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**14. ABSTRACT**

Overexpression of EGFR is frequently linked to more aggressive tumor behavior, including increased proliferation, metastasis, and therapeutic resistance. Here, we identified a molecular linkage between IKKα and EGFR signaling in breast cancer cells. Inhibition of IKKs activity elevates EGFR tyrosine phosphorylation. In addition, IKKα forms a specific interaction with EGFR in Golgi apparatus and catalyzes EGFR S1026 phosphorylation. We found that EGFR S1026A possess a stronger tumorgenesis phenotype compare with wild type EGFR suggesting a negative regulation of IKKα in EGFR signaling. In agreement with an earlier finding where conditional ablation of IKKα in the mice keratinocytes elevates the autocrine loop of EGFR, our results further provide a potent role of IKKα kinase activity in preservation of EGFR activity.

**15. SUBJECT TERMS**
EGFR signaling, inflammatory kinase, IKKalpha, tumorgenesis, tumor suppressor

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**Title: The role of IKKα in EGFR signaling regulation**

## 1. INTRODUCTION:

EGFR is one of the most well studied receptor tyrosine kinases (RTK). This cell surface molecule plays an essential and fundamental role in dictating cell proliferation and differentiation, cell cycle control, biological development, tumorigenesis, and malignant development (1-3). To date, EGFR has been extensively investigated in all aspects of biomedical researches. EGFR regulates many signaling pathways including JAK-Stat3/5, PI3K-Akt, and MAPK kinase pathways (4-7). Upon ligand stimulation, EGFR forms homodimer or heterodimer with one of other three family members. The dimerization subsequently triggers the autophosphorylation of EGFR and/or Src kinase mediated transphosphorylation (8).

The phosphorylated EGFR provides docking sites for binding downstream adaptor proteins and thereafter activates several downstream signaling pathways. Several tyrosine residues in the intracellular domain of EGFR such as Y992, 1068, 1086, and 1173 have been well characterized. They provide docking sites for adaptor proteins such as Shc, Grb2, and Gab and result in the activation of PI3K/Akt and Ras/MAPK signaling pathways (6). The activation of PI3K/Akt and /or Ras/MAPK pathways provides survival signals promote tumorigenesis in various cancers (1,9,10). On the other hand, Src-induced transphosphorylation of Y845 on EGFR provides docking site to recruit Stat3/5 and subsequently activated Stat3 and/or Stat5 through the formation of homo- or hetero-dimers. The dimerized Stat3 or Stat5 translocates into the nucleus, binds to its cogent DNA to regulate cell proliferation, differentiation, cell cycle, and migration.

In this study, we found a major inflammation regulator, IKKα inhibits EGFR activity through a novel signaling pathway in breast cancer cells. IKKα binds to and phosphorylated EGFR at S1026. Inhibits of IKK activity led to hyperphosphorylation of EGFR Y845 and STAT3 Y705. Consistent with an earlier finding that IKKα serves as a tumorsuppressor inducing skin cancer (11), our study provides novel mechanistic insight of IKKα mediated EGFR suppression.

## 2. RESEARCH ACCOMPLISHMENTS BODY

### Part I: Functional analysis of EGFR S1026A

In the previous report, we have showed that EGFR S1026A process higher pY845 and pY705 STAT3 status in NIH3T3 cells. The higher activity induces a faster cell growth rate in MTT assay. In the current study, we provided more evidence that EGFR S1026A stable expression is indeed process higher pY845 and pYSTAT3 in Cho cells (Fig.1A) and MCF7 cells (Fig.1D).

To understand how EGFR S1026 phosphorylation affects its function, EGFR stable transfectants were subject to a series of cell-based function assays. To observe whether EGFR S1026 is indeed affected cellular function, *In vitro* cell growth and BrdU incorporation assay showed that phospho-deficient EGFR-S1026A significantly gain its ability to stimulated cell growth compared to EGFR-WT (Fig.1B). To test whether EGFR S1026 phosphorylation could impact the tumorigenesis, we performed clonogenic assay to observe *in vitro* cell proliferation rate using MCF7 stable cell clones (Fig. 1C). In addition, we investigated whether EGFR S1026A could support breast cancer MCF7 cell

to grow tumor *in vivo* using orthotopic animal model. MCF7 stable clones expressing either EV (empty vector), EGFR WT, EGFR DN (dominant negative), or EGFR S1026A mutant were injected into mammary fat pads of nude mice and tumor sizes were measured at indicated time points. As shown in Figure 1D, EGFR S1026A stimulates MCF7 cells to grow tumor in compare with EGFR WT. Together, our *in vitro* and *in vivo* results supported that EGFR S1026 phosphorylation plays an essential role in regulating cell growth, DNA synthesis, and tumorigenesis.

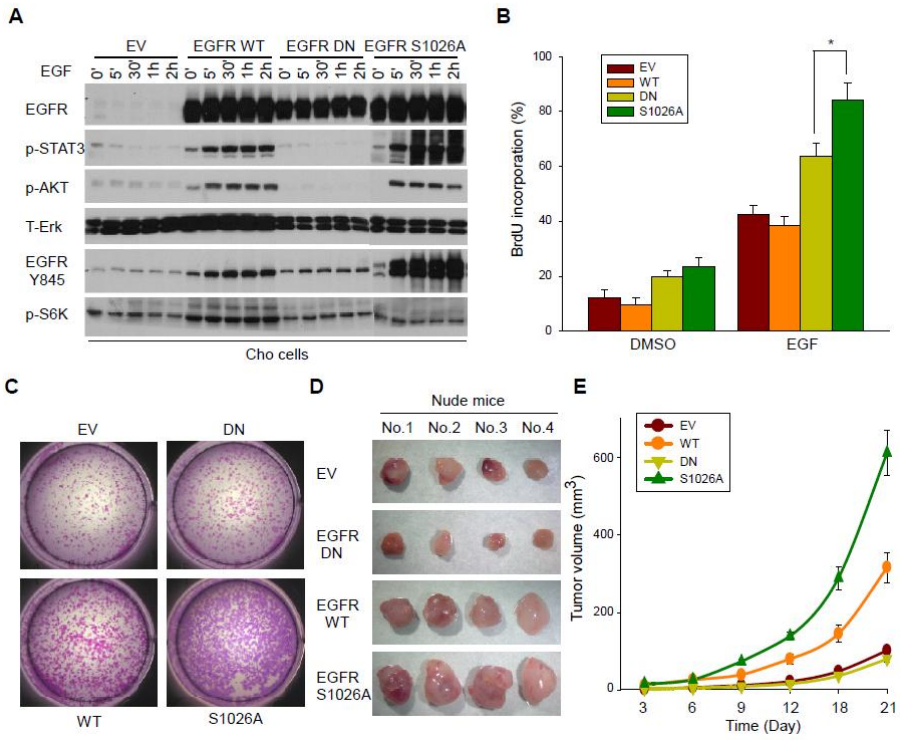


**Figure 1. EGFR S1026A exhibits higher tumorigenesis activity** (A) EGFR S1026A shows hyperactivation of p-EGFR Y845 and p-STAT3 Y705. EGFR stable clone were serum starved for overnight and treated with 30 ng/μl EGF for indicated time points and analyzed by western blot. (B) EGFR S1026A induces higher DNA synthesis rate. MCF7 stable transfectants were seeded in 6-well culture plates and cultured to reach 90% confluence. The cells then were serum-starved and treated with Bromodeoxyuridine (BrdU) for 18 hr prior to assay. (C) Colongenic assay showing EGFR S1026A bears higher tumorigenesis ability. (D) MCF7 stable clone cells were inoculated into mammary fat pads of nude mice. The tumor size was measured and statistically analyzed by student's t-Test in (E).

## Part II: IKKα disrupts the oncogenic synergy between Src and EGFR

As we proposed in potential pitfall and alternative plan, we would like to investigate whether Src is involved in IKKα meditated EGFR inhibition. Because EGFR Y845 transphosphorylation is primarily mediated by Src kinase (see introduction), it is possible that IKKα affects its protein local structure results in the blockage of Src-mediated Y845 phosphorylation. Src Y416 is located at the activation loop of Src kinase domain usually indicates its enzyme activity; we therefore test this hypothesis using Bay 11-7082 treated MDA-MB-468 cells. Interestingly, Src Y416 but not Src Y527 was elevated together with EGFR Y845 and STAT3 Y705 phosphorylation upon Bay 11-7082

treatment (Fig. 2A). Inhibition of IKK using Bay 11-7082 enhances EGFR and Src physical interaction (Fig. 2A). In addition, stably expression of IKKα in MDA-MB-468 cells suppresses Src activity by preventing Src and EGFR association (Fig. 2B). To recapitulate EGFR and Src interaction, a DuoLink assay was performed under confocal microscopy. MDA-MB-468 cells stained with either EGFR or Src antibody showed no signal (data not shown). Co-staining with both EGFR and Src antibodies significantly amplified the signal of Texas red reporter (Fig. 2C, left panel) suggesting that they are in close proximity. Similar to earlier observation, stably expressing IKKα reduces EGFR and Src interaction (Fig. 2C, right panel).

We also compare the interaction between EGFR WT and S1026A. As EGFR S1026A induces better tumorigenesis potential, the binding affinity toward Src also gets increased (Fig. 2D). To target EGFR Y845 hyperactivation, we treated the cells with EGFR TKI (AG1478 and Iressa) and Src inhibitor (PP2 and Dasatinib). Interestingly, inhibition of EGFR kinase activity does not completely block Y845, whereas treatment of Src inhibitor completely abolishes Bay 11-7082 mediated activation (results will be included in the next progress report). These results indicate that the direct interaction between EGFR and IKKα abrogates EGFR and Src interaction, thereby affecting EGFR Y845 and STAT3 activations.

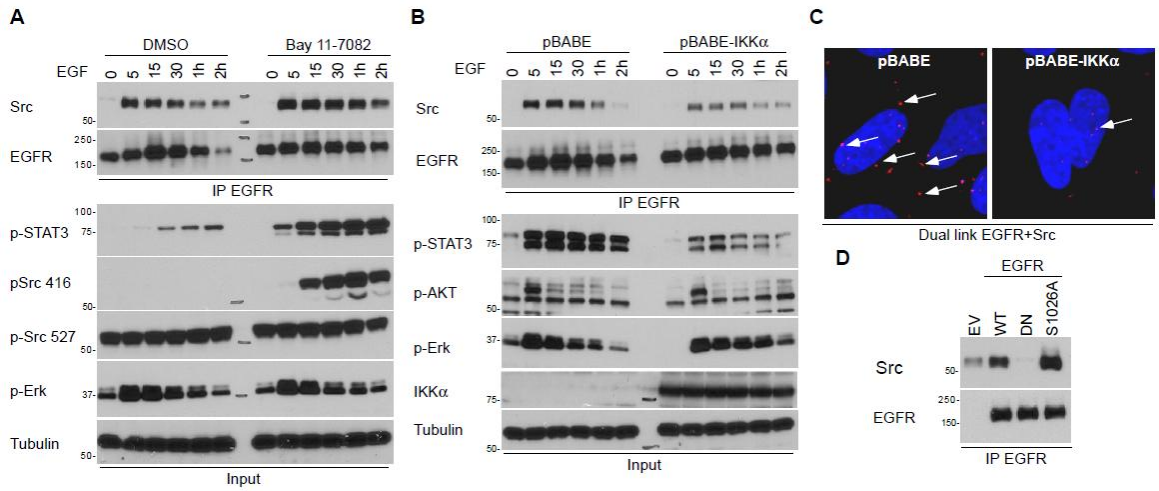


**Figure 2. IKKα inhibits EGF-induced EGFR-Src association and p-STAT3 activation.** (A) Inhibition of IKKα enhances EGF-induced EGFR-Src association. MDA-MB-468 cells were pretreated with Bay 11-7082. EGFR and Src interaction were analyzed using IP and Western blot. (B) IKKα interferes EGF induced EGFR-Src association. MDA-MB-468 cells stably expressing empty vector (pBABE) or IKKα were subject for IP/Western analysis. (C) Dual link analysis showing IKKα inhibits EGFR/Src interaction. (D) EGFR S1026 phosphorylation is required for IKKα mediated EGFR-Src disruption.

## Part III: IKKα does not affect EGFR ubiquitination and degradation

Phosphorylation of EGFR at Y1045 triggers Cbl-mediated ubiquitination and induces EGFR ubiquitination and proteasome-dependent protein degradation (12-14). It is therefore of interest to know whether IKKα affects EGFR signaling pathway by modulating protein turnover. To do this, we measure EGF mediated EGFR ubiquitination in both inhibitor and knockdown experiment. As shown in the Figure 3A, manipulating of

IKKα does not influence EGFR ubiquitination. In addition, overexpression of IKKα failed to induce EGFR ubiquitination (Fig. 3B and 3C). Consistent with our hypothesis, IKKα specifically affects EGFR Y845/Src interaction but not EGFR Y1045/Cbl mediated protein turnover.

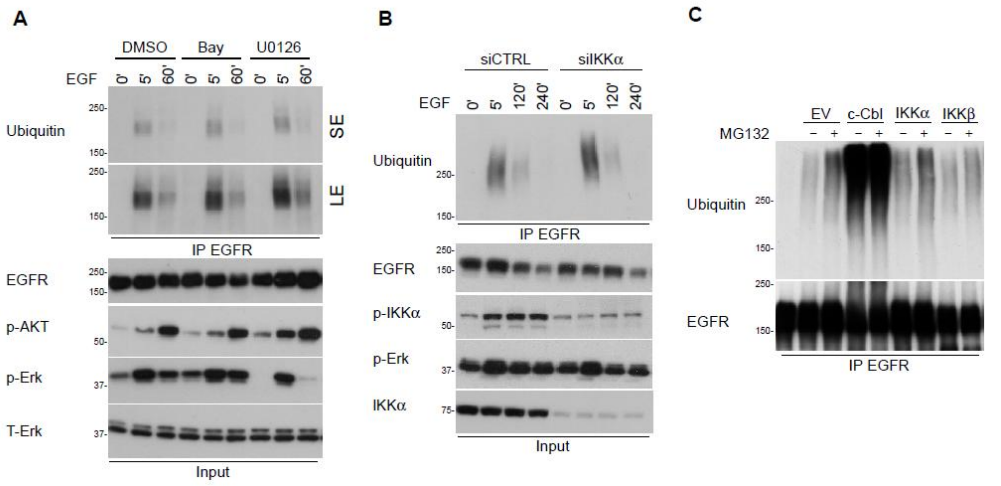


**Figure 3. IKKα does not affect EGFR Y1045 mediated ubiquitination.** (A) HeLa cells were pretreated with indicated inhibitors for 45 min and subject to EGF stimulation. Endogenous EGFR were than IP and Western blotted with anti-ubiquitin (P4D1) antibody. (B) HeLa cells with or without siIKKα were subject to ubiquitination assay. (C) HEK 239 cells were transient transfected with EGFR and c-Cbl, IKKα, or IKKβ. Ubiquitination were detected as described in (A).

## Part IV: IKKα specific phosphorylates EGFR at S1026

Because EGFR S1026 phosphorylation remains unidentified, we found EGFR S1026 is highly conserved across species (Fig. 4A). To recapitulate IKKα mediated EGFR S1026 phosphorylation, we purified and analyzed the phospho-EGFR S1026 antibody. As shown in Figure 4B, IKKα induce a nice phosphorylation of EGFR using a p-EGFR S1026 antibody. Mutation of S1026 to analine (S1026A) abolishes IKKα mediated EGFR phosphorylation. We next confirm the membrane localization of EGFR S1026 phosphorylation in MDA-MB-468 cells. Using a confocal microscopy, we detect a non-overlapped membrane colocalization between endogenous EGFR and p-EGFR S1026 expression (Fig. 4C). To test S1026 phosphorylation at physiological conditions, MEF cells were treated with RANKL at indicated time points. Ablation of IKKα abrogate RANKL mediated EGFR S1026 phosphorylation (Fig. 4D). To identify a physiological correlation, we investigated IKKα and p-EGFR S1026 expression in 13 human breast cancer cell lines. A positive correlation between IKKα and p-EGFR S1026 expression (correlation coefficient $r=0.63$, $p<0.05$) was found, suggesting that high IKKα promotes EGFR phosphorylation in breast cancer cells (Fig. 4E).

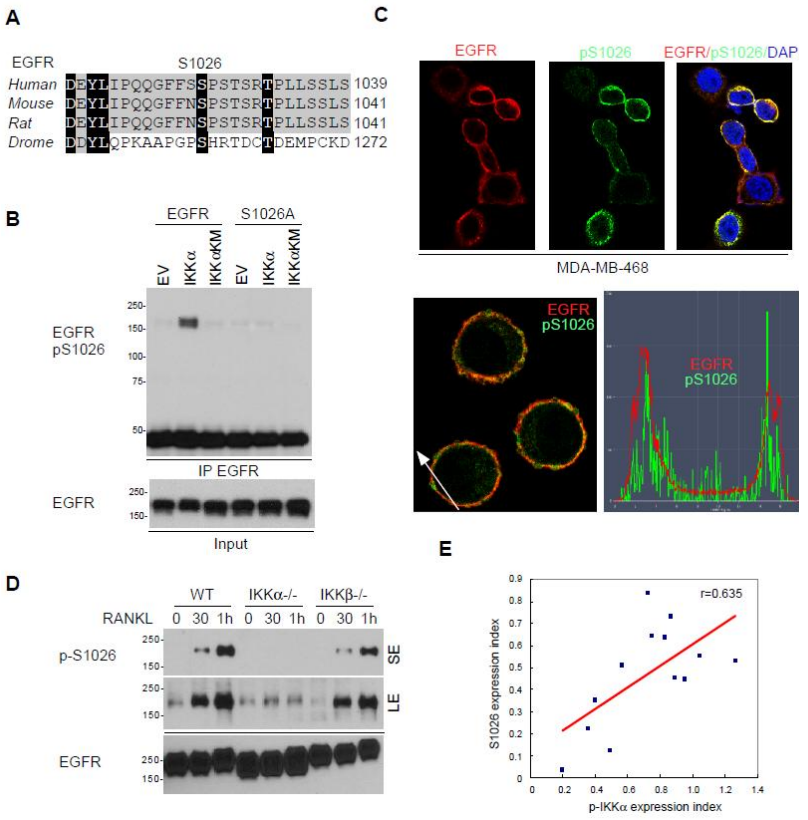


**Figure 4. Detection endogenous EGFR S1026 phosphorylation.** (A) Amino acid sequence alignment of EGFR among different species. (B) IKKα phosphorylates EGFR at S1026. HEK 293 cells were transfected with indicated plasmids. EGFR was IP and western blotted with p-EGFR S1026 antibody. (C) Membrane non-overlapped localization of EGFR and pS1026. MDA-MB-468 cells were fixed and subject to immunofluorescence staining using mouse anti-EGFR (AB13) and rabbit anti-p-EGFR S1026 antibody. (D) IKKα is required for EGFR S1026 phosphorylation. RANKL induced EGFR S1026 phosphorylation is abrogated in IKKα deficient MEF cells. (E) A positive correlation between p-IKKα and p-EGFR S1026 expression from 14 cell lines.

**Part V: The tumor suppressor function of IKKα in triple negative breast cancer**

To investigate IKKα-mediated STAT3 downregulation via EGFR, a luciferase reporter assay was performed. We found a STAT3 reporter, Lye6-Luc, responds to STAT3 CA (constitutive activate)-induced stimulation in HeLa cells, whereas overexpression of STAT3 DN fails to do so (data not shown). Moreover, co-expression of IKKα, but not IKKβ or IKKγ, significantly reduces STAT3 CA mediated reporter activity (Fig. 5A). Similar experiment were performed in HeLa-shCTRL and HeLa-shEGFR cells, we found that IKKα mediated STAT3 repression requires EGFR (Fig. 5B). To identify the potential STAT3 downstream target that regulated by IKKα, we examine gene expression profile of IKKα (NCBI gene ID: Chuk) using public data set generated from 917 cancer cell line (CCLE) (15). We compared the expression profile of IKKα and 60 STAT3 downstream targets in breast cancer cells (16). Among then, 12 genes show negatively correlated with IKKα expression using CCLE. Nonsupervised hierarchical clustering analysis was performed based on Erbb2, ERα (ESR1), PR (PgR) and EMT profile. Strikingly, the gene list was able to distinguish basal-like from luminal type breast cancer cells with high accuracy (90% properly segregated) (Fig. 5C). To identify the specific STAT3 downstream target that regulated by IKKα/EGFR signaling, the 14 gene expression profile was determined in EGFR stable clone and IKKα MEF cells by real-time PCR. Interestingly CCL2 was significantly increased in EGFR S1026A cells and IKKα -/- cells (Fig. 5D). These results indicate that CCL2 is the specific

STAT3 target downregulated by IKKα through EGFR.

We next asked if clinical distinct group of patient samples also shared the differential expression pattern of IKKα. First, we analyzed IKKα genes expression from Netherlands Cancer Institute (NKI) data set, *n*=295 (17). To do this, patients in the NKI cohort were first dichotomized according to expression levels of IKKα. As expected, two groups of breast cancer patients showed a significant difference in recurrence-free survival (RFS; Figure 5E). When the patients were dichotomized according to expression level of IKKα, RFSs of patients with higher expression of IKKα were significantly better than that of those with lower expression of IKKα (Fig. 5E).

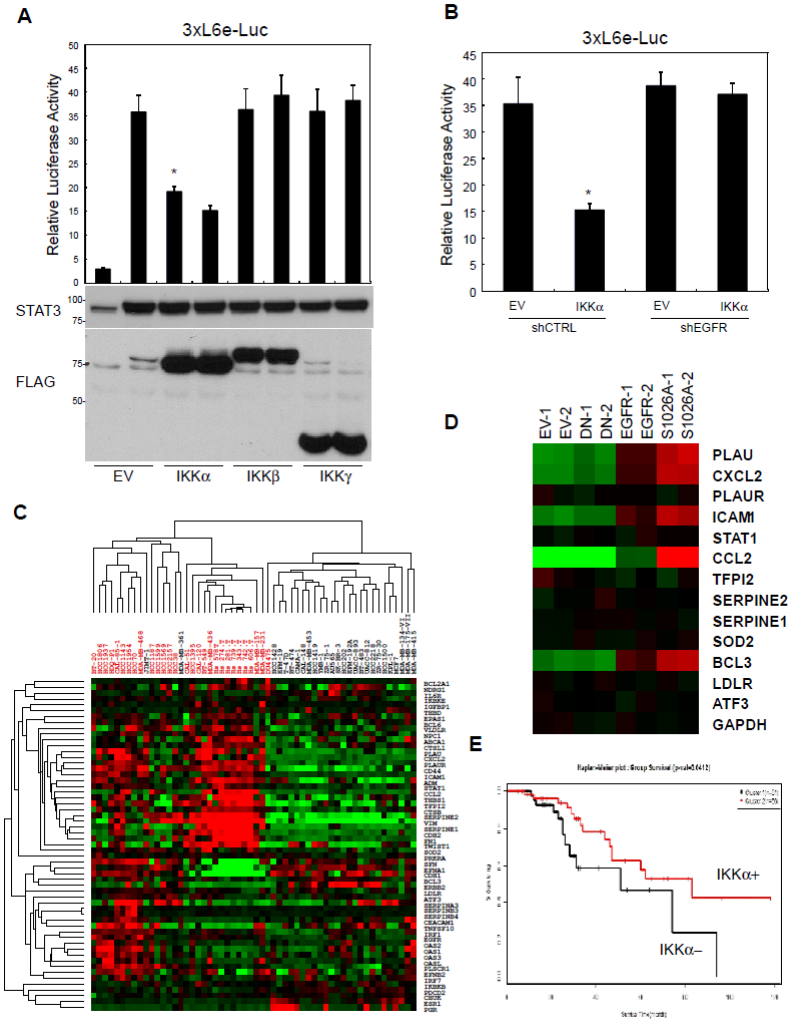


**Figure 5. IKKα downregulates CCL2 expression.** (A) IKKα inhibits STAT3 transcriptional activity. HEK-293 cells were transfected with the lye6-Luc together with indicated plasmids. The luciferase activity was measured and normalized according to *Renilla* luciferase activity. (B) EGFR is required for IKKα mediated STAT3 inhibition. (C) Heat map generated using 56 breast cell lines from the CCLE panel showing the levels of expression of IKKα (chuk) and STAT3 downstream targets. (D) Real time PCR showing S1026A induced STAT3 target gene expression. (E) Kaplan-Meier overall survival curves of IKKα in breast cancer patient data set.

**Part VI: IKKα depletion induces EGFR mediated tumorigenesis**

To determine the biological significance of homozygous IKKα-loss in MMTV-EGFR-induced mammary tumorigenesis, IKKα floxed MMTV-Cre mice were crossed with transgenic MMTV-EGFR mice to generate IKKα$^{-/-}$/EGFR mice. IKKα$^{-/-}$/EGFR and MMTV-EGFR virgin females were monitored for mammary tumor formation by weekly palpation. Hyperplasia occurrence in IKKα$^{-/-}$/EGFR mice and MMTV-EGFR mice was

followed up for a period of up to 48 weeks. We found that time-to-hyperplastic lesion development was shorter for IKKα$^{-/-}$/EGFR verse MMTV-EGFR mice. The earliest onset of hyperplastic lesion in the IKKα$^{-/-}$/EGFR was 40 weeks, whereas it was 48 weeks in the MMTV-EGFR mice. Therefore, IKKα-deficiency leads to accelerate hyperplastic lesion onset of MMTV-EGFR-induced mammary tumorigenesis. Whole-mount analyses of carmine-stained non-tumor-bearing glands were from different ages of IKKα$^{-/-}$/EGFR and MMTV-EGFR mice. Arrows indicate atypical hyperplastic lesion; LN, lymph node. We are still in the process of assessing whether loss of IKKα affecting EGFR-dependent tumor formation in a xerograph model (Fig. 6).

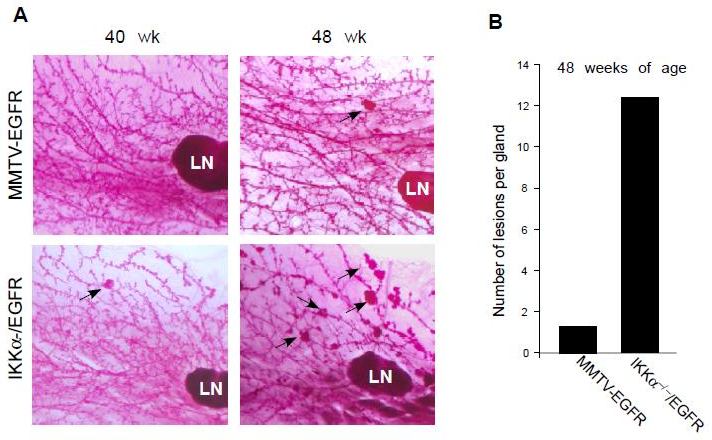


**Figure 6. IKKα-deficiency leads to accelerate tumor onset of MMTV-EGFR-induced mammary tumorigenesis.** (A) Whole-mount analyses of carmine-stained non–tumor-bearing glands were from different ages of IKKα$^{-/-}$/EGFR and MMTV-EGFR mice. Arrows indicate atypical hyperplastic lesion; LN, lymph node. (B) Quantification of hyperplastic mammary lesions.

**Part VII: Tumor necrosis factor alpha-induces EMT required p65-mediated transcriptional upregulation of Twist1. Supported by DoD funding, the PI has accomplished another project unraveling tumor microenvironment mediated breast cancer metastasis (see attached paper).**

In the past two DoD funding years, we also identify that chronic treatment with TNFα in breast cancer cells induces EMT phenotypic changes and stemness, and subsequently identified Twist1 as a novel modulator of this regulation. Our results establish a signaling axis by which tumor microenvironment elicits Twist1 expression that fosters cancer metastasis. Therefore, targeting NFκB-mediated Twist1 upregulation may provide favorable therapeutic strategies for breast cancer treatment (18).

**Part VIII: Phosphorylation of Twist1 by AKT1 Modulates Epithelial-Mesenchyme Transition in Breast Cancer Cells. Supported by DoD funding, the PI also serves as first author of another manuscript related to Triple Negative Breast Cancer (TNBC) treatment.**

Accumulating evidence from both cellular and genetic studies suggests AKT1/PKBα serves as a negative regulator of EMT during breast cancer metastasis. In this study, we found that AKT1 induced a phosphorylation-dependent ubiquitination and degradation of Twist1, engages the proteasome to Twist1-mediated EMT regulation. Our findings reveal a novel molecular concept by which non-specific inhibition of AKT may result in Twist1 stabilization to increase the metastatic potential in breast cancer cells. This manuscript is in submission to Cancer Cell. An abstract is attached. Manuscript is

available upon request.

## 3. CONCLUSION

EGFR, as an essential growth and survival factor, plays an important role in cancers of the lung, breast, brain, ovary, skin, and colon. The modification patterns of EGFR are critical for its function and the understanding of these EGFR modifications could help us design the optimal therapeutic strategies for targeting various EGFR-associated cancers and/or non-cancerous diseases. In current study, we identified that EGFR serine phosphorylation as a novel posttranslational modification playing an indispensable role in regulation of EGFR signaling pathways. We identified that IKKα is a serine/theronine kinase responsible for EGFR S1026 phosphorylation. Our data suggest that EGFR S1026 phosphorylation mainly affects its synergic interaction with Src. Similar to other serine/theronine phosphorylation, phosphorylation by IKKα downregulates EGFR signaling and thereby diminishes cell growth and tumorigenesis.

Our results also provide the first mechanistic evidence of how IKKα could serve as a tumor suppressor. Although conditional ablation of IKKα in keratinocyte resulting in skin cancer formation, the tumor suppressor function of IKKα remains elusive. Here, both knock-down and inhibitor analysis show that inhibition of IKKα augments EGFR tyrosine phosphorylation (mainly Y845), Src Y416 and STAT3 Y705. Src-activated signal pathway through Y845 is so called transphosphorylation activation pathway, whereas other EGFR activation pathway is autophosphorylation signal pathway. To our knowledge, IKKα binds to and phosphorylates EGFR. Expression of IKKα interferes with EGFR and Src interaction, and therefore diminishes EGFR Y845 transphosphorylation. Interestingly, inhibition of IKKα mediated hyperactivation is reversible. Using Src inhibitor, PP2, phosphorylation of Y845 on EGFR was reduced, suggesting that two types of EGFR activations are intrinsically correlated and interacted.

To look for possible tumor suppressor function of IKKα, we analysis human breast cancer cell line dataset from CCLE. We found that IKKα negative correlated with triple negative breast cancer phenotype. We also analysis two patient data set and found out IKKα relative to poor prognosis. This result provides the first evidence suggests the tumor suppressor function of IKKα in patient sample.

## 4. FUTURE WORKS:

The MMTV-hEGFR transgenic mice developed mammary epithelial hyperplasias, hypertrophy, or slight dysplasias in about 55% of mammary glands of animals examined. Since the inhibition of IKKα results in hyperactivation of EGFR to provide a survival advantage for cancer cells, we plan to create conditional knock out of IKKα in mammary gland and cross with EGFR overexpression mice to measure tumor onset. The age of the mouse in which mammary tumor is first palpable will be recorded and tumor size will be measured. Although our preliminary data indicate that mice lack of IKKα accelerate hyperplastic lesion, deletion of IKKα enhances EGFR mediated tumorigenesis remains unknown. We are now breading more $IKK\alpha^{-/-}$/EGFR mice to reach statistical significance. In the meantime, we will keep observing the tumorigenesis of mice for

longer period. Biopsies of tumor tissue will be obtained. To see if IKKα mediated EGFR phosphorylation is important in enhancing the malignant phenotype of EGFR induced tumor progression. Mice tumor section will be stained with EGFR S1026 antibody. Downstream signaling such as p-EGFR 845 and p-STAT3 will also be included to test our hypothesis.

## 5. KEY RESEARCH ACCOMPLISHMENTS: 2011-2012

a) Investigate the impact of EGFR S1026A in regulating EGFR Y845 and p-STAT3 phosphorylation in Cho cells (NIH3T3 cells have been done in the earlier year). As shown in Figure 1, EGFR S1026A shows an elevated phosphorylation of p-Y845 and p-STAT3.

b) Investigate the biological function of EGFR S1026A *in vivo* using orthotropic mammary mouse model.

c) Identification of IKKα as negative regulator in EGFR/Src synergetic activation.

d) Purification and characterization of phospho-EGFR S1026 antibody. Endogenous S1026 phosphorylation was detected using phospho-EGFR S1026 antibody. This phosphorylation is IKKα dependent and S1026 specific.

e) Identify CCL2 as IKKα/EGFR/STAT3 downstream target.

f) Two papers related to breast cancer metastasis (PI is the first author) have either been publish or submitted.

## 6. REPORTABLE OUTCOMES

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## 8. APPENDICES:

A. Cancer Research (2012) 72(5): 1290-1300
B. ABSTRACT of recent manuscript

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Cancer Research

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*Tumor and Stem Cell Biology*

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# Epithelial–Mesenchymal Transition Induced by TNF-α Requires NF-κB–Mediated Transcriptional Upregulation of Twist1

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## Abstract

Proinflammatory cytokines produced in the tumor microenvironment facilitate tumor development and metastatic progression. In particular, TNF-α promotes cancer invasion and angiogenesis associated with epithelial–mesenchymal transition (EMT); however, the mechanisms underlying its induction of EMT in cancer cells remain unclear. Here we show that EMT and cancer stemness properties induced by chronic treatment with TNF-α are mediated by the upregulation of the transcriptional repressor Twist1. Exposure to TNF-α rapidly induced Twist1 mRNA and protein expression in normal breast epithelial and breast cancer cells. Both IKK-β and NF-κB p65 were required for TNF-α–induced expression of Twist1, suggesting the involvement of canonical NF-κB signaling. In support of this likelihood, we defined a functional NF-κB–binding site in the *Twist1* promoter, and overexpression of p65 was sufficient to induce transcriptional upregulation of Twist1 along with EMT in mammary epithelial cells. Conversely, suppressing Twist1 expression abrogated p65-induced cell migration, invasion, EMT, and stemness properties, establishing that Twist1 is required for NF-κB to induce these aggressive phenotypes in breast cancer cells. Taken together, our results establish a signaling axis through which the tumor microenvironment elicits Twist1 expression to promote cancer metastasis. We suggest that targeting NF-κB–mediated Twist1 upregulation may offer an effective a therapeutic strategy for breast cancer treatment. *Cancer Res; 72(5); 1290–300. ©2012 AACR.*

## Introduction

The transcriptional factor NF-κB was initially characterized as a central regulator in response to pathogens and viruses. Subsequently, studies found that NF-κB is activated in a range of human cancers and to promote tumorigenesis via the regulation of target genes expression. In mammals, NF-κB binds to their target gene promoters as homo- or heterodimers composed of 5 subunits: RELA (p65), RELB, c-REL, NFκB1 (p105/p50), and NFκB2 (p100/p52). NF-κB activation is exclusively regulated by 2 independent pathways. In the canonical pathway, NF-κB activation is induced by various inflammatory stimuli, including TNF-α, interleukin-1 (IL-1); bacterial products, such as lipopolysaccharide (LPS); chemical inducers, such as phorbol-12-myristate-13-acetate (PMA); and reactive oxygen species, such as $H_2O_2$ through the IKKα/IKKβ/IKKγ complex. Upon stimulation, activated IKKβ phosphorylates the NF-κB inhibitor, IκBα, at Ser32 and Ser36 and triggers its rapid degradation through the β-TrCP–mediated 26S proteasome proteolysis, resulting in the liberation of the NF-κB. As a consequence, the NF-κB heterodimer translocates to the nucleus, binds to its cognate DNA motifs in the promoters, and induces a myriad of gene expression involved in immune response (TNF-α, IL-1, and cyclooxygenase 2), cell proliferation (cyclin D1 and c-MYC), angiogenesis (VEGF, IL-6, and IL-8), cell survival (XIAP, BCL-xL, and c-IAP2), invasion (matrix metalloproteinase-9), and EMT (Snail; refs. 1, 2). In contrast, the noncanonical pathway is activated by different types of inflammatory stimuli via IKKα homodimers that modulates of B-cell development and adaptive immune response (3).

Epithelial–mesenchymal transition (EMT), a complex reprogramming process of epithelial cells, plays an indispensable role in tumor invasion and metastasis (4). The well-defined features of EMT include loss of epithelial markers (E-cadherin and α- and γ-catenin), gain of mesenchymal cell markers

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

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(fibronectin, vimentin, and N-cadherin), and the acquisition of migratory and invasive properties (5). Currently, studies show that EMT is controlled by a group of transcriptional repressors, such as Zeb-1/2, Twist1, Snail, and Slug. Upon activation, these repressors recruit histone deacetylases to the E-box elements of the E-cadherin promoter, resulting in transcriptional silence of E-cadherin expression (6). Twist1, known as a master regulator of morphogenesis, induces EMT to facilitate breast tumor metastasis (7). The role for Twist1 in EMT regulation has also been reported in many other cancer types, including those of the prostate (8) and uterus (9). In addition to that in patients with breast carcinoma, high expression of Twist1 also correlates with tumor invasion and metastasis in patients with esophageal squamous cell carcinomas (10), hepatocellular carcinoma (11), and gliomas (12).

Inflammation, hypoxia, and tumor–stroma interactions are the major activators of metastatic cascade. This tumor microenvironment, which consists of infiltrated immune cells and their secretory cytokines and/or chemokines, facilitates cancer cell motility, invasiveness, and metastatic potential (13, 14). To date, extensive studies have pointed to NF-κB signaling as a critical inflammatory mediator in the response to invading pathogens. In addition, drugs and inhibitors aimed at targeting NF-κB have shown promising clinical implications (15). Therefore, determining how NF-κB mediates high malignancy to enhance cancer cell invasion, migration, and subsequent metastasis may provide novel therapeutic value. Indeed, activation of the NF-κB pathway is required for induction and maintenance of Ras- and TGF-β–dependent EMT (16). NF-κB also binds to the promoter of the E-cadherin repressor ZEB-1/2 resulting in regulation of the EMT phenotype (17). A recent study further suggested that inflammation-induced cell migration and invasion occur via NF-κB–mediated stabilization of Snail (18). Despite the presence of antiapoptotic cross-talk between Twist1 and NF-κB (19), the exact regulatory mechanism of NF-κB in EMT regulation has yet to be determined. Here, we examine the role of NF-κB activation in the EMT process and elucidate an important, but underdeveloped, proinflammation cytokine TNF-α–mediated breast cancer metastasis through the initiation of EMT. We show that rapid activation of NF-κB by TNF-α upregulates Twist1 expression through nuclear translocation of p65, which in turn activates *Twist1* gene expression, is an essential node for the chronic inflammation–induced EMT.

## Materials and Methods

Detailed information is included in Supplementary Information.

### Cell culture, stable transfectants, and transfection

MCF10A, MCF-12A, MDA-MB-453, HBL-100, BT-549, and HEK-293 cells were obtained from American Type Culture Collection. GP293 cells were purchased from Clontech. IKKα$^{-/-}$, IKKβ$^{-/-}$, and p65$^{-/-}$ mouse embryonic fibroblasts (MEF) were maintained as previous described (20–22). MCF10A was cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 5% horse serum, 10 μg/mL insulin, 20 ng/mL epidermal growth factor (EGF), 100 ng/mL cholera toxin, and 500 ng/mL hydrocortisone. IKKβ stable transfectants in MDA-MB-435 cells were selected using blasticidin S as described previously (20). For transient transfection, cells were transiently transfected with DNA using an SN liposomes (23), Lipofectamine 2000 (Invitrogen), or electroporation by a Nucleofector 1 device (Amaxa Biosystems) with electroporation buffer (137 mmol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L $Na_2HPO_4$, 6 mmol/L glucose, and 20 mmol/L HEPES, pH 7.0). For analysis of ligand-dependent Twist1 expression, cells were serum starved overnight and harvested directly or after stimulation at different time points.

### Mouse model of lung metastasis

Tumor metastasis assays were done using an intravenous breast cancer mouse model. The murine mammary tumor cell line 4T1-Luc was infected with lentiviral-based short hairpin RNA (shRNA) stable clones. Cells ($1 \times 10^5$) were then injected into the lateral tail vein of BALB/c mice (The Jackson Laboratory; 5 mice per group). Two weeks later, mouse were injected intraperitoneally either with PBS or 10 mg/mouse LPS in PBS. Lung metastasis was detected using an IVIS-100 imagining system (Xenogen). To measure lung metastases, animals were weighed before each experimental endpoint, and lung nodules were stained with India ink, excised, and counted immediately.

### Immunohistochemistry of human breast tumor tissue Samples

Immunohistochemistry (IHC) was done as described previously (20, 24, 25). Human tissue specimens were incubated with antibodies against IKKβ, p65, or Twist1 and a biotin-conjugated secondary antibody and then incubated with an avidin–biotin–peroxidase complex. Visualization was done using amino-ethylcarbazole chromogen. The human breast tumor samples used in cell fractionation and Western blots were provided by the breast tumor bank at The University of Texas MD Anderson Cancer Center. For statistical analysis, Fisher exact test and Spearman rank correlation coefficient were used, and a $P$ value less than 0.05 was considered statistically significant. According to histologic scoring, the intensity of staining was ranked into 4 groups: high (score 3), medium (score 2), low (score 1), and negative (score 0).

## Results

### TNF-α induces a rapid expression of Twist1

To study TNF-α–mediated EMT regulation, mammary epithelial cells derived from normal tissue, MCF10A, and HBL-100 cells were treated with TNF-α in the presence or absence of TGF-β for several passages (Supplementary Fig. S1A). As expected, we found that chronic exposure to TNF-α enhanced TGF-β–induced EMT signaling as indicated by E-cadherin expression. However, continuous treatment with TNF-α to passage 4 alone (2 days per passage) led to a loss of E-cadherin expression and promoted late EMT morphologic changes compared with that of TGF-β treatment (Supplementary Fig. S1A and S1B). To identify the genetic signatures that are involved in modulation of TNF-α–mediated EMT, RT$^2$ Profiler PCR array (SuperArray Bioscience Corporation) containing 84 well-characterized EMT mediators was done. Between 2 tested cell lines, Twist1 mRNA was the only one found to be

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significantly upregulated upon TNF-α stimulation (Supplementary Fig. S1C). Various growth factors and cytokines, including EGF, IGF-1, TGF-α, TGF-β, Wnt3a, TNF-α, IFN-γ, HB-EGF, and IL-1β, were tested to validate their ability to induce Twist1 expression. When MCF10A cells were treated with various ligands for 2 hours, we found that TNF-α rapidly induced Twist1 expression to a degree similar to that in cells treated with IL-1β (Fig. 1A). Next, we measured the time-dependent expression of Twist1 and found that it increased significantly after 1 hour of TNF-α stimulation and reached maximal level after 2 to 4 hours (Fig. 1B). This regulation is present not only in mammary epithelial cells derived from normal tissue such as MCF10A and HBL-100 but also in breast cancer cells (BT-549 and MDA-MB-435), suggesting that TNF-α–induced Twist1 expression might be a general phenomenon (Fig. 1B). Next, to determine whether NF-κB is responsible for the TNF-α/IL-1β–induced Twist1 expression, several NF-κB inducers, such as LPS, PMA/Inomycin, and $H_2O_2$ as well as the IKKβ small molecule inhibitor TPCA-1 were used to test their effects on Twist1 expression. As shown in Fig. 1C, Twist1 expression was upregulated in response to NF-κB inducers with a similar degree of increase at 2-hour treatment. Similarly, Twist1 expression correlated with the activation status of NF-κB (using phosphorylated IκBα as readout) in both MCF10A and HBL-100 cells (Fig. 1C and Supplementary Fig. S1D). Given that TNF-α activation induces p65 nuclear translocation, we examined endogenous p65 and Twist1 localization in BT-549 (Fig. 1D and Supplementary Fig. S2) and MCF10A (Supplementary Fig. S1E) cells and found that both TNF-α and IL-1β induced nuclear translocation of p65 2 hours after treatment. Meanwhile, under the same exposure condition, we observed an increase in the level of nuclear Twist1 by confocal microscopy (Fig. 1D, middle). To further confirm the upregulation of Twist1 and p65 nuclear translocation, nuclear and cytoplasmic

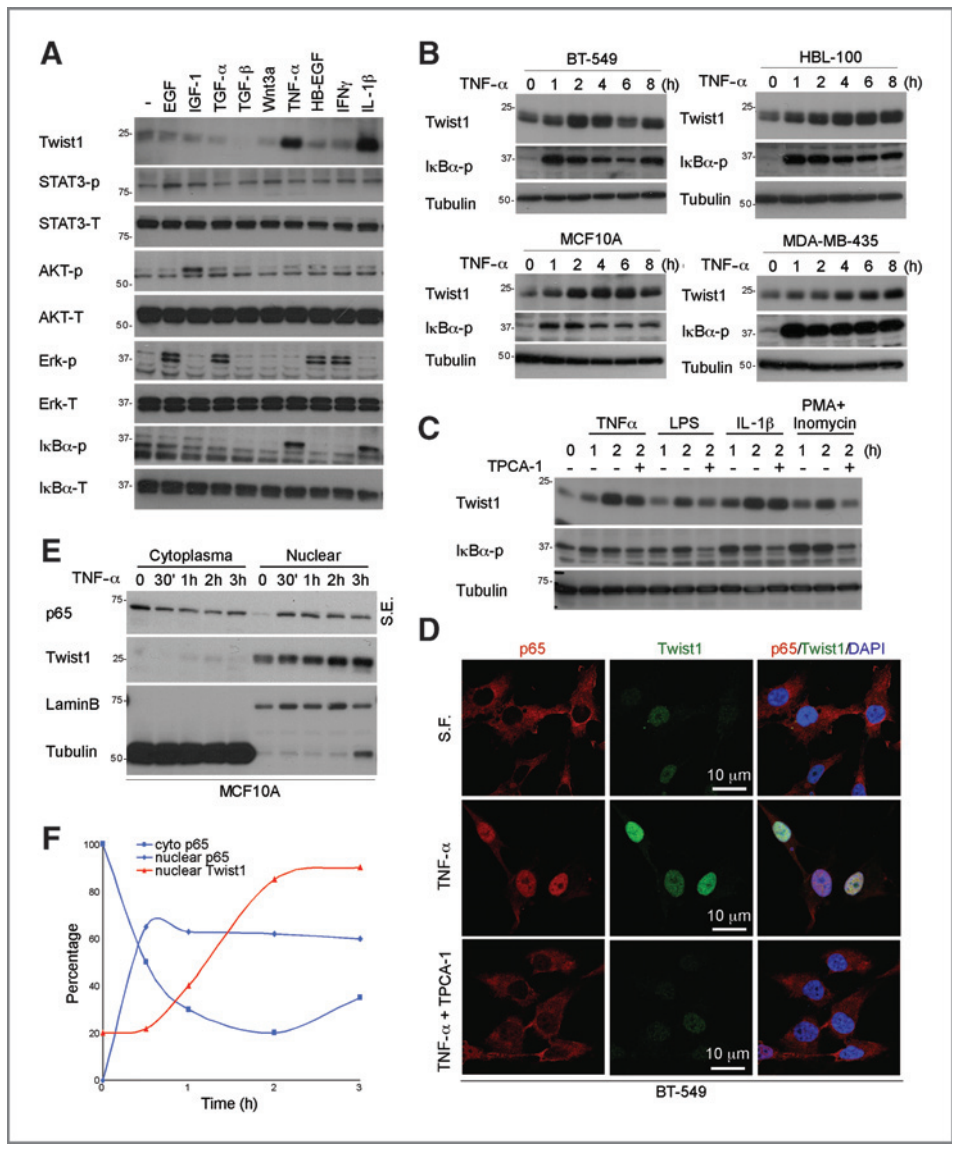


Figure 1. Activation of NF-κB induces Twist1 expression. A, cells were serum starved overnight and then treated with 30 ng/mL EGF, 25 ng/mL IGF-1, 1 μg/mL TGF-α, 100 nmol/L TGF-β, 30 ng/mL Wnt3a, 10 ng/mL TNF-α, 10 ng/mL HB-EGF, and 50 ng/mL IFNγ for 2 hours. Protein expression were analyzed by Western blot. B, BT-549, HBL-100, MCF10A, and MDA-MB-435 cells were serum starved overnight and then treated with 10 ng/mL TNF-α for the indicated periods. C, serum-starved MCF10A cells were treated with various NF-κB activators, TNF-α (10 ng/mL), LPS (1 ng/mL), IL-1β (10 ng/mL), and PMA/Inomycin (PMA, 10 nmol/L and Inomycin, 100 nmol/L) at indicated time point. TPCA-1 was applied 30 minutes before the experiment. D, MCF10A were treated with TNF-α for 2 hours. After fixation, the cellular location of endogenous p65 (red) and Twist1 (green) were analyzed by confocal microscopy. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, blue). S.F., Serum-free. E, MCF10A cells were treated with TNF-α at different time point. Cytosolic and nuclear p65 and Twist1 protein were separated using hypotonic buffer. Tubulin and lamin B indicate cytosolic and nuclear fraction, respectively. S.E., short exposure. F, densitometric analysis of the Western blot.

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fractions of MCF10A cells were isolated at different time points upon treatment with TNF-α (Fig. 1E). We observed that TNF-α induced nuclear translocation of p65 at 30 minutes, whereas the nuclear expression of Twist1 began to increase 1 hour after treatment (Fig. 1F). These results suggested that TNF-α triggers a dynamic interaction between nuclear translocation of p65 and nuclear expression of Twist1.

### IKKβ is also required for TNF-α–induced Twist1 expression

Because TNF-α can induce activation of various signaling pathways, we wanted to determine which signaling cascade is responsible for TNF-α–mediated Twist1 expression. To do so, MCF10A cells were serum starved overnight and pretreated with various inhibitors prior to TNF-α stimulation. We found that upregulation of Twist1 by TNF-α was not affected by mitogen-activated protein kinase/extracellular signal–regulated kinase, mTOR, p38, or JNK kinases inhibitors. In contrast, IKKβ inhibitors, BAY 11–7082 and parthenonlide, both abrogated TNF-α–induced Twist1 expression (Fig. 2A). To diminish the off-target effect of these chemical inhibitors and further validate the role of IKKβ in TNF-α–induced Twist1 expression, we introduced a lentiviral-based IKKβ shRNA into MCF10A cells. Consistently, silencing IKKβ expression level also attenuated TNF-α–induced Twist1 expression (Fig. 2B). Interestingly, we showed that activation of IKKα via receptor activator of NF-κB ligand treatment (Supplementary Fig. S3A) or silencing IKKα expression (Supplementary Fig. S3B) had no effect on TNF-α–induced Twist1 expression. We also conducted experiments using previously established IKKβ and IKKα knockout MEFs (20). As shown in Supplementary Fig. S3C, we detected TNF-α–induced Twist1 expression in wild-type MEFs but not in IKKβ-deficient MEFs. Reexpression of wild-type IKKβ but not an IKKβ kinase-dead mutant (KA)

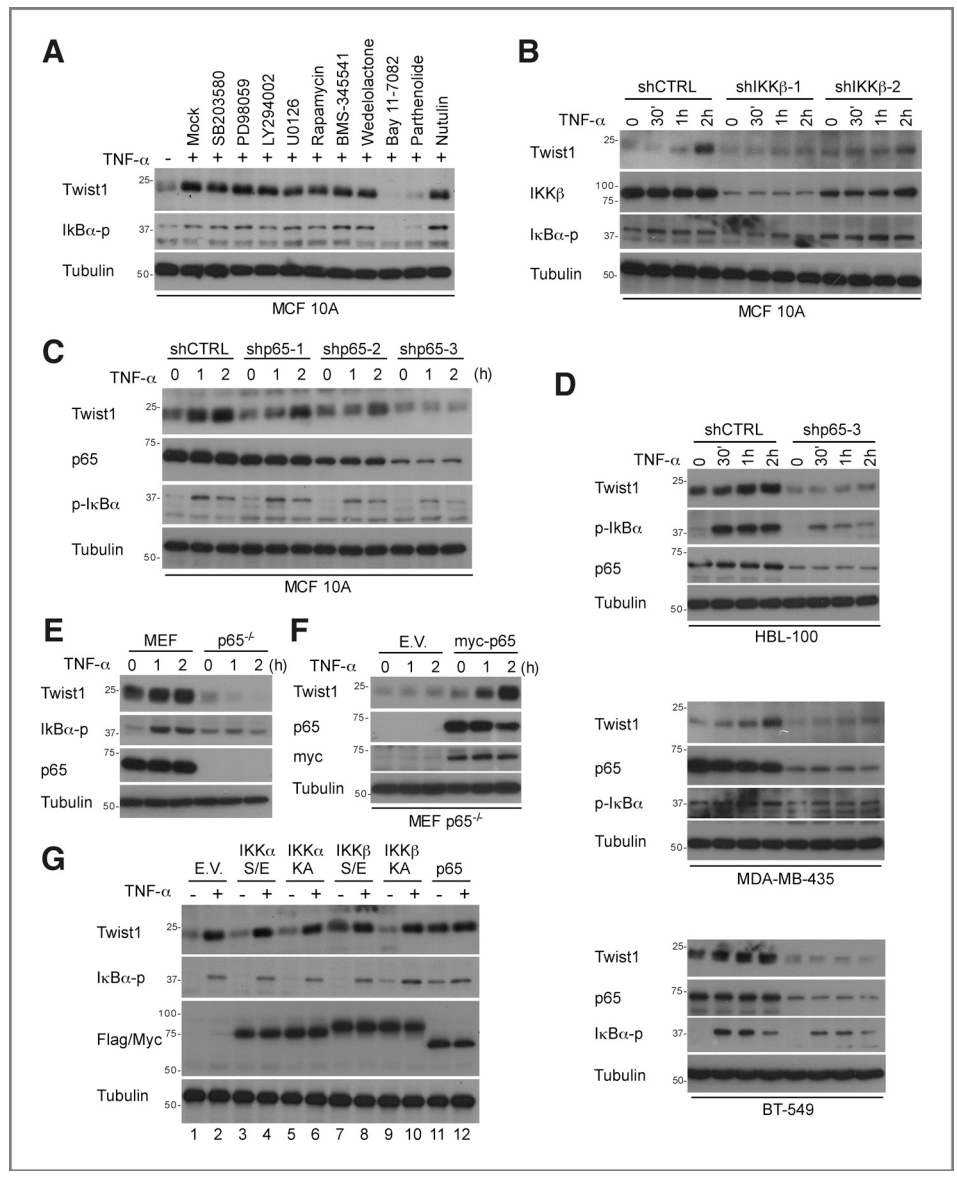


Figure 2. NF-κB is required for TNF-α–mediated Twist1 expression. A, MCF10A cells were treated with SB203580, PD98059, LY294002, U0126, rapamycin, BMS-345541, wedelolactone, 40 μmol/L Bay 11–7082, 80 μmol/L parthenolide, and nutulin for 30 minutes. B, 2 individual shIKKβ stable clones of MCF10A cells were serum starved overnight and treated with TNF-α at various time points. C, ShCTRL and shp65 stable clone of MCF10A cells were serum starved overnight and treated with TNF-α at various time points. D, control or shp65-3 was expressed in HBL-100, BT-549, and MDA-MB-453 cells followed by treatment with TNF-α or a vehicle for up to 2 hours. The protein expression of Twist1, p65, and p-IκBα was analyzed using Western blot. E, MEF and $p65^{-/-}$ MEF cells were serum starved overnight and then treated with 10 ng/mL TNF-α or a vehicle. F, Myc-p65 was transiently transfected into $p65^{-/-}$ MEFs to restore p65 expression. TNF-α–mediated Twist1 expression was analyzed using Western blot. G, flag-tagged IKK or Myc-tagged p65 was transiently expressed in HBL-100 cells. The protein expression of Twist1 and p-IκBα were examined using Western blot.

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restored TNF-α–induced Twist1 expression (Supplementary Fig. S3D), suggesting that the kinase activity of IKKβ is required. Similarly, in low–IKKβ-expressing MDA-MB-453 cells, Twist1 expression was not affected by TNF-α; however, reintroducion of IKKβ by stable transfection elevated the TNF-α–induced Twist1 expression (ref. 20; Supplementary Fig. S3E). Altogether, we concluded that the canonical IKKβ-dependent NF-κB signaling is required for TNF-α–induced Twist1 expression.

### TNF-α–mediated Twist1 expression is dependent on p65 activation by IKKβ

Because activation of NF-κB cascade usually results in nuclear translocation and activation of p65, we hypothesized that p65 might be involved in TNF-α–induced Twist1 expression. To elucidate the causal relationship between p65 and Twist1, p65 was stably knocked down using 3 independent shRNAs in MCF10A cells. We found that knockdown of endogenous p65 expression attenuated TNF-α–induced Twist1 expression (Fig. 2C). Moreover, stable clones harboring high levels of p65 expression showed a higher Twist1 expression in response to TNF-α treatment. These results also ruled out the off-target effects due to shRNA-mediated gene silencing (Fig. 2C). Consistently, knockdown of p65 expression also inhibited TNF-α–induced Twist1 expression in BT-549, HBL-100, and MDA-MB-435 cells (Fig. 2D). In addition, TNF-α rapidly induced Twist1 expression in wild-type ($p65^{+/+}$) MEFs but not in p65-deficient ($p65^{-/-}$) MEFs (Fig. 2E). Restoration of myc-tagged p65 in $p65^{-/-}$ MEFs rescued TNF-α–induced Twist1 expression, further supporting that p65 is required for TNF-α–mediated Twist1 expression (Fig. 2F). To further confirm this finding, we expressed constitutively active or kinase-dead IKKα, IKKβ, or p65 in HBL-100 cells and then treated with TNF-α. Expression of both constitutively active IKKβ (Fig. 2G, lane 7) and p65 (Fig. 2G, lane 11) was sufficient to induce Twist1 expression to a degree similar to that of TNF-α treatment. To establish a clinical relevance of inhibition of NF-κB–mediated Twist1 expression, both MCF10A and HBL-100 cells were pretreated with nonsteroidal anti-inflammatory drugs and subjected to TNF-α stimulation. When these cells were pretreated with another commonly used NF-κB inhibitor, sanguinarine, and tosyl phenylalanyl chloromethyl ketine 1 (TPCK-1), TNF-α–induced Twist1 expression was abolished (Supplementary Fig. S3F and S3G). Therefore, targeting NF-κB–mediated Twist1 expression implicates a novel aspect for breast cancer therapy.

### TNF-α–induced Twist1 expression is transcriptionally regulated by p65

Because the TNF-α–induced Twist1 expression requires p65, it would be of interest to determine whether TNF-α–induced Twist1 expression is transcriptionally regulated. Indeed, TNF-α elevated Twist1 mRNA expression at 1 hour of treatment in MCF10A and HBL-100 cells (Fig. 3A and data not shown). Consistent with this finding, Twist1 expression induced by TNF-α, LPS, or IL-1β was abrogated when cells were pretreated with a transcription inhibitor (actinomycin D) or a protein synthesis inhibitor (cycloheximide; Fig. 3B). Because Twist1 undergoes protein degradation via 26S proteasome machinery (26), we also tested whether the activation of NF-κB affects Twist1 protein stability. As shown in Supplementary Fig. S4C and S4D, the Twist1 protein half-life was not influenced by TNF-α treatment or coexpression of p65, suggesting that TNF-α induces Twist1 expression exclusively via transcriptional regulation.

### p65 Binds directly to the *Twist1* promoter to regulate its expression

The p65 protein is a multifunctional transcription factor that elicits its physiologic function by regulating target gene expression upon NF-κB activation. To investigate the molecular mechanism by which TNF-α induces Twist1 expression, we used 3 bioinformatics programs to identify the putative binding sites for p65 on the *Twist1* promoter. We found that the *Twist1* promoter sequence from −970 to +1 contains 4 p65-binding sites, 2 of which represent a consensus among 3 predications (Supplementary Fig. S4A), suggesting that p65 might regulate Twist1 expression by directly binding to its promoter. Using a luciferase reporter construct, Twist1-Luc responded to TNF-α stimulation in HEK-293 (Fig. 3C) and MCF10A cells (Supplementary Fig. S4E). In contrast, treatment with TPCA-1 (an IKKβ inhibitor) abrogated TNF-α–mediated *Twist1* promoter activities. Moreover, coexpression of p65 and Twist1-Luc significantly enhanced the reporter activity but not IKKα or dominant negative IKKβ (Supplementary Fig. S4B).

Furthermore, to locate the authentic p65-binding sites, a nested deletion of Twist1-Luc (D1, D2, D3, and D4) was generated. Among the 5 constructs, a p65-4 element alone on the *Twist1* promoter maintained high reporter activity by p65 induction, indicating that the critical p65 DNA-binding elements are located in the 120-bp region of the promoter (Fig. 3D). To pinpoint the exact binding motifs, we introduce point mutations into the p65-3 and p65-4 elements of Twist1 D4-Luc (Fig. 3E, left panel). Ablation of the p65-4–binding site on the Twist1 promoter abrogated p65-mediated Twist1 expression (Fig. 3E). We also transient transfected Twist1 D4-Luc into a stable clone of MCF10A-expressing p65 shRNA and showed that cells harboring high level of p65 posses higher reporter activity, confirming that endogenous p65 is critical for Twist1 expression (Supplementary Fig. S4F and S4G).

To further examine the binding of p65 to the Twist1 promoter *in vivo*, a chromatin immunoprecipitation (ChIP) assay was done using stable MCF10A-p65 cells. Upon TNF-α stimulation, nuclear p65 bound to the human *Twist1* gene promoter at 30 minutes. In contrast, immunoglobulin G did not associate with the *Twist1* promoter at a detectable level. The binding of p65 to the *Twist1* promoter was released by treatment with TPCA-1 (Fig. 3F). Moreover, gel sift assay was also conducted to confirm that p65 is bound to the *Twist1* promoter *in vitro* (data not shown). Collectively, these results suggest that p65 regulates Twist1 transcription by directly binding to the *Twist1* promoter in a TNF-α–dependent fashion.

### p65-mediated Twist1 expression results in EMT

To determine the functional consequences of p65 activation in breast cancer cells, ectopic expression of p65 in

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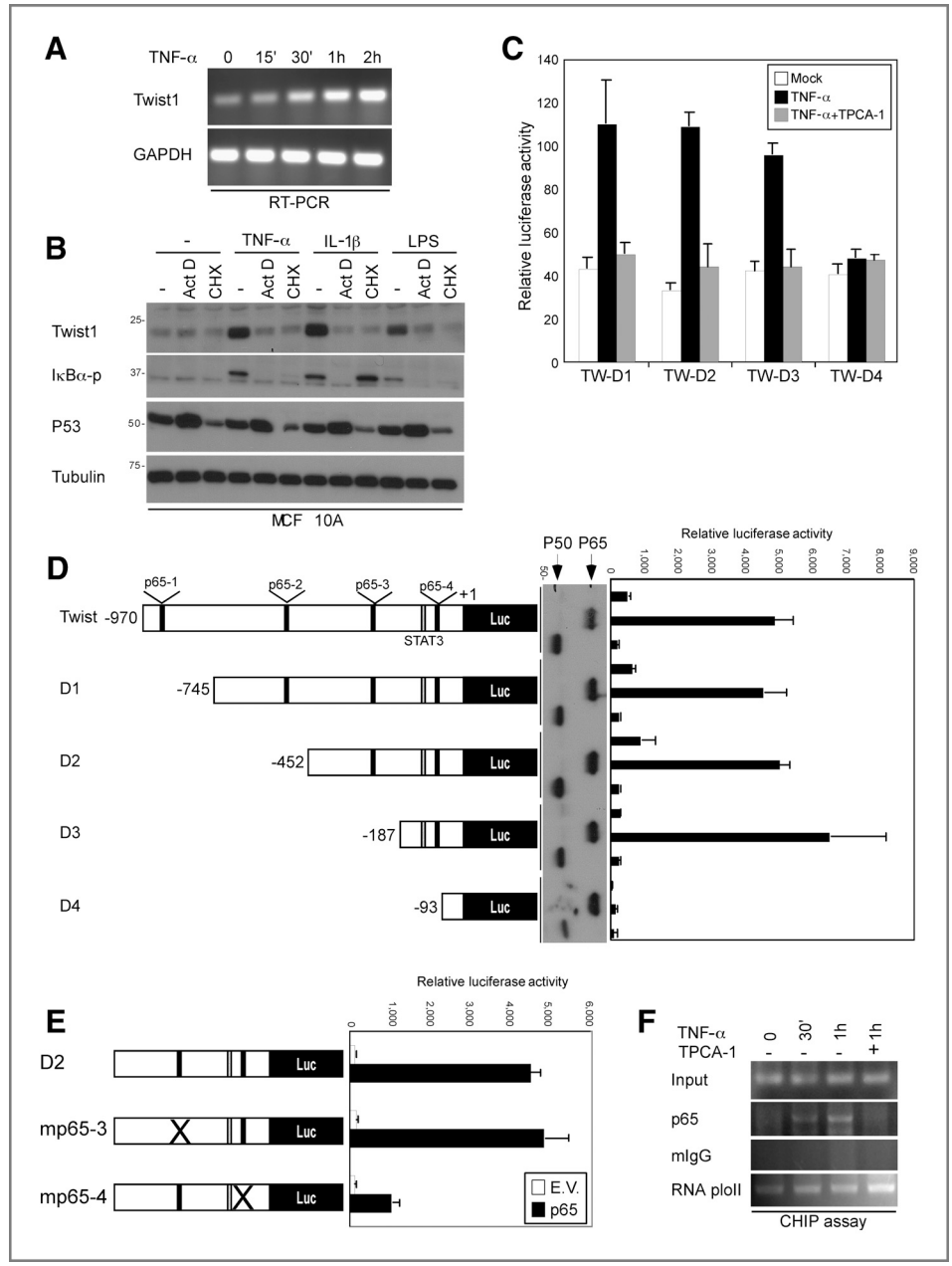


**Figure 3.** p65 transcriptionally regulates Twist1 expression. A, mRNAs isolated from TNF-α–treated MCF10A cells were subjected to RT-PCR using primer sets specific against Twist1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, MCF10A cells were pretreated with 500 ng/mL Act D and 10 μg/mL cycloheximide (CHX) for 1 hour, stimulated with various agents for 2 hours, and subjected to Western blot with the indicated antibodies. C, HEK-293 cells transfected with the indicated *Twist1* promoter were treated in the presence or absence of 10 ng TNF-α and TPCA-1 for 2 hours. The luciferase activity was measured and normalized according to *Renilla* luciferase activity. D, a series of deletion mutants of the *Twist1* promoter were introduced to HEK-293 cells together with or without p65 and p50 (expression showed in the middle panel). E, identification of p65-binding site on *Twist1* promoter. Wild-type and p65-binding element–mutated *Twist1* promoter luciferase was transiently expressed in HEK-293 cells. The relative luciferase activity is present as the means ± SE from 3 independent experiments. F, ChIP of p65 in response to TNF-α treatment.

MCF10A cells was accomplished using a retroviral infection. H-RasV12, which is known to induce EMT in various types of cells (27), was used as a positive control. Compared with empty vector-infected cells (pBABE), p65-expressing cells exhibited spindle-like morphology, loss of cell contact, and formation of vimentin fibers reminiscent of EMT (Fig. 4A, phase contrast micrograph). The EMT-like phenotypic changes were confirmed by detecting expression of characteristic molecular markers using immunofluorescence (Fig. 4A, immunostaining) and Western blot (Fig. 4B). In p65-expressing cells, the expression of mesenchymal markers fibronectin, N-cadherin, and vimentin was significantly upregulated, whereas that of the epithelial marker E-cadherin was downregulated. We observed similar results using MCF-12A cells infected with a p65-expressing retrovirus (Supplementary Fig. S5A–S5C). MCF10A-p65 cells showed increased cellular migration and invasion abilities as measured by a wound healing assay and Boyden chamber assay in media lacking EGF, respectively (Fig. 4E and F). Interestingly, we observed a significant upregulation of Twist1 expression in p65 overexpressing MCF10A and MCF-12A cells. The increase in Twist1 expression was further enhanced by treatment with TNF-α (Supplementary Fig. S5D).

To test whether upregulation of Twist1 expression is required for p65-induced EMT, Twist1 expression was knocked

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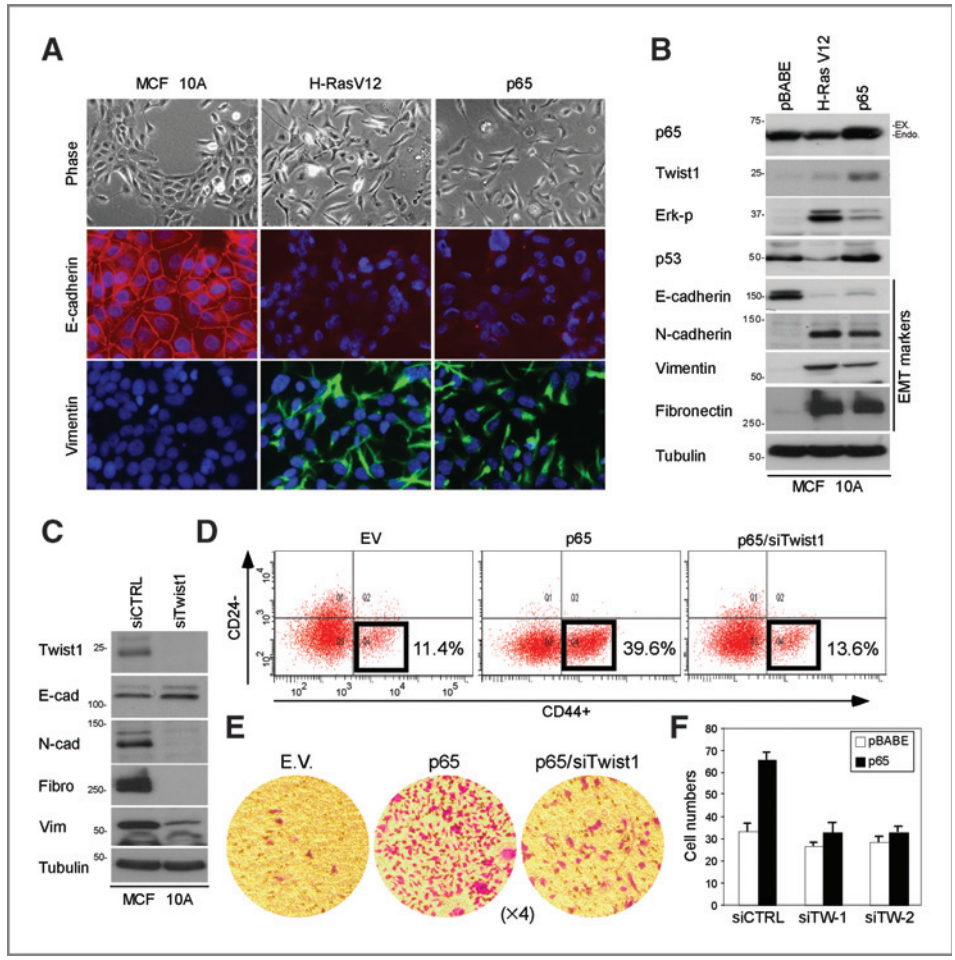


**Figure 4.** p65 overexpression upregulates Twist1 expression and induces changes in epithelial cell morphology. A, phase-contrast and immunofluorescent micrographs showing the morphologic appearance of MCF10A cells infected with pBABE (empty vector) as compared with that of cells infected with pBABE-H-Ras V12 and pBABE-p65. B, Western blot analysis of the protein expression for p65, Twist1, and EMT markers in MCF10A stable clones shown in A. C, Western blot analysis of mesenchymal markers in MCF10A-p65 cells with Twist1 siRNA. D, abrogation of p65-mediated cancer stem cell population by Twist1 suppression. E, reduction of p65-mediated cell invasion by Twist1 suppression. F, reduction of p65-mediated cell migration by Twist1 suppression.

down in the MCF10A-p65 stable cells. This knockdown inhibited cell migration, invasion, and formation of EMT phenotype (Fig. 4C), suggesting that Twist1 is required for p65-mediated EMT phenotypic changes. It has been documented that Twist1 modulates breast cancer stem cells via transcriptional suppression of CD24 expression (28). Therefore, we asked whether the p65–Twist1 axis regulates breast cancer cells side population. Indeed, p65 overexpression could induce $CD24^{-}/CD44^{+}$ population in 2 different breast epithelial cell lines (MCF10A in Fig. 4D and MCF-12A in Supplementary Fig. S5C) and that downregulation of Twist1 expression by siRNA partially reversed the stem cell molecular signature by reducing p65-induced cancer stem cell population (Fig. 4D, right). In addition, we found that Twist1 is required for p65-mediated mammosphere formation (Supplementary Fig. S5E). Together with cell migration and invasion assays (Fig. 4E and F), these results identified a prerequisite role for Twist1 in p65-mediated breast tumor progression.

### Inflammation-induced Twist1 upregulation increases metastatic potential

To test whether constitutive Twist1 expression contributes to TNF-α–induced EMT, the endogenous Twist1 was knocked down in MCF10A cells using a lentivirus-based shRNA (shTwist1). We found that 3 independent shRNA constructs efficiently knocked down endogenous Twist1 expression, as confirmed by Western blot (Fig. 5B). Inhibition of Twist1 expression in MCF10A cells significantly reduced TNF-α–mediated EMT at passage 3, whereas cells infected with shRNA against luciferase (shCTRL) exhibited EMT (Fig. 5A). Moreover, Twist1 knockdown resulted in increased E-cadherin and reduced fibronectin expression. Thus, suppression of Twist1 expression in MCF10A cells partially reversed TNF-α–induced EMT (see above). Consistent with these phenotypic changes, TNF-α–induced breast cancer stem cell population was abolished by Twist1 inhibition (Fig. 5C). We then confirmed our finding using a xenograft lung metastasis model in which administration of the inflammation inducer LPS enhances lung metastasis in mice. Our *in vivo* metastasis assay showed that knockdown of Twist1 expression in 4T1-Luc cells antagonized LPS-induced metastasis by measuring the number of lung nodules formed in mice (Fig. 5D and E). Although Twist1 had little effect on intrinsic metastatic potential, it had a significant impact on inflammation-induced metastasis (82% lower in lung nodules vs. shCTRL in LPS-treated mice). Thus, these results suggest that inflammation-induced upregulation of Twist1 expression plays an essential role in breast cancer metastasis.

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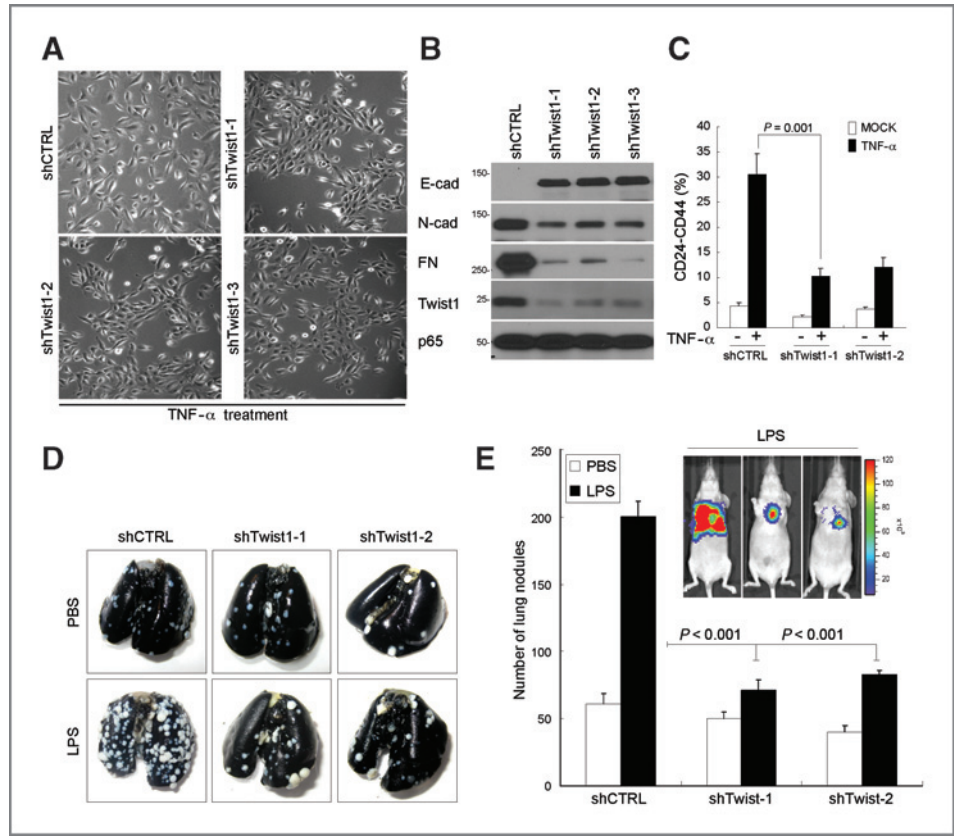


Figure 5. Twist1 is required for inflammation-induced metastasis. A, phase-contrast images of EMT morphotypic changes in MCF10A shCTRL and shTwist1 stable cells treated with TNF-α. B, Western blot of the EMT markers from cells in A. C, abrogation of TNF-α–mediated cancer stem cell population by Twist1 suppression. D, representative photograph of metastatic lung nodules. 4T1-Luc cells with control and 2 shTwist1 stable clones were injected into BALB/c mice via the tail vein. The mice then received intraperitoneal injection of saline or 10 μg LPS. Seven days later, the mice were sacrificed and the entire lungs were stained with India ink and resected. E, quantification of the lung nodules in C. The error bars represent SD for $n = 5$.

### IKKβ/nuclear p65 associates positively with Twist1 in cancer cell lines and primary breast carcinomas

To elucidate the clinical relevance of NF-κB activation and Twist1 expression, the association of their cDNA expression was examined by reanalyzing NCI-60 microarray databases from a total of 60 various cancer cell lines. A strong correlation was found between Twist1, p65, and IKKβ expression (data not shown). To determine the significance of p65/Twist1 in the EMT, we selected 37 cell lines from the NCI-60 panel and found that expression of the Twist1 was inversely correlated with that of E-cadherin (correlation coefficient $r < -0.8$; Fig. 6A, Supplementary Fig. S6A and S6B), indicating the functional significance of the Twist1 in these cell lines. As shown in Fig. 6A, the expression of Twist1 was significantly correlated with that of p65 ($r = 0.529$; Supplementary Fig. S6D) and IKKβ ($r = 0.630$; Supplementary Fig. S6C).

We next asked whether overexpression of Twist1 in the breast cancer cells might be a result from NF-κB activation. Because nuclear p65 reflects the active state of NF-κB (18) and the functional Twist1 is known to localize in the nucleus, we measured the expression of p65 and Twist1 in nuclear extracts from 14 different cancer cell lines (Fig. 6B). As expected, the nuclear fraction of p65 level was highly correlated with the nuclear Twist1 ($r = 0.804$, $P = 0.0013$; Fig. 6D). These results are also consistent with the earlier finding that nuclear Twist1 expression is associated with p65 nuclear translocation (Fig. 1D–F). To determine the clinical correlation of p65 and Twist1 protein expression in human breast cancer, we examine their expression in 14 freshly isolated low- and high-grade breast tumor samples. On the basis of our data, p65 and Twist1 expression levels were elevated in high-grade tumors, indicating that coexpression of p65 and Twist1 enhances the aggressive phenotype of breast cancer cells (Fig. 6C).

### Clinical significance of activation of the IKKβ–p65–Twist1 axis in a cohort of primary breast carcinomas

To further examine our findings in human primary tumors, we studied the expression of IKKβ, p65, and Twist1 in 115 human primary breast tumor specimens using IHC analysis. Twist1 was detected in 67 (51%) of the 82 specimens with high p65 expression but in only 10 (7.6%) of the 49 specimens with low p65 expression, indicating that p65 expression associates with high levels of Twist1 expression ($P < 0.0001$; Table 1). Consistent with this finding, we found that IKKβ expression associates with Twist1 ($P < 0.023$; Table 1) and p65 expression ($P < 0.017$; Supplementary Table S1) expression. Next, we analyzed their expression with other clinical records and found strong activation of IKKβ–p65–Twist1 axis in patients with lymph node metastasis (Supplementary Table S2). We also analyzed the expression of p65 and Twist1 in breast tumor tissues and correlated the findings with patient survival data. The Kaplan–Meier overall survival curves showed that high p65 and Twist1 expression levels were associated with poor survival (Supplementary Fig. S6E and S6F). However, the

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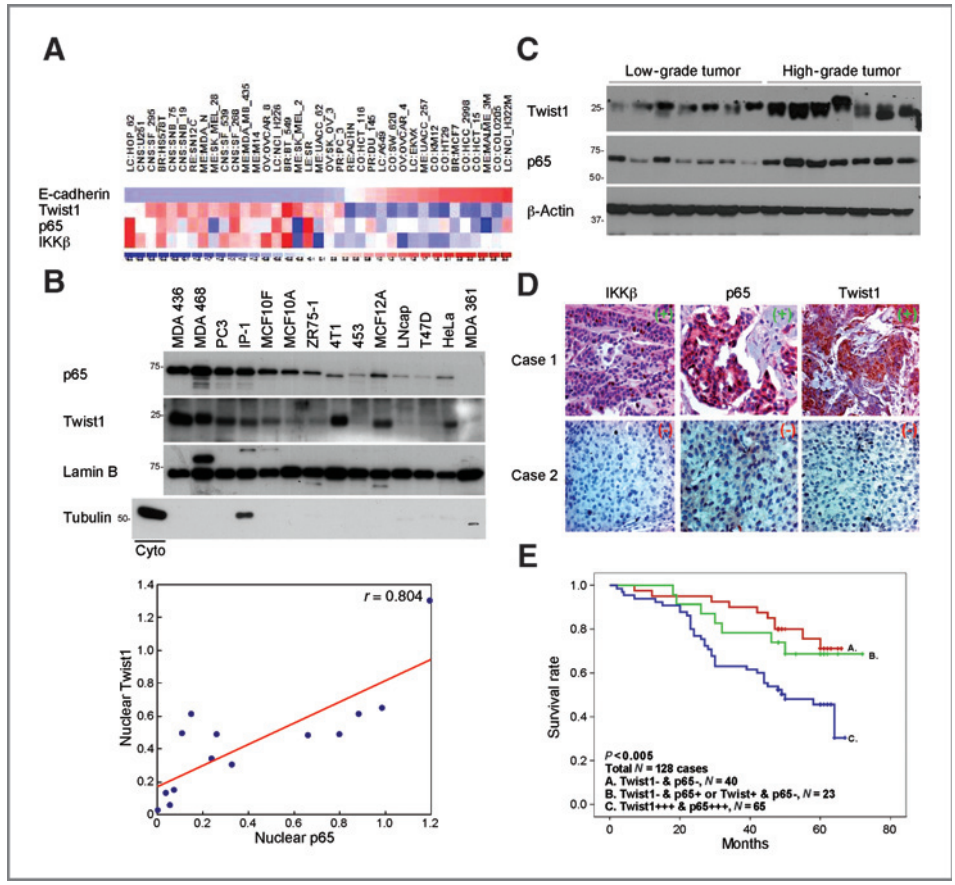


Figure 6. Clinical association of IKKβ, p65, and Twist1 expression with survival of breast cancer patients. A, heatmap generated using 37 cell lines from the NCI-60 panel showing the levels of expression of E-cadherin, Twist1, p65, and IKKβ. B, Western blot analysis of p65 and Twist1 expression in nuclear fraction isolated from 14 cell lines. C, Western blot analysis of p65 and Twist1 expression in high- and low-grade human breast tumor samples. D, IHC staining of human breast cancer samples showing the expression of IKKβ, p65, and Twist1. E, Kaplan–Meier overall survival curves of p65 and Twist1.

combination of p65 and Twist1 expression was a better predictor of survival than was either factor alone ($P < 0.02$ vs. $P < 0.005$; Fig. 6E). Taken together, the IHC staining data further strengthened the notion that activation of the IKKβ complex induces nuclear translocation of p65 and subsequently upregulation of Twist1 expression, which contributes to the promotion of EMT phenotype and is associated with poor clinical outcome in breast cancer patients.

## Discussion

Chronic inflammation–induced metastasis has long been considered as a major challenge in cancer therapy and is a primary cause of mortality in many cancers. Understanding the underlying mechanism governing the metastatic nature is therefore critical and may uncover therapeutic interventions. In this study, we investigated an important, but underdeveloped, signaling axis that controls inflammatory cytokines and

**Table 1.** Relationships between expression of Twist1, NF-κB/p65, and IKKβ in surgical specimens of breast cancer

| | | Expression of Twist1 | | | |
|---|---|---|---|---|---|
| | | −/+ | ++/+++ | Total | *P* |
| NF-κB | −/+ | 39 (29.8) | 10 (7.6) | 49 (37.4) | |
| | ++/+++ | 15 (11.5) | 67 (51.1) | 82 (62.6) | |
| | Total | 54 (41.2) | 77 (58.8) | 131 (100) | $P < 0.0001$ |
| IKKβ | −/+ | 39 (30.5) | 42 (32.8) | 81 (63.3) | |
| | ++/+++ | 13 (10.2) | 34 (26.6) | 47 (36.7) | |
| | Total | 52 (40.6) | 76 (59.4) | 128 (100) | $P < 0.023$ |

NOTE: Positive correlation between Twist1, NF-κB/p65, and IKKβ calculated using the Pearson $\chi^2$ analysis. All the values within parentheses are percentages.

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promotes EMT. Despite the essential role of TGF-β–dependent Smad regulation in EMT, we discovered a novel aspect by which p65 transactivation of Twist1 expression is required for TNF-α–induced EMT. On the basis of our findings, we propose a model in which elevated TNF-α from macrophages or the tumor microenvironment upregulates the canonical NF-κB signaling through the activation of IKKβ but not IKKα. The liberated cytoplasmic p65 then translocates to the nucleus, recognizes a cognate sequence on the Twist1 promoter, induces Twist1 expression, and promotes tumor metastasis (Supplementary Fig. S6G).

IKKβ is a component of the classic IKK complex, which is composed of 3 subunits: 2 catalytic kinases (IKKα and IKKβ) and a regulatory scaffold partner (IKKγ)). Upon stimulation by either TNF-α or IL-1β, activated IKKβ phosphorylates the NF-κB inhibitor IκBα and disrupts the nuclear retention of NF-κB. In fact, IKKβ does more than simply induce IκBα degradation for its tumorigenesis activity. For example, IKKβ directly phosphorylates p65 to promote its interaction with transcriptional coactivators and enhance its transactivation (29). Moreover, IKKβ-induced TSC1 phosphorylation inhibits its association with GTPase-activating protein (TSC2), alters mTOR activity, allows VEGF-A expression, and promotes tumorigenesis (20). In this study, we found that both IKKβ and p65 are mutually exclusively important in TNF-α–mediated Twist1 regulation. Both stable knockdown and overexpression of IKKβ affects Twist1 expression. Given that constitutive active IKKβ induces EMT in EpRas cells (16), the involvement of IKKβ in EMT supports our hypothesis. Here, we identified a mechanism for IKKβ-mediated tumor metastasis via upregulation of Twist1 expression. In addition to the requirement of IKKβ for TNF-α–mediated Twist1 expression, constitutively activated IKKβ promotes Twist1 expression, which may in turn contribute to the EMT phenotype.

Twist1 is a bHLH transcription factor that has been known as an essential player in the aggressive phenotype of EMT (7). Given that EMT is usually accompanied by an increase in stem cell–like properties to facilitate metastatic colonization as well as drug resistance (30, 31), researchers recently showed that Twist1 induces cancer stem cell ability by inhibiting CD24 gene expression (28). Surprisingly, we found that p65-induced EMT is also accompanied by the acquisition of cancer stem cell properties. In addition, downregulation of Twist1 expression suppressed p65-mediated malignancy, including EMT and stemness, suggesting that Twist1 is a central modulator downstream from NF-κB. By *in vivo* metastasis experimental model, suppression of Twist1 expression reduced LPS-meditated lung metastasis. Therefore, this study strongly supports the notion that p65 and Twist1 oncoproteins interact to regulate the expression of a series of target genes involved in aggressive cancer behavior. This regulation may likely contribute to inflammation-induced breast cancer metastasis.

Despite frequent reports of Twist1 overexpression in human cancers, transcriptional regulation of the human *Twist1* genes remains largely unknown. Previously, we showed that EGF receptor cooperates with STAT3 to induce EMT in breast cancer cells via upregulation of *Twist1* gene expression (24). In addition, STAT3 has been shown to transcriptionally activate Twist1 expression, resulting in AKT2-mediated oncogenic properties (32). A recent study showed that knockdown of STAT3 expression in murine 4T1 mammary tumor cells led to altered expression of Twist1 (32). Moreover, regulation of the murine Twist genes has involved NF-κB (33) and Wnt1/TCF/h–catenin pathways (34). However, the NF-κB and TCF/h-catenin response elements found in the mouse *Twist1* gene promoters are not present in the human *Twist1* gene. Herein, we provide the first evidence to show that TNF-α stimulates p65 to bind to the human *Twist1* promoter and regulate its transcription. Using TF Search and TESS transcription factor search tools together with biochemical analysis, we identified a p65-binding site on the *Twist1* promoter in response to TNF-α treatment. Because the murine *Twist1* promoter also contains the p65 consensus site, this novel axis is reminiscent of an evolutionarily conserved mechanism.

Given that Twist1 undergoes caspase-mediated cleavage and proteasome-mediated degradation under apoptotic stimuli (26), investigation of the Twist1 protein stability in response to NF-κB activation is conceivable. To date, p65 has been shown to enhance Snail protein stability by recruiting COP9 signalosome 2 (CSN2) complexes to inhibit β-TRCP–mediated degradation (18). In contrast, our result exclude the possibility that p65 affects Twist1 protein stability, albeit over a short period. We report herein that expression of the human *Twist1* gene is directly upregulated by p65-mediated transcriptional activation in response to chronic inflammation.

Several lines of evidence show that TNF-α–mediated Twist1 expression in breast cancer cells contributes to their aggressive phenotype. We showed in this study that (i) TNF-α and various NF-κB activators induce Twist1 expression in both normal breast epithelial and breast cancer cells; (ii) both canonical modules of NF-κB signaling, IKKβ, and p65, are required for TNF-α–mediated Twist1 expression; (iii) Twist1 expression is required and correlates with p65-mediated cancer progression; and (iv) downregulation of Twist1 expression reduces TNF-α–mediated EMT and tumor metastasis. Because *Twist1* promoter also contains a functional p65-binding motif, we propose that breast cancer cell metastasis induced by proinflammatory cytokine TNF-α is coordinated by a canonical NF-κB signaling involved in Twist1 activation. The in-depth analysis of this novel axis may improve understanding of breast cancer signaling and therefore introduce a therapeutic strategy for targeting breast cancer malignancy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Phosphorylation of Twist1 by AKT1 Modulates Epithelial-mesenchyme Transition in Breast Cancer Cells

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## Abstract

Epithelial-to-mesenchyme transition (EMT) is an essential physiological process that promotes cancer cell migration, invasion, and metastasis. Accumulating evidence from both cellular and genetic studies suggest AKT1/PKBα serves as a negative regulator of EMT and breast cancer metastasis while AKT2 and AKT3 serve generally as an oncogene to promote tumorgenesis. However, the underlying mechanism by which AKT1 suppresses EMT remains poorly defined. In studies with AKT1/2 association complex, Twist1, a master regulator of EMT, was identified as an AKT1 interacting partner connecting to AKT1-mediated EMT suppression. We found that AKT1 binds to Twist1 and phosphorylates it at three serine/threonine residues *in vitro* and *in vivo*. Phosphorylation by AKT1 facilitates β-TrCP-mediated Twist1 ubiquitination and degradation. Ablation of these residues on Twist1 enhances Twist1 stability, reduces E-cadherin expression, and changes in EMT morphology, suggesting that Twist1-induced EMT is suppressed by AKT1-mediated phosphorylation. Interestingly, Twist1 stabilization was found to be involved in MK-2206 (possesses higher inhibition toward AKT1) mediated EMT in breast cancer cells. Targeting Twist1 stability using a β-TrCP inducer, Resveratrol, attenuates MK-2206-mediated metastatic lesion. Altogether, our findings reveal a novel molecular mechanism by which non-specific inhibition of AKT may result in Twist1 stabilization to increase the metastatic potential in breast cancer cells.