MLK3 Regulates Paxillin Phosphorylation in Chemokine-Mediated Breast Cancer Cell Migration and Invasion to Drive Metastasis

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Abstract
MLK3 kinase activates multiple mitogen-activated protein kinases and plays a critical role in cancer cell migration and invasion. In the tumor microenvironment, prometastatic factors drive breast cancer invasion and metastasis, but their associated signaling pathways are not well-known. 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. Cancer Res; 72(16); 4130–40. ©2012 AACR.

Introduction
Recent decreases in breast cancer mortality are primarily because of improved diagnosis and treatment. However, approximately 40,000 deaths annually in the United States 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, 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).

Mitogen-activated protein kinase (MAPK) signaling contributes to breast cancer cell migration, invasion, and metastasis. MLK3 is a MAPKKK that regulates the 3 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 proline-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 showed 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 multidomain adaptor protein that 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

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-12-0655
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Published OnlineFirst June 13, 2012; DOI: 10.1158/0008-5472.CAN-12-0655

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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, whereas Rac controls formation of lamellipodia. Rho triggers formation of stress fibers and regulates focal adhesions (25). Phosphorylation of paxillin on Tyr 31 and Tyr 118 leads to enhanced Rac activity and decreased RhoA activity (27, 28).

Herein, we show that MLK3 is required for 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 that, 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. On the basis of these findings, we propose that targeting the 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 American Type Culture Collection. MDA-MB-231-luc2-tdTomato cells were from Caliper Life Sciences. Cell line authentication was carried out using short tandem repeat and amelogenin profiling. The p-paxillin (S178) antibody was from Bethyl Laboratories. All other phosphoantibodies 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 or from Epitomics. Recombinant human CXCL12 was from R&D systems. Collagen I and Matrigel were from Becton Dickinson. Pharmacologic inhibitors SP600125, U0126, and SB203580 were from Calbiochem. CEP-1347 was kindly provided by Cephalon, Inc., a wholly-owned, indirect subsidiary of Teva Pharmaceutical Industries Ltd. Flag-MLK3 or MLK3 K144R constructs were described (14). GFP-FAK was a gift from Dr. Jun-Lin Guan (University of Michigan, Ann Arbor, MI). HA–PaxS178A construct was generated from wild-type HA-Pax construct (a gift from Dr. Ravi Salgia, University of Chicago, Chicago, IL) using site-directed mutagenesis (Stratagene) following manufacturer’s instructions. #1 MLK3 siRNA (5′-GGGCGAGUAGAUAGUAGUU-3′) and #2 MLK3 siRNA (5′-CUUGAGGAGCUAAGCAUG-3′) were from Dharmacon (11, 15). JNK1/2 siRNA (5′-AAAGAAUGCUACCUCU-3′) was from Qiagen (29). AP21967 was provided by Ariad Pharmaceuticals.

Stable cell populations and transfections

MCF10A-MLK3 cells (16) were treated with ± 50 nmol/L AP21967 to induce MLK3 expression. MDA-MB-231 cells expressing pSuper or MLK3 short hairpin RNA (shRNA) have been described (16). Transfection of DNA constructs was carried out using Lipofectamine 2000 (Invitrogen). Transfection of siRNA (30–100 nmol/L) was carried out using INTERFERin (Polyplus transfection). Forty-eight hours posttransfection, cells were subjected to migration, invasion assays, immunofluorescence, or immunoblotting.

Immunoblotting, coimmunoprecipitations, 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). Coimmunoprecipitation experiments were carried out 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 1.5× 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 chemotractant 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% bovine serum albumin, 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 ImageJ software.

Spontaneous metastasis model

All experiments involving animals were carried out in accordance with standard protocols approved by All University Committee on Animal Use and Care at Michigan State University. Female athymic nu/nu mice (6-week-old; Harlan Laboratory) were maintained in microisolation cages under specific pathogen-free conditions. MDA-MB-231 cells-pSuper or -shMLK3 (2 × 10⁶ cells/site) were subcutaneously injected into SCID mice. Tumors were measured using a caliper twice weekly. Mice were euthanized after 7 weeks, primary tumors were excised and lysed using radioimmunoprecipitation...
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 carried out 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 intraperitoneal 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 toward 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 (Fig. 1A). As shown in Fig. 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 (Fig. 1A). In addition, transient silencing of MLK3 in BT549 cells reduced CXCL12-induced migration (Fig. 1B).

In a Matrigel invasion assay using CXCL12 as a chemoattractant, CEP-1347 reduced invasion of MDA-MB-231 cells by approximately 5-fold (Fig. 1C). In addition, CEP-1347 blocked invasion of BT549 cells (Fig. 1D). Silencing of MLK3 in BT549 cells also largely inhibited invasion (Fig. 1E). MLK3 silencing had negligible effect on proliferation of BT549 cells (Supplementary Fig. S1). Taken together, these results supported 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 (3.2-fold, respectively), which was attenuated by silencing with MLK3 siRNA (Fig. 3A), suggesting MLK3 is a major mediator of JNK signaling to paxillin. As 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 (Fig. 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 (Fig. 3C). MLK inhibition with CEP-1347 also reduced both JNK activation and Ser 178 paxillin phosphorylation (Fig. 3D). The requirement of MLK3 in paxillin phosphorylation was confirmed using a different siRNA sequence (Supplementary Fig. S3A 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). Because 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 showed 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 Tyr 118, which is involved in its association with focal adhesion kinase through its association with focal adhesion kinase.
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).

As 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, showing that active MLK3 drives Tyr 118 phosphorylation of paxillin (Fig. 4A). In response to serum stimulation in MDA-MB-231 cells, CEP-1347 reduced Tyr 118 phosphorylation of paxillin by 2-fold at 5 minutes and 3.5-fold at 30 minutes, paralleling the effects of JNK inhibition (Fig. 4B). Consistently, HGF-induced Tyr 118 phosphorylation of paxillin was dramatically reduced in MDA-MB-231 cells expressing shMLK3 (Fig. 4C). From these data, we concluded 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 (Fig. 4D). EGF has previously been shown to facilitate the interaction between FAK and paxillin in 293T cells (32). Our data confirmed that the association between GFP-FAK and HA-paxillin is enhanced by EGF (Fig. 4D). However, pretreatment with CEP-1347 abrogated the EGF-induced association of GFP-FAK and HA-paxillin (Fig. 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 (Fig. 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 (Fig. 4F). These data provided 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 (Fig. 5A and B). Similar effects were observed using 2 different MLK3 siRNA sequences. Silencing of MLK3 had no effect on total vinculin protein levels (Fig. 5C). These data suggested 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 wild-type 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 Fig. S4A). In 293T cells, expressing control vector or wild-type 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 Fig. S4B). Likewise, in BT549 cells, ectopically expressed wild-type paxillin, but not paxillin S178A, was phosphorylated on Tyr 118 (Fig. 6A).

Increased cellular stress fibers are observed in keratinocytes upon expression of the paxillin S178A mutant (24). As shown in Fig. 6B, a similar phenotype was observed upon silencing of MLK3 in BT549 breast cancer cells. As 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 (Fig. 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 (Fig. 6D and 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 Fig. 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 (Fig. 6G). These data revealed 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 MDA-MB-231-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 (Fig. 7A). Seven weeks postinoculation, 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 Fig. 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 (Fig. 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 Fig. S6). These data provided 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 (Fig. 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 after 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 (Fig. 7D). Furthermore, CEP-1347 inhibits both CXCL12- and HGF-induced Ser 178 phosphorylation of paxillin (Fig. 7E).
phosphorylation of paxillin in Lu cells (Fig. 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 Fig. S7). These data, taken together, suggested 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 showed 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 (Figs. 2–4), phosphorylation events that are essential in cell migration (24, 35). MLK3 modulates interactions between 2 key focal adhesion proteins, paxillin and FAK (Fig. 4). MLK3–JNK–paxillin signaling negatively regulates Rho activity to promote focal adhesion turnover in cell migration (Figs. 5 and 6). Finally, MLK3 is critical for formation of breast cancer lung metastases in a mouse xenograft model (Fig. 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 suggest that MLK3-JNK signaling is an important regulator of breast cancer cell migration and invasion and that targeting this pathway may be a promising therapeutic strategy for treating breast cancer metastasis.
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 (Figs. 1–3). Thus, MLK3 emerges as an important signaling node that relays extracellular cues to JNK to control paxillin phosphorylation. Although it is possible that other MAPKKKs contribute to paxillin phosphorylation through JNK, MLK3 seems 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). 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 (Fig. 4). Indeed,
MLK3 silencing increases the number of focal adhesions in breast cancer cells (Fig. 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, because 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 (Fig. 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 (Fig. 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). Although 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 axis negatively regulates Rho activity (49). Ablation of Ser 178 paxillin might be a predictive biomarker for metastasis. Our novel findings show that prometastatic factors found in the tumor microenvironment converge on MLK3 to promote breast cancer cell migration and invasion. Due to the fact that 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 show 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J. Chen, K.A. Gallo
Development of methodology: J. Chen, K.A. Gallo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Chen, K.A. Gallo
Writing, review, and/or revision of the manuscript: J. Chen, K.A. Gallo
Study supervision: K.A. Gallo

Acknowledgments
The authors thank Eva Miller and Jonathan Kasper for assisting with mouse surgery and Sandra O'Reilly (MSU-RTSF) for help with in vivo imaging.

Grant Support
This work was supported by grants to K.A. Gallo from the Department of Defense Breast Cancer Research Program (W81XWH-09-1-0049) and the Elsa U. Pardee Foundation. J. Chen was the recipient of the MSU-Barnett Rosenberg Fellowship in Biological Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 6, 2012; revised May 16, 2012; accepted May 31, 2012; published OnlineFirst June 13, 2012.

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doi:10.1158/0008-5472.CAN-12-0655

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