BT549 cells were treated with control or Mlk3 siRNA for 48 h, stained with phalloidin (F-actin) and DAPI (nucleus) and imaged using confocal microscopy. Bar, 50 μm. (C) Immunoblots from Rhotekin pulldown assay of MDA-MB-231-pSuper or -pSuper-Mlk3 (left panel) and BT549 cells treated with control or Mlk3 siRNA (right panel). (D) Immunoblots from Rhotekin pulldown assay of MDA-MB-231 cells treated -/+ CEP-1347 (E) Immunoblots from Rhotekin pulldown assay of MDA-MB-231 cells treated -/+ SP600125 for 6 h. (F) Rhotekin pulldown assay and immunoblots from MDA-MB-231 cells expressing HA-paxillin (Wt) or HA-paxillin S178A mutant. (G) Transwell migration assay of cells from (F) was performed with corresponding immunoblots shown.

Figure 7. Depletion of MLK3 prevents formation of lung metastases. (A) Tumor growth curve of MDA-MB-231-pSuper or -pSuper-shMlk3 inoculated into mouse mammary fat pads. (B) Immunohistochemistry of lung sections using a human-specific CD44 antibody. Magnification, 400x. CD44-positive nodules were quantified in 10 sections per mouse. Statistical analysis was done using GraphPad Prism 5. (C) Schematic model showing MLK3-JNK-pSer 178 paxillin signaling axis, activated through prometastatic factors CXCL12 and HGF, leading to FAK-mediated Tyr118 phosphorylation of paxillin, and suppression of Rho activity. (D) Cultured cells derived from lung metastases of MDA-MB-231-Luc2-tdTomato were designated as Lu. Immunoblots from serum-deprived parental MDA-MB-231-Luc2-tdTomato cells (designated as Pa) and Lu cells treated with CXCL12 or HGF for 20 min. (E) Serum-deprived Lu cells treated -/+CEP-1347 overnight, and treated with CXCL12 or HGF as in (D).
Figure 1

Relative invasive activity (%)

MDA-MB-231

Relative migratory activity (%)

CXCL12

Relative invasive activity (%)

shMK3

Relative migratory activity (%)

siCont

siMlk3

MDA-MB-231 BT549

CXCL12

MDA-MB-231

BT549

actin

JNK

pJNK

CEP-1347

pSuper

CEP-1347

siCont

siMlk3

American Association for Cancer Research
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Figure 2

A  MCF10A-MLK3
   AP21967
   - - SP U SB k252a
   MLK3
   p-JNK
   JNK
   p-paxi S178
   paxillin
   actin

B  MDA-MB-231
   vec wt k144r mkl3
   MLK3
   p-MLK3
   p-paxi S178
   paxillin
   p-JNK
   actin

C  MDA-MB-231
   con jnk siRNA
   JNK
   p-paxi S178
   paxillin
   actin
Figure 3

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Figure 4

A. BT549
- vec
- Mlk3

MLK3
p-paxi Y118
p-paxi S178
paxillin

B. MDA-MB-231

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C. MDA-MB-231
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E. MDA-MB-231
- - CEP-1347

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F. MDA-MB-231
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Relative intensity in Paxillin/FAK binding
-vec CEP1347
Figure 5

(A) Images showing cell morphology after siRNA treatment with siCont, siMlk3 #1, and siMlk3 #2.

(B) Bar graph showing the number of focal adhesions per cell. siMlk3 treatments result in a decrease in focal adhesions compared to siCont.

(C) Western blot analysis of BT549 cells showing reduced levels of MLK3 and Vinculin with siMlk3 treatments.
Figure 6

A. BT549

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B. siCont vs siMlk3

C. MDA-MB-231

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![Graph showing relative migratory activity](https://example.com/graph.png)
Figure 7

A

MDA-MB-231

Tumor volume (mm^3)

Days

0

10

17

24

31

39

49

control

shMlk3

B

pSuper

shMlk3

p=0.0065

Number of metastatic nodules/mouse

C

CXCL12

CXCR4

HGF

MET

MLK3

MLK3

FAK

JNK

p-paxi Ser178

+ + +

+ + +

+ + +

CEP-1347

p-paxi Ser178

+ + +

+ + +

+ + +

actin

paxillin

D

MDA-MB-231-Luc2-tdTomato

- CXCL12 HGF

Pa Lu Pa Lu Pa Lu

p-paxi Ser178

paxillin

actin

E

MDA-MB-231-Luc2-tdTomato-Lu

- CXCL12 HGF CEP-1347

- + - + - +

p-paxi Ser178

paxillin