MAPK kinase 3 is a tumor suppressor with reduced copy number in breast cancer

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Abstract

Cancers are initiated as a result of changes that occur in the genome. Identification of gains and losses in the structure and expression of tumor suppressor genes and oncogenes lies at the root of the understanding of cancer cell biology. Here we show that the MAPK kinase MKK3 suppresses the growth of breast cancer where it varies in copy number. A pervasive loss of MKK3 gene copy number in breast cancer patients is associated with an impairment of MKK3 expression and protein level in malignant tissues. To assess the functional role of MKK3 in breast cancer, we showed in an animal model that MKK3 activity is required for suppression of tumor growth. Active MKK3 enhanced expression of the cyclin-dependent kinase inhibitors p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1}, leading to increased cell cycle arrest in G\textsubscript{1} phase of the cell cycle. Our results reveal the functional significance of MKK3 as a tumor suppressor and improve understanding of the dynamic role of the MAPK pathway in tumor progression.

Precis: Findings reveal the functional significance of a MAPK kinase as a tumor suppressor in breast cancer and improve our understanding of the dynamic role of the MAPK pathway in tumor progression.
Introduction

Since its initial description, endogenous suppression of tumorigenesis has been a cornerstone and topic of intensive investigation in cancer research (1). Today, we appreciate that cells employ a growing assortment of genes to restrict proliferation and prevent tumor growth, including those that function in cell cycle checkpoints and mitogenic signalling (2-4). Identification of changes to the genetic material encoding critical regulators of these processes is a central theme in efforts toward understanding cancer development, as all cancers carry tumorigenic somatic mutations (5, 6).

Current thought on tumor suppressor loss suggests a more fluid continuum model, in which subtle, but critical, changes in tumor suppressor levels can have drastic effects on the development of cancer, rendering haploinsufficiencies and partial losses just as tumorigenic as “two hits” (7). Chromosome 17 has emerged as a lightning rod of cancer-associated genetic mutations and rearrangements, with the 17p arm in particular a noted hotspot for deletions (8-10). However, the identity of specific genes with tumor suppressive function within these regions has remained elusive. The M KK3 gene is located on chromosome 17p11.2, and recent proteome-profiling work has highlighted M KK3 as a senescence-promoting and generally downregulated protein in immortalized mammary epithelial cells (11), adding weight to the pursuit of mechanistic insight into the functional role of M KK3 activity in breast cancer.

Breast cancer is the most commonly diagnosed cancer in women and second-leading cause of cancer-related deaths, statistics which strongly advocate for a better understanding of the mechanisms that underlie mammary carcinogenesis (12). In this report, we show that M KK3 expression is impaired in malignant mammary tissues and identify a significant copy number loss of genomic M KK3 in human breast cancer patients. In a model of breast cancer, active
MKK3 significantly restricted tumor growth both in vivo and in an in vitro cell line expression system, while impaired MKK3 signals led to dramatically enhanced tumor growth. Cyclin-dependent kinase inhibitors p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1}, mediators of G\textsubscript{1} cell cycle arrest, were both significantly upregulated in an MKK3 activity-dependent mechanism, resulting in inhibition of cell cycle progression in breast cancer cells. These findings identify new molecular functions for MKK3-dependent signalling and position MKK3 as a novel tumor suppressor that is altered in human breast cancer.
Materials and Methods

Pathology samples from breast cancer patients

The Ethics Committee of Chinese PLA General Hospital in Beijing approved this study, and informed consent was obtained from the patients. All pathology samples and clinical information were collected in accordance with institutional guidelines and regulations.

Animals

Immunodeficient Rag1−/− mice were from Jackson (B6.129S7-Rag1tm1Mom/J). Protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Antibodies and Reagents

Antibodies to cyclin-dependent kinase inhibitors p21, p27, p16, p15, p18, and p57 as well as phospho-ATF2, phospho-p53 (Ser6; Ser9; Ser15; Thr81; Ser20; Ser33; Ser37; Ser46; Ser392), phospho-Akt, total Akt, and c-Jun were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies for MKK3 (C-19), actin, and HRP-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for phospho-p21 (Ser130) was from Biorbyt (San Francisco, CA). FITC-conjugated antibodies to Flag were from Sigma (Oakville, ON). Alexa 594-conjugated goat anti-rabbit secondary was from Invitrogen (Carlsbad, CA). Recombinant stem cell factor (SCF) was from PeproTech Inc. (Rocky Hill, NJ).

TissueScan cDNA arrays and quantitative PCR

Quantitative PCR cDNA arrays were conducted using cDNA arrays (Origene, Cancer Survey cDNA Array 96 – I, lot number 0312) according to the manufacturer’s instructions. Primer
sequences were: MKK3, For 5’- CTTGGTGACCATCTCAGAACTGG -3’ and Rev 5’- CTTCTGCTCCTGTGAGTTCACG -3’ and PPIA, For 5’- ACCGCCGAGGAAACCGTGT -3’ and Rev 5’- CTGTCTTTGGGACCTTGTCTGCAA -3’.

For correlative analysis, total RNA was extracted from clinical tumor sample tissue using Trizol and reverse transcribed using Reverse Transcriptase M-MLV (TakaRa), according to the manufacturer’s instructions. The primers used in qPCR for MKK3 and PPIA were as noted above and for GAPDH were: For 5’- ACATCATCCCTGCCTCTACTG -3’ and Rev 5’- ACCACCTGGTGCTCAGTGTA -3’. For additional detail see Supplementary Materials and Methods.

**Immunohistochemistry**

Slides with sections of formalin-fixed paraffin-embedded (FFPE) normal mammary tissues and patient-derived tumor tissues (Origene and study patients) were incubated in 10 mM citrate-tween buffer at 97°C for 20 mins for antigen unmasking. Specimens were blocked in 2% goat serum/5% BSA in PBS-tween for 2 hrs, and incubated overnight at 4°C in rabbit anti-MKK3 (C-19, Santa Cruz) at 1:100 in 5% BSA PBS-tween. Slides were then washed and incubated with Alexa 594-conjugated goat anti-rabbit (Invitrogen) at 1:2000 in blocking buffer. Specimens were mounted in DAPI (Vectasheild) and imaged using equivalent settings on a Nikon E600 microscope equipped with 20X or 40X objective lens using ACT-1 software (Nikon).

**Quantitative PCR assessment of genomic MKK3 in clinical tumor samples**

Human genomic DNA was isolated from random breast cancer patient tumor samples at SinoGenoMax Co. (Beijing, China). Normal female genomic DNA samples were from a random control panel (Sigma, HRC1). Ct values for two distinct genomic sites in the MKK3 gene and
five reference genomic sites (BCMA, SDC4, NUDT6, FGF2, and MYOD1) were determined for each DNA sample. Seg-1 and NCI-H774 (ATCC, Manassas, VA) cell lines were used as internal controls as NCI-H774 cells have a previously reported double deletion of the M KK3 gene (13). A 1:1 mixture of Seg-1 and NCI-H774 is thus expected to have a half level of genomic M KK3. For additional detail see Supplementary Materials and Methods.

**Fluorescence in situ hybridization (FISH)**

Slides with sections of formalin-fixed paraffin-embedded (FFPE) patient-derived tumor tissues (study patients) were analyzed using the Histology FISH kit (Dako, Denmark) according to the manufacturer’s instructions. Probe with specificity for the M KK3 gene (SureFISH Chr17 CEP, Red, Human Chr17: 20893971-21327887, Agilent Technologies, Cedar Creek, TX) was used according to the manufacturer’s instructions (Dako). For additional detail see Supplementary Materials and Methods.

**Generation of stable cell lines**

MDA-MB-468 cells were obtained via the ATCC and resuscitated from early passage liquid Nitrogen vapour stocks as needed and cultured in DMEM supplemented with 10% FBS. Cells were cultured for less than 3 months before re-initiating cultures and were routinely inspected microscopically for stable phenotype. A concentration of 0.8 mg/ml G418 was used to generate stable lines. MDA-MB-468 cells were transfected with Flag-MKK3dn or Flag-MKK3ca plasmids (Addgene, Cambridge, MA) using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable colonies of cells were plated under selection by limiting dilution to isolate stable clones. Plates were incubated for 3 weeks allowing colonies to form.
Immunofluorescence

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde for 20 min, and permeabilized with a 0.2% solution of Triton-X in PBS for 10 min. Samples were blocked with 10% BSA (Sigma, Oakville, ON) for 30 mins followed by a 2 hr incubation with anti-Flag antibody (1:500) in a 3% BSA solution in PBS and finally with Alexa 594-conjugated goat anti-mouse IgG F(ab)_2 (Molecular Probes) at 1:200 for 1 hr. Coverslips were mounted with DAPI-containing Vectasheild (Vector Laboratories, Burlingame, CA).

Flow cytometry

Cells (5 ×10^5) were collected using trypsin (Invitrogen), fixed, and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) for 20 min. Cells were stained in 100 μl with FITC-conjugated Flag antibody (1:1000 or 1 μg/ml) for 2 hr, washed, and analyzed on a FACS Aria (BD Biosciences).

Tumor growth in vivo

For xenotransplantation studies, 1 ×10^7 log-phase growing untransfected, MKK3dn, or MKK3ca MDA-MB-468 cells were trypsinized, washed twice in PBS, and injected into immuno-comprimised Rag1-deficient mice at the mammary fat pad (200 μl/injection in PBS). At endpoint, tumors were surgically excised, collected, and imaged using an iPhone 4S (Apple). Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E).
Thymidine incorporation and WST-1 assay

5 x10³ cells were seeded in quadruplicate in a 96-well plate. 48 hrs later, cells were pulsed with 0.25 μCi of ³H-thymidine for 3 hrs. Incorporation was measured on a Wallac 1409 scintillation beta counter. For WST-1 assays, cells were cultured as above for 4 days and WST-1 proliferation reagent (Clontech) was added at 1:10 dilution. Plates were read 90 mins later.

Quantitative real-time PCR array

1-2 x10⁶ cells were collected and processed using Trizol (Invitrogen) and purified using RNeasy (Qiagen). Breast Cancer RT-PCR Arrays (Qiagen) were conducted in triplicate according to the manufacturer’s protocol and qPCR was performed using an ABI 7000 Sequence detector (PE Applied Biosystems, Foster City, CA). For additional detail see Supplementary Materials and Methods.

Western blotting and p53 siRNA

Cells were lysed in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors. For siRNA knockdown of p53, WT MDA-MB-468 and MKK3-mutant cells were used in reverse transfection (Life Technologies, Ambion, TP53 s607 and s605) according to the manufacturer’s instructions. For additional detail see Supplementary Materials and Methods.

Chromatin immunoprecipitation (ChIP)

WT MDA-MB-468 and MKK3dn, or MKK3ca transfected cells were plated in 10 cm dishes. The following day, chromatin-protein complexes were prepared according to the manufacturer’s
instruction (ChIP-IT Express Enzymatic, Active Motif). For additional detail see Supplementary Materials and Methods.

**Cell cycle analysis**

Cell cycle staging was conducted using Vybrant DyeCycle Green (Invitrogen) by flow cytometry according to the manufacturer's instructions. Data was analyzed using WinList 5.0 software.

**Nuclear protein preparation and electrophoretic mobility shift assay (EMSA)**

Nuclear protein extracts were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. EMSA was performed as previously described (14). Double-stranded oligonucleotides for AP-1 and SP-1 consensus binding sequences were from Promega.

**Statistical Analysis**

The paired Student’s *t* test was used for statistical evaluation of data. Results were considered significant when *p* < 0.05. Data are expressed as means ± SEM unless otherwise indicated.
Results

**MKK3 expression and genomic copy number is reduced in human breast cancer.** To assess the mRNA expression level of **MKK3** in primary tumor tissues compared to normal tissues, a cDNA array with 9 tumor and 3 normal tissues from eight types of cancer was employed in a quantitative PCR (qPCR) assay. **MKK3** gene expression was significantly downregulated in the tumor tissue in five of eight cancers examined, including breast, colon, liver, lung, and thyroid cancer, and was decreased in kidney cancer, while prostate and ovary tumor tissues showed normal **MKK3** expression (Fig. 1A). This is the first report of widespread **MKK3** expression loss as a feature in several types of cancer, most notably in breast and liver cancers. Following up on this, we examined MKK3 protein levels in cancerous versus normal mammary tissue by immunohistochemistry. A similar remarkable impairment in MKK3 protein expression in cells of the ductal walls was detected in breast tumor tissues (Fig. 1B).

Genomic copy number variation has emerged as a pervasive phenomenon and an important consideration in a broad range of disease causality including breast cancer (15, 16), resulting in gene copy number imbalances or loss of heterozygosity (LOH) that can ultimately lead to altered gene expression and protein function (8). To investigate the copy number status of **MKK3** in clinical samples from breast cancer patients, we designed a qPCR assay to assess relative genomic MKK3 content in patient-derived samples. DNA from the NCI-H774 cell line was used to evaluate specificity and create an internal assay control as NCI-H774 cells have a natural double deletion of the **MKK3** gene (13). Remarkably, our analysis revealed a significantly decreased genomic **MKK3** level in eight of ten breast cancer patients (Fig. 1C). Additional patient tumor data from clinical evaluation of common relevant breast cancer markers is provided (Supplementary Table S1). Comparison of genomic and mRNA levels for **MKK3** in
patients, suggests a positive correlation between gene loss and gene expression level (Supplementary Fig. S1). Further evaluation of tissues from these patients by fluorescence in situ hybridization (FISH) confirmed that M KK3 copy number was heterogeneously reduced, reflecting intra-tumoral genomic heterogeneity at the M KK3 locus (Fig. 1D). These results are the first to identify a specific M KK3 copy number deficiency and strongly suggest that copy number loss may contribute to reduced M KK3 expression in breast cancer. These findings prompted us to pursue the functional role of M KK3 activity in breast cancer.

**M KK3 activity regulates tumor growth in vivo via differential regulation of cell proliferation.** To determine the function of M KK3 signalling activity in breast cancer, we employed the human breast cancer cell line, MDA-MB-468, which had a relative genomic M KK3 level of 1.44 by qPCR, similar to those patients who displayed genomic loss in Fig 1C. This cell line was transfected plasmids encoding either a dominant negative (M KK3dn) or constitutively active (M KK3ca) Flag-tagged mutant M KK3 gene, in which the activation site is mutated at two amino acids, rendering the resulting protein either permanently inactive or active (17). Colonies were selected and assessed for degree of expression and purity by immunofluorescence and flow cytometry and were monitored weekly for stable expression (Supplementary Fig. S2). To assess the role of M KK3 activity on tumor growth in vivo, we employed an immunodeficient (Rag1-/-) mouse model permitting xenotransplantation studies with our human cell lines. M KK3dn, M KK3ca, and untransfected MDA-MB-468 breast cancer cell lines were transplanted subcutaneously at the mammary fat pad of female Rag1-deficient mice. Interestingly, a significant and opposing effect on tumor growth was observed in M KK3-mutant tumors. While M KK3ca tumors grew significantly slower than those resulting from
untransfected MDA-MB-468 cells, MKK3dn tumors grew significantly faster (Fig. 2A). Also noteworthy, was that while all mice that received MDA-MB-468 cells and MKK3dn cells developed tumors, only 50% (p = 0.049) of mice receiving MKK3ca cells developed a palpable tumor. At experimental endpoint, tumor tissues were surgically excised and three representatives from each group are shown (Fig. 2B). Following the trend in tumor growth, a remarkable MKK3 activity-dependent difference in the density of intratumoral MDA-MB-468 cells was observable by histological staining (Fig. 2C). These results indicate that MKK3 activity is suppressive of breast cancer tumor growth, and conversely, that impaired MKK3 signals lead to significantly enhanced breast cancer tumor growth.

To directly determine the role of MKK3 activity on tumor cell proliferation, in vitro studies were conducted using five independently derived MKK3dn and MKK3ca cell lines. MKK3 activity had a significant impact on basal proliferation, with MKK3ca cells at 64% and MKK3dn cells at 229% of the proliferative rate of untransfected cells (Fig. 3A). These results were further confirmed using a WST-1 proliferation assay (Fig. 3B) and by counting equivalently seeded cells in culture (Fig. 3C-D). In accordance with our observations on in vivo tumor growth, these findings indicate that MKK3 activity inhibits tumor cell proliferation, and conversely, that a lack of MKK3 signalling may enhance breast cancer tumor growth via enhanced tumor cell proliferation.

**MKK3 activity promotes p21 and p27 expression in breast cancer cells.** To investigate the mechanism of MKK3 activity-dependent impairment of cell proliferation, a qPCR array was used to assess the expression of 84 breast cancer-related genes in these cells. Of the nine genes found to be differentially/oppositely regulated in MKK3dn and MKK3ca cells, the most
significantly oppositely regulated was cyclin-dependent kinase inhibitor 1A (CDKN1A), the gene encoding the inhibitor of cell cycle progression p21<sup>Cip1/Waf1</sup> (Fig. 4A). Quantitative analysis showed that CDKN1A expression in MKK3ca was 2.56-fold while that of MKK3dn cells was 0.66-fold compared to control cells (Fig. 4A). The full array data set is presented in Supplementary Table S2.

To confirm our gene expression analysis at the protein level, Western blots were conducted to analyze the expression of a panel of cyclin-dependent kinase (CDK) inhibitors. Indeed, opposing expression of p21 protein was detected in MKK3-mutant cell lines in accordance with mRNA levels detected by qPCR (Fig. 4B). Notably, another CDK inhibitor, p27<sup>Kip1</sup> (CDKN1B), was similarly oppositely deregulated in mutant MKK3 cell lines (Fig. 4B). Expression of family member p16 (CDKN2A) was in accordance with qPCR data and, as in the case of p15 (CDKN2B), was not significantly oppositely regulated by MKK3 (Fig. 4B), while remaining members p18 and p57 were not detectable (data not shown). Phosphorylation of p21 in a p38-dependent mechanism has been reported to stabilize p21 protein (18). We analyzed p21 at Ser130 in our cell lines and could not detect any phosphorylation at this site, suggesting that this mechanism is not playing a role in our system.

**MKK3 activity regulates CDK inhibitors independently of p53 and AP-1.** These results prompted an investigation into the mechanism of MKK3 activity-dependent expression of p21 and p27. It is well-established that MKK3 signalling through the p38 MAPK protein family culminates in the activation of downstream transcription factors, including activating transcription factor 2 (ATF2) (19). Monitoring of ATF2 phosphorylation confirmed constitutive activity in MKK3ca cells and impaired signalling in MKK3dn cells, detectable in cells
stimulated for activation of the MAPK pathway through the stem cell factor receptor c-kit (Fig. 5A). The tumor suppressor p53 is a major regulator of p21 and p27 gene expression, however p53-dependent and p53-independent mechanisms are known (20-22). Notably however, MDA-MB-468 cells express a mutant p53 protein with the R273H mutation which results in defective DNA contact and binding, suggesting that the MKK3 activity-dependent effect is unlikely to be mediated by p53 (23), but to confirm this was case, we conducted further analysis of p53 activation. To determine if MKK3 signaling activity modifies p53 phosphorylation and activation in MKK3ca cells, Western blotting for activation of the p53 protein was conducted. No MKK3-dependent activation of p53 consistent with activation of p21 and p27 was detectable. In fact, MKK3 signals led to dephosphorylation of p53 at Ser9, and in general, inconsistent with a p53-dependent mechanism, total p53 protein expression was slightly enhanced in MKK3dn cells (Fig. 5B). In addition to those shown in Figure 5B, phosphorylation analysis was also conducted at Ser6 and Thr81, but no phosphorylation was detected at these sites. These results suggest that MKK3-activity regulates p21 and p27 expression independently of p53 phosphorylation.

We then further examined the role of p53 in our MKK3 transfected cell lines by siRNA-mediated knockdown of p53. Knockdown of p53 resulted in enhanced expression of both p21 and p27 in all cell lines, and did not impair the enhanced p21 and p27 expression in MKK3ca cells (Fig. 5C). Overall enhanced expression in the knockdowns is likely an effect of removal of the dominant negative and/or gain-of-function effects exerted by the mutant protein on basal p53 target gene expression. These results suggest that in our cell lines, mutant p53 is inhibitory to basal expression of p21 and p27, and that MKK3 can direct p21 and p27 expression in the absence of functional p53. Finally, we examined our cell lines by chromatin
immunoprecipitation to determine if MKK3ca cells might yet have enhanced p53 association with the p21 promoter via another mechanism. However, our findings show similar p21 promoter binding in in WT and MKK3ca cells, and an enhancement in binding in the MKK3dn cells. It worth noting however, that the level of binding to the p21 promoter in this assay was between 0.03 - 0.06% of input DNA, an extremely low overall level that indicates considerably weak interactions between p53 and the p21 promoter, which is not surprising given a functionally defective p53. Altogether, these results suggest that MKK3 activity driven expression of p21 and p27 is occurring via p53-independent mechanism in our model.

The AP-1 transcription factor can also control p21 gene expression through activation of SP-1 promoter binding (24). Gene expression array data indicated that c-Jun, a component of AP-1, was oppositely regulated in MKK3-mutant cells, consistent with p21 (Fig. 4A). MKK3-dependent protein expression of c-Jun was confirmed by Western blotting (Fig. 6B). However, investigation of the DNA-binding activity of AP-1 and SP-1 revealed that their activities were not notably altered in the MKK3-mutant cells (Fig. 6C), suggesting that this mechanism is unlikely to be contributing to MKK3-directed p21 expression.

**MKK3 activity promotes p21/p27-mediated G₁ cell cycle arrest, inhibiting tumor growth.**

Cell cycle regulators p21 and p27 inhibit cell cycle progression at the G₁ checkpoint (25), a considerably attractive mechanism to target in cancer (26-30). Recent studies in glioblastoma proliferation have shown that a natural plant product, β-elemene, inhibits tumor growth by arresting cells in the G₀/G₁ phase through a mechanism dependent on MKK3 activation (31). To determine if MKK3-mutant cells indeed have deregulated cell cycle progression, flow cytometric analysis of cell cycle staging was conducted. Consistent with enhanced p21 and p27 expression,
MKK3ca cells showed significant cell cycle arrest at the G1 phase, while MKK3dn cells had significantly reduced numbers in G1 indicating a more rapid entry into the S phase. Accordingly, MKK3ca cells had significantly fewer numbers in the G2/M mitotic phase, while MKK3dn cells had enhanced numbers in S and G2/M (Fig. 7A). Collectively, these results identify MKK3 as a novel tumor suppressor in breast cancer that is downregulated in a range of cancer types, and suggest that active MKK3 promotes G1 cell cycle arrest and restricts tumor cell proliferation by enhancing p21 and p27 expression, resulting in suppressed tumor growth, while impaired MKK3 signals have the opposing effect on both gene expression and tumor growth (Fig. 7B).

Discussion

Addressing the significance of MKK3 in breast and other types of cancer has garnered increasing interest in recent years as efforts continue to decipher the elusive role of the MAPK pathway in carcinogenesis and tumor suppression (3). In this study, we have identified a new tumor suppressive role for the mitogenic signalling protein MKK3 in the regulation of breast cancer tumor growth. We have characterized this function mechanistically to be mediated by MKK3 signalling activity-dependent regulation of the expression of cell cycle inhibitors, p21 and p27, and subsequent restriction of cell cycle progression, as shown in our proposed model (Fig. 7B). Importantly, we demonstrate that MKK3 expression is significantly impaired in breast cancer tissues, and describe a novel loss of MKK3 genomic copy number in breast cancer patients. This is the first specific detection of MKK3 copy number variation in human cancer and suggests that MKK3 is a suppressor of mammary carcinogenesis with potential prognostic, predictive, and/or therapeutic value.
In breast cancer, inactivation of the MAPK phosphatase Wip1 inhibits mammary carcinogenesis. Wip1 functions to inactivate signals mediated by MKK3 through the p38 MAPK pathway, supporting a suppressive role for MKK3 signalling in breast cancer pathogenesis (32, 33). Our mechanistic results expand significantly on how MKK3 activity inhibits carcinogenesis, through promotion of cell cycle arrest. Inhibition of cell cycle progression is clearly an attractive mechanism to target and promote in the development of anti-cancer therapies, and the significance of p21 and p27 expression in this mechanism has not been understated in the literature (26-28, 34). In breast cancer, p21 and p27 have received considerable attention as clinical indicators and potential targets in therapeutic development (28-30). In agreement with the findings we present here, previous work in leukemia showed that impaired MKK3 signals enhanced prostaglandin PGJ2-induced proliferation, and described impaired p21 and p27 expression in treated THP-1 cells (35). Additionally, a natural fungal product (FTY720) induced MKK3 activity and p21 expression in prostate cancer cells, leading to cell cycle arrest (36), while studies in muscle cell differentiation have shown that dominant negative MKK3 inhibits p21 and p27 expression (37). Downstream of MKK3, p38 MAPK activity is linked to p27 expression, leading to contact inhibition in mouse embryonic stem cells (38, 39), and it is also well-known that p38 can activate p53-dependent p21 expression (40), and further stabilize p21 protein by direct phosphorylation (18). While these reports suggest MKK3 signals are linked to p21 and p27 expression, we report the first direct evidence that active MKK3 is a vital regulator of these two cycle cell inhibitors, independent of p53 activation, with ramifications in tumor progression, and may represent a novel approach to targeting cell cycle inhibition in cancer.

Induction of p21 transcription and resultant cell cycle arrest is associated with both p53-dependent and p53-independent mechanisms (20, 22, 34). The DNA damage response is known to
activate p21 expression in a p53-dependent manner inducing transient senescence preceding DNA repair or apoptosis (41), whereas p53-independent p21 induction is more associated with the induction of cellular senescence, differentiation, and development (20). The p38 MAPK, a direct substrate of MKK3, can itself activate p53 at Ser15, Ser33, and Ser46, leading to p21 transcription (3, 42, 43). To determine if MKK3 activity-dependent p21 expression was regulated by p53, we examined p53 phosphorylation at a range of sites, including Ser46, Ser15, and Ser392, which are known to lead to p53-dependent p21 transcription (44-46), and found no altered p53 activation. Notably, total p53 protein was actually slightly enhanced in MKK3dn cells. We also examined the contribution of p53 by knockdown and by ChIP analysis of promoter-association. Altogether, these results support a p53-independent transcriptional regulation of p21 by MKK3 activity. Interestingly, our results may have additional relevance in cases of p53 mutation, which occur in up to 50% of cancers, given that our results describe a scenario of p53 mutation in which p21 and p27 expression is actively repressed by the R273H mutant p53.

Interestingly, p21 and p27 expression are known to be upregulated by inhibition of the PI3K/Akt signalling pathway leading to G1 cell cycle arrest (47), and indeed MKK3ca MDA-MB-468 cells had impaired activation of this pathway when Akt phosphorylation was examined (Supplementary Fig. S3), suggesting that MKK3 signals can inhibit Akt activation thereby supporting p21 and p27 expression. Aside from a transcription factor activation-mediated mechanism acting directly on the p21 and p27 promoters, an alternative mode of action could be through mRNA stability. MKK3 signalling is associated with increased mRNA stability of transcripts with adenosine/uridine-rich elements in the 3′UTR in breast cancer cells (48), and p21 and p27 expression are known to be regulated through RNA stabilization mechanisms (49-51). It
remains unclear precisely how MKK3 activity can direct p21 and p27 expression leading to cell cycle arrest.

Despite extensive interest in a tumor suppressive role for MAPK signalling, this study is, to our knowledge, the first direct assessment of MKK3 activity on tumor growth in an in vivo xenograft model. We report a novel p53-independent mechanistic regulation of cell cycle inhibitors p21 and p27, dependent on MKK3 activity, leading to cell cycle arrest at G₁ and significantly impaired tumor growth in vivo. Moreover, we identify a prevalent loss of MKK3 genomic copy number in breast cancer patients, and significantly impaired gene and protein expression in malignant mammary tissues. Collectively, these results position MKK3 as a novel tumor suppressor that is altered in human breast cancer.
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References


Figure Legends

Figure 1. Loss of MKK3 expression in human breast cancer patients. (A) MKK3 gene expression was evaluated by qPCR of normal tissue and tumor tissue using cDNA arrays. Data are expressed relative to the 3 normal controls ±SEM for \( n = 9 \) tumor samples per tissue type. Significant p values were as follows: breast \( p = 0.0008 \); colon \( p = 0.029 \); liver \( p = 0.000009 \); lung \( p = 0.024 \); and thyroid \( p=0.042 \). (B) Sectioned specimens of normal mammary tissue or tumor tissue were analyzed for MKK3 protein level by immunohistochemistry using anti-MKK3 antibodies (red) and DAPI (blue) to visualize nuclei. Representative images from \( n = 4 \) tumor specimens and a normal mammary tissue specimen are shown. (C) Human genomic DNA from breast cancer patient tumor samples and normal female control samples were analyzed by qPCR to assess copy number. DNA from Seg-1 and NCI-H774 cell lines (alone or mixed 1:1) were used as an internal assay control. Data is expressed as ±SEM for \( n = 10 \) tumor and 9 normal controls; \( p = 0.009 \) between groups. (D) Breast cancer patient-derived tissues were analyzed for MKK3 gene copy number variation by fluorescence in situ hybridization (FISH). Red punctuations indicate the presence of a genomic copy of MKK3, while cell nuclei are stained blue with DAPI. A representative image from patient number 9 in panel C is shown, confirming patient tumor cells display a heterogeneous loss of MKK3 genomic DNA. Evaluation of five fields of view had an average of 1.10 copies per nuclei. Boxed area is magnified from the original 1000× magnification.

Figure 2. MKK3 activity is suppressive of tumor growth in vivo and proliferation in vitro. (A) Immunodeficient Rag1\(^{-/-}\) mice were transplanted with MKK3dn, MKK3ca, or untransfected MDA-MB-468 cells at the mammary fat pad. Tumor volume was measured over time for \( n = 7 \),
5, and 8 respectively, and data are expressed as ± SD. * p < 0.05; ** p < 0.01; *** p < 0.001. (B) At experimental end point, tumors were surgically removed from mice and 3 representative tumors are shown for each cell type. (C) Histological staining of tumor tissue with H&E. Images are representative of n = 5 tumors per group.

Figure 3. MKK3 activity directs proliferation of MDA-MB-468 cells. (A) Untransfected MDA-MB-468 cells and five independently derived lines for MKK3dn and MKK3ca cells (5 x10^3) were seeded in 96-well plates in quadruplicate. The following day, cells were pulsed with ^3H-thymidine for 4 hours, incorporation was measured, and data were expressed as ±SEM. *** p < 0.001; n = 5 independently derived clones each. (B) Untransfected MDA-MB-468 cells and five independently derived lines for MKK3dn and MKK3ca cells (5 x10^3) were seeded in 96-well plates in quadruplicate. Four days later proliferation was measured by colorimetric WST-1 assay, expressed as the OD at 440 nm minus background. ** p < 0.01; *** p < 0.001; n = 5 independently derived clones each. (C-D) Untransfected MDA-MB-468, MKK3dn, and MKK3ca cell lines were seeded at 1 x10^5 cells per well and on days 2, 4, and 7, three wells per group were counted by trypan blue exclusion. * p < 0.05; ** p < 0.01 for n = 3. Representative images of cells in culture at 48 and 96 hrs are shown.

Figure 4. MKK3 activity differentially regulates p21 and p27 cyclin-dependent kinase inhibitor expression in MDA-MB-468 cells. A quantitative real-time PCR array was used to examine cell lines for the expression of 84 breast cancer markers. (A) List of all breast cancer array genes oppositely regulated by MKK3dn and MKK3ca mutant proteins. * p < 0.05; ** p < 0.01; *** p < 0.001. Green indicates >1.5 fold decrease and blue indicates 1.5 fold increase.
compared to WT. Data is expressed as a summary of \( n = 3 \) experiments. *** \( p < 0.001 \). Aside from CDKN1A/p21, additional analysis was conducted on Jun (c-Jun; Fig. 6), IL-6, and CST6. MKK3-dependent IL-6 production (48 hr) was measured by ELISA and was consistent with mRNA, however MKK3-dependent IL-6 production did not remarkably alter cell-free culture supernatant levels (not shown), while CST6 protein was undetectable by Western blot. (B) Cell lines were examined for expression of a panel of cyclin-dependent kinase inhibitors by Western blotting. CDK inhibitors p21 and p27 were similarly differentially regulated. Actin is shown as a loading control and MKK3 mutant protein expression is shown using a Flag antibody. Representative blots from \( n = 4 \) experiments are shown.

**Figure 5. MKK3-directed proliferation in MDA-MB-468 is p53-independent.** (A) Western blot analysis of MKK3 activity-dependent ATF-2 phosphorylation in MDA-MB-468 cells with actin as a total protein loading control. Cells were untreated (NT) or stimulated with SCF for 120 min to monitor downstream activation of the p38 MAPK pathway via ATF-2 activation. A representative of \( n = 4 \) experiments is shown. (B) Western blot analysis of MKK3 activity-dependent p53 activation by phosphorylation at Ser 9, Ser 15, Ser 20, Ser 33, Ser 37, Ser 46, and Ser 392 and total MKK3 activity-dependent p53 protein expression in MDA-MB-468 cells with actin as a loading control. Representative blots from \( n = 4 \) experiments are shown. (C) Knockdown of p53 by siRNA in WT, MKK3ca, and MKK3dn cells and subsequent Western blot analysis of p21 and p27 expression. Representative blots (p53 siRNA s607) from \( n = 4 \) experiments with two different p53 siRNAs showing similar results is shown with detection of p53 to confirm knockdown and actin as a loading control. (D) Chromatin immunoprecipitation was used to measure relative binding of p53 to the promoter of p21 in WT, MKK3dn, and
M KK3ca cells. Data were calculated as % input and expressed as relative p21 promoter binding ±SEM for $n = 3$ independent experiments; * $p < 0.05$.

**Figure 6. MKK3 activity regulates c-Jun expression, but not AP-1 or SP-1 transcription factor activity.** (A) Western blot analysis of MKK3-directed c-Jun protein expression with actin as a total protein loading control in cell untreated (NT) or stimulated with stem cell factor (SCF) for 120 mins; WT = untransfected MDA-MB-468 cells. A representative blot from $n = 4$ experiments is shown. (B) EMSA analysis of MKK3 activity-dependent AP-1 and SP-1 transcription factor activity in MDA-MB-468 cells.

**Figure 7. MKK3 activity is tumor suppressive in MDA-MB-468 cells by enhancing p21 and p27-mediated G1 cell cycle arrest.** (A) MKK3 activity enhances cell cycle arrest. Untransfected (control) MDA-MB-468, MKK3dn, and MKK3ca cells were analyzed for cell cycle progression staging by flow cytometry following Vybrant DyeCycle Green staining. Data is expressed at % of total cells ±SEM for $n = 3$ experiments. * $p < 0.05$; ** $p < 0.01$. (B) Model of MKK3 activity as a p53-independent tumor suppressor regulating p21 and p27 expression and subsequently leading to G1 cell cycle arrest and inhibited breast cancer tumor growth.
Figure 1
Figure 2
Figure 3
**Figure 4**

### Table A

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**Differential**

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† in DN, ‡ in CA

**Figure 4**

**B**

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**Figure 5**

### A

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### B

**p53 phosphorylation sites**
- p-p53 (Ser9)
- p-p53 (Ser15)
- p-p53 (Ser20)
- p-p53 (Ser33)
- p-p53 (Ser37)
- p-p53 (Ser46)
- p-p53 (Ser392)
- p53
- actin

### C

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### D

**Relative binding of p53 on p21 promoter**
- **WT**: ![Graph](image)
- **MKK3dn**: ![Graph](image) *
- **MKK3ca**: ![Graph](image)

* Significant difference compared to WT.
Figure 6
Figure 7

A

![Graph showing cell cycle phase distribution](image)

Cell cycle phase

- G0/G1
- S
- G2/M

Legend:
- WT
- MKK3dn
- MKK3ca

B

Stress signal / growth factor

- MKK3
- p38
- p53
- ATF2
- G0/G1 cell cycle arrest
- CDKN1A
- CDKN1B

Tumour growth

CDKN1A

CDKN1B

p21

p27

G1 cell cycle arrest
MAPK kinase 3 is a tumor suppressor with reduced copy number in breast cancer


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