Tid1 Negatively Regulates the Migratory Potential of Cancer Cells by Inhibiting the Production of Interleukin-8

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Abstract

Tid1 is the human homologue of the *Drosophila* tumor suppressor, Tid56. Reducing the expression of Tid1 in MDA-MB231 breast cancer cells enhanced their migration without affecting their survival or growth rate. From microarray screening, we discovered that after Tid1 depletion, the mRNA level of interleukin-8 (IL-8) was significantly increased in these cancer cells, which consequently increased secretion of IL-8 protein by 3.5-fold. The enhanced migration of these Tid1-knockdown cells was blocked by reducing the IL-8 expression or by adding an IL-8 neutralizing antibody to the culture medium, suggesting that enhancement of cell motility in these Tid1-deficient cells is dependent on the *de novo* synthesis of IL-8. Subsequently, we found that abrogating the nuclear factor κB binding site in the IL-8 promoter completely blocked the Tid1 depletion–induced IL-8 expression in the breast cancer cells. As increased IL-8 levels are known to promote tumor metastasis, we tested the effect of Tid1 knockdown on tumor metastasis and found that Tid1 depletion enhanced the metastasis of breast cancer cells in animals. Together, these results indicate that Tid1 negatively regulates the motility and metastasis of breast cancer cells, most likely through attenuation of nuclear factor κB activity on the promoter of the *IL8* gene. (Cancer Res 2005; 65(19): 8784-91)

Introduction

Tumor metastasis is the major reason for morbidity and death in cancer patients. Metastasis is the process by which tumor cells spread from their site of origin to distant sites after gaining access to the circulatory system. Active cell migration is required for a cancer cell to establish itself at a second site. The migratory capacity of a cell can be regulated by a number of physiologic factors, including growth factors, extracellular matrix components, and cytokines. Therefore, understanding molecular mechanisms modulating the migratory processes of tumor cells should provide important information in developing therapeutic interventions for tumor metastasis.

The *Drosophilal(2)tid* gene, *Tid56*, is the first and only member of the DnaJ cochaperone family that has been classified as a tumor suppressor. The null mutation of the *Tid56* gene not only keeps imaginal discs from differentiating but also leads to lethal tumorigenesis during the early developmental larval stage (1). For Tid1, the only mammalian counterpart of Tid56, it has been reported that increased expression of Tid1 in human lung adenocarcinoma cell lines reduced the potential for colony formation in soft agar (2) and Tid1-depleted U2OS cells showed augmented colony formation in soft agar (3). Recently, Canamasas et al. showed that loss of Tid1 expression was associated with human basal cell carcinoma but not with normal keratinocytes (4). Similarly, Trentin et al. also reported a Tid1 mutation in human glioma cells and introduction of wild-type Tid1 into these cancer cells induced their apoptosis (5). These observations suggest that Tid1 plays a role in human carcinogenesis. However, the molecular mechanisms involved in these phenomena are poorly understood.

As a DnaJ protein, Tid1 serves as a cochaperone and regulatory factor for the Hsp70 family of molecular chaperones, and is characterized by a J-domain, a highly conserved tetrahelical domain that binds to Hsp70s to regulate their activity and provide substrate specificity. Recently, we showed that the cochaperonic and regulatory function of Tid1 on Hsp70 is required for Tid1 to reduce the malignant signals derived from ErbB2/Her2 by promoting ubiquitination and consequent degradation of ErbB2 (6). By this mechanism, increased expression of Tid1 induces apoptosis in ErbB2-overexpressing breast carcinoma cells and inhibits the growth of ErbB2-dependent tumors in animals. However, increased expression of Tid1 has no effect on the uncontrolled proliferation of breast carcinoma cells with low expression levels of ErbB2 (MDA-MB231). To investigate the role of Tid1 in these cells, we depleted endogenous Tid1 in these cancer cells using Tid1-specific short interfering RNA (siRNA) oligos and found that the migratory potential of these cells was significantly increased. Using microarray screening, we found that reducing Tid1 protein in MDA-MB231 cells up-regulated the *de novo* synthesis of interleukin-8 (IL-8), which is known to modulate cancer cell migration and metastasis. Blocking the production of IL-8 or neutralizing the activity of IL-8 in these Tid1-depleted MDA-MB231 cells almost completely abolished the increase in their motility induced by Tid1 depletion, indicating that Tid1 depletion–induced enhancement of cell migration was caused by increased production of IL-8. Induction of IL-8 expression is also known to be the reason for the promotion of breast cancer cell migration stimulated by the tissue factor-factor VIIa (FVIIa) pathway (7). We investigated whether Tid1 has a role in regulating FVIIa-mediated IL-8 production and consequent up-regulation of cancer cell migration, and found that increased expression of Tid1 in breast carcinoma cells effectively blocked both FVIIa-induced IL-8 synthesis and the resulting enhancement of cell migration. Because increased IL-8 levels are associated with increased metastasis of tumor cells (8, 9), we examined whether Tid1 knockdown could promote tumor metastasis and found that depletion of cellular Tid1 in breast carcinoma cells enhanced their metastatic capability.

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Materials and Methods

Reagent and antibodies. FVIIa was purchased from Enzyme Research Laboratories (South Bend, IN) and thrombin was kindly provided by Dr. W. Ruf at The Scripps Research Institute (La Jolla, CA). Antibodies against β-actin (C-11) and nuclear factor κB (NF-κB) p-65 (SC-109) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human IL-8 neutralization antibody and ELISA kit for detecting IL-8 production were purchased from R&D Systems (Minneapolis, MN). Anti-Tid1 antibody was kindly provided by Dr. Maria Rozakis-Adcok (McMaster University, Hamilton, Ontario, Canada).

RNAi and transfection. RNAi-mediated gene knockdown was done with the 19-nucleotide targets using siRNAs as follows:

siTid1-1 sense, 5′-GGAGUUCACCGUGAACAUCdTdT-3′
siTid1-1 antisense, 5′-GAUGUUCACGGUGAACUCCdTdT-3′
siTid1-5 sense, 5′-CAGCUACGGCUACGGAGACdTdT-3′
siTid1-5 antisense, 5′-GUCUCCGUAGCCGUAGCUGdTdT-3′
IL-8 sense, 5′-ACCACCGGAAGGAACCAUCdTdT-3′
IL-8 antisense, 5′-GAUGGUUCCUUCCGGUGGUdTdT-3′
siControl sense, 5′-UUCUCCGAACGUGUCACGUdTdT-3′
siControl antisense, 5′-ACGUGACACGUUCGGAGAAdTdT-3′

MDA-MB231 (2×10^5) cells were transfected with a final concentration of 100 nmol/L siRNAs using GenePORTER I (San Diego, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were split, incubated for 8 hours with DMEM containing 10% fetal bovine serum, and then starved in DMEM containing 0.1% fetal bovine serum overnight for the following migration assays.

Recombinant adenoviruses and infections. A full-length cDNA encoding human $Tid1_S$ and its J-domain mutant (Tid1HQ) were cloned into the *Bgl*II and *Hin*dIII sites of pAdTrack-CMV adenovirus shuttle vector (Q-Biogene, Carlsbad, CA) as described previously (4). Adenoviruses containing $Tid1_S$ was used to infect breast cancer cell lines in 24-well plates (1×10^4/well) for 6 hours. Thereafter, the growth medium was replaced every 2 days. Infection efficiency was checked by green fluorescence at a multiplicity of infection of 5×10^4, which was used to reach a 100% infection rate. For generation of Ad-shTid1, a Tid1-specific sequence containing a hairpin loop (5′-GATCCCCAGCTACGGCTACGGAGACTTCAAGAGAGTCTCCGTAGCCGTAGCTGTTTTTGGAAA-3′) was cloned into pSuper vector (10), then moved to pShuttle vector (Q-Biogene) at *Xba*I and *Hin*dIII site as described previously (4).

Cell growth assay. Twenty-four hours posttransfection, cells were split and plated onto polystyrene six-well plates (5×10^4/well) from Corning (Corning, NY) in normal growth medium. Every 24 hours, cells were collected and counted by hemocytometer.

Migration assay. Serum-starved cells were collected by limited trypsin treatment followed by the addition of soybean trypsin inhibitor as described previously (11). The MilliCell membrane (8 μm pore size, polycarbonate filter, 12 mm diameter) from Millipore (Bedford, MA) was precoated with 1 μg/mL rat-tail collagen (Boehringer Mannheim, Indianapolis, IN) for 2 hours and then were air-dried. Cells (1×10^6/well) were added to the upper chamber containing 300 μL of DMEM with 0.5% bovine serum albumin. The upper chamber was placed into the lower chamber containing 400 μL of DMEM with 0.5% bovine serum albumin. For FVIIa-induced migration assay, FVIIa or other stimulants were added to the upper chamber. These chambers were incubated at 37°C containing 5% CO_2 for 6 hours. Nonmigratory cells on the upper surface of the membrane were removed by swiping with a damp cotton swab. The cells that migrated across the filter were fixed in 37% formaldehyde and 25% glutaraldehyde in PBS and stained with 0.1% crystal violet in PBS for 30 minutes. The number of migratory cells per membrane was measured by light microscopy. Each data point is the average of cells in three random fields. Each determination represents the average of at least three individual wells.

Immunofluorescence. MDA-MB231 cells were plated on poly-D-lysine–coated glass coverslips in 12-well plates from BD BioCoat (Bedford, MA) overnight, fixed and permeabilized the next day as described previously (12). After blocking in 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 hour, the cells were incubated with antibody against p65 (Santa Cruz Biotechnology) at 1:200 dilution for 1 hour to detect NF-κB. Subsequently, the cells were incubated with Alexa Fluor 568 goat anti-rabbit (red-orange; Molecular Probes, Inc., Eugene, OR) for 30 minutes at a 1:1,000 dilution. At the same time, nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (0.1 μg/mL) for 30 minutes. These cells were viewed and photographed with a Zeiss Axiovert S100TV microscope.

ELISA. ELISA assays were done using commercial IL-8 ELISA kits from R&D Systems. Conditioned medium from treated or nontreated MDA-MB231 cells were collected from 12-well plates, aliquoted, and stored at

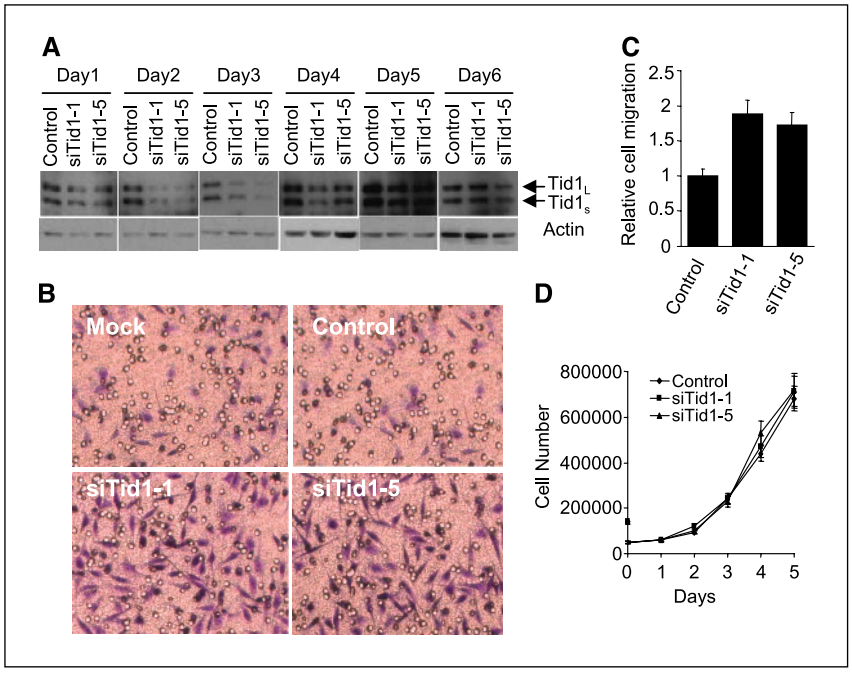
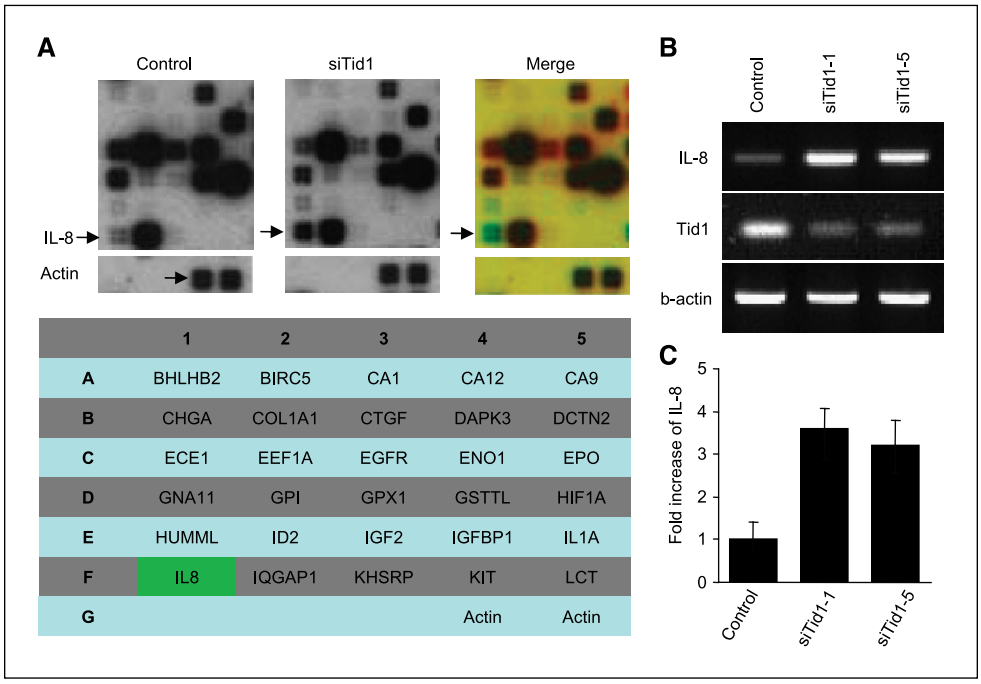


Figure 1. Sequence-specific silencing of *Tid1* gene increases cancer cell motility. MDA-MB231 cells were transfected with the sequence-specific dsRNA oligonucleotides for Tid1 (siTid1-1 or siTid1-5) or control siRNA (negative control). *A,* after transfection, cells were lysed at the indicated day and analyzed by Western blotting using anti-Tid1 antibody. *B,* 1 day after transfection, the subconfluent transfected cells were starved overnight in DMEM containing 0.1% fetal bovine serum and then plated on MilliCell. After 6 hours at 37°C, cells that migrated to the underside of the membrane were stained with crystal violet as described in Materials and Methods. Phase contrast images of migrated cells are shown. *C,* relative cell migration was determined by comparing the number of migratory cells obtained from Tid1-specific dsRNA transfectants to that from cells transfected with control oligos whose value was taken as 1. *D,* MDA-MB231 cells were transfected with control, siTid1-1, or siTid1-5 oligos as described in (*A*). These treated MDA-MB231 cells were grown in normal growth medium and their proliferation was monitored by counting cell numbers at the indicated day using a hemocytometer.



	1	2	3	4	5
A	BHLHB2	BIRC5	CA1	CA12	CA9
B	CHGA	COL1A1	CTGF	DAPK3	DCTN2
C	ECE1	EEF1A	EGFR	ENO1	EPO
D	GNA11	GPI	GPX1	GSTTL	HIF1A
E	HUMML	ID2	IGF2	IGFBP1	IL1A
F	IL8	IQGAP1	KHSRP	KIT	LCT
G				Actin	Actin

Figure 2. Depletion of Tid1 up-regulates IL-8 expression in cancer cells. *A,* MDA-MB231 cells were transfected with Tid1-specific siRNA or control siRNA, and 2 days later, total RNA was isolated and used to produce biotin-labeled cDNA probes. These probes were used separately in hybridization with the microarray membranes (details in Materials and Methods) to analyze the alteration in gene expression profiles in cancer cells after Tid1 knockdown. Each gene in this cDNA array was spotted four times. Actin cDNA (positions G4/5) was spotted as an internal control. Control blot (using probe generated from control cells, *left*) and Tid1-knockdown blot (using probe from Tid1-depleted cells, *middle*) were merged, and green spots indicate the positions of those genes whose expression levels were up-regulated by 2.0-fold or more after Tid1 depletion (*right*). *IL8* gene (position F1) was one of them. *B,* MDA-MB231 cells were transfected with Tid1-specific siRNA or control siRNA, and 2 days later, the expression levels of *IL8* and *Tid1* gene in control or Tid1-depleted cells were examined by semiquantitative reverse transcription-PCR with IL-8 or Tid1-specific primers. *C,* 1 day after transfection with Tid1-specific siRNAs (siTid1-1 or siTid1-5) or control siRNA, MDA-MB231 cells were starved overnight in DMEM containing 0.1% fetal bovine serum. The medium was collected and assayed for IL-8 protein by ELISA. Results are presented as fold increase by taking the value of IL-8 protein in medium of cells transfected with control oligos as one. *Columns,* mean; *bars,* ± SD.

−80°C until assayed. Samples were diluted 5- to 320-fold in deionized water before assaying. Assays were done in triplicate, and readings were compared with standard curves obtained with human recombinant IL-8 provided in the kit.

RNA extraction, reverse transcription-PCR, and cDNA microarray. mRNA was isolated from MDA-MB231 cells using oligo(dT)-conjugated magnetic beads. mRNA (0.5 μg) was used to generate cDNA by reverse transcription using DNA polymerase from Superarray (Frederick, MD). These cDNAs were then used to produce biotin-labeled cDNA probes separately using the Tumor Metastasis Gene Array kit or the Human Hypoxia Signaling Pathway Gene Array kit (Superarray) following the manufacturer's protocol. These arrays were hybridized, washed, and developed according to the manufacturer's instructions. Signal intensities for all genes were quantified and compared using GEArray Analyzer software (Superarray) after normalization to the signals from the housekeeping genes. Genes were identified as up-regulated if the signal from the siTid1-treated cells was >2-fold than that from the siControl-treated cells.

Semiquantitative reverse transcription-PCR assay. MDA-MB231 cells were used to generated mRNA and cDNA as described above. PCR was done using primer pairs specific for Tid1, IL-8, or β-actin:

Tid1 sense: 5′-AAGCAGTACGATGCCTACGG-3′
Tid1 antisense: 5′-TGGCCATCCTCGACTCCTGC-3′
IL-8 sense: 5′-ATGACTTCCAAGCTGGCCGT-3′
IL-8 antisense: 5′-CCTCTTCAAAAACTTCTCCACACC-3′
β-actin sense: 5′-TCCGGAGACGGGGTCA-3′
β-actin antisense: 5′-CCTGCTTGCTGATCCA-3′

The cycling conditions for PCR were as follows: 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and extension at 72°C for 10 minutes. Ten percent of the PCR products were analyzed on 1.5% agarose gels containing ethidium bromide (0.1 μg/mL). The expression levels of Tid1 or IL-8 were normalized by β-actin expression in the same cells.

Plasmids and reporter gene activity assay. IL-8 promoter corresponding to −1,481/+44 bp was cloned from MDA-MB231 cells and was used for site-directed mutagenesis to mutate activator protein (AP-1), CAAT/enhancer binding protein (C/EBP), and NF-κB sites as described previously. MDA-MB231 cells (1.5×10^5/well) were grown in 12-well plates (Nunc, Naperville, IL) and transiently transfected with 100 ng of the reporter plasmids along with siTid1s or siControl using GenePORTER I. The pRL Renilla luciferase expression vector (10 ng/well) were also cotransfected for normalizing transfection efficiency. The activities of firefly and Renilla luciferase were measured in 20 μL of total cell lysate by using the dual-luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's instructions.

Real-time quantitative reverse transcription-PCR assay. Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) during 38 cycles. For relative experimental metastasis, specific primers for human hypoxanthine phosphoribosyltransferase (HPRT) message, which do not cross-react with its mouse counterpart were designed (forward, 5′-TTCCTTGGTCAGGCAGTATAATCC-3′; reverse, 5′-AGTCTGGCTTATATCCAACACTTCG-3′). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

***In vivo* experimental metastasis studies.** One day after infection, Ad-shTid1 or Ad-Sc-treated MDA-MB-231 breast carcinoma cells (10^6 cells) were injected into the tail vein of CB-17 severe combined immunodeficiency (SCID) mice. Lungs were collected on day 28. Lung tumor burdens were determined by wet lung weights. Afterward, these lungs were fixed or snap-frozen for immunohistochemistry and RNA extraction.

Results

Depletion of Tid1 protein by siRNA increases cancer cell migration. To clarify the function of Tid1 in tumor development, we transfected the breast carcinoma cell line, MDA-MB231, with double-stranded small inhibitory RNA oligonucleotides (siTid1-1 or siTid1-5) designed to specifically silence the *Tid1* gene, which led to significant reduction of the expression levels of both forms of Tid1, $Tid1_L$, and $Tid1_S$, to <20% of control cells (Fig. 1*A* and *B*). Maximal reduction of Tid1 was reached 2 to 3 days after transfection, after which, the expression of Tid1 gradually recovered (Fig. 1*A* and *B*). Next, we determined whether depletion of Tid1 would have any effect on malignant properties such as cell migration and cell growth. We found that migration of siTid1-transfected MDA-MB231 cells was increased 2-fold over that of siControl-transfected cells (Fig. 1*B* and *C*). On the other hand, silencing the *Tid1* gene did not affect the growth of MDA-MB231 cells under normal growing conditions (Fig. 1*D*). These results suggested that Tid1 negatively regulates the motility of MDA-MB231 cells.

Tid1-knockdown enhanced the expression and secretion of IL-8. Cell migration is one of the critical factors for tumor progression and metastasis and a number of physiologic factors could modulate the ability of the cell to migrate (13). To analyze the molecular mechanism responsible for enhanced cell motility induced by Tid1 depletion, microarray-based assays were used to examine the alteration in gene expression profiles in MDA-MB231 cells after suppressing the expression of *Tid1* gene (Fig. 2*A*). We analyzed this microarray data by searching for tumor progression and metastasis-associated genes whose expression levels were altered >2-fold after Tid1 depletion. We found that the message of the *IL8* gene, a common tumor progression and metastasis-associated gene (9, 14), was significantly up-regulated in Tid1-depleded MDA-MB231 cells (Fig. 2*A*). Using semiquantitative reverse transcription-PCR, we further confirmed that the level of IL-8 mRNA was elevated in the cells transfected with siTid1-1 or siTid1-5 compared with that in the cells transfected with siControl (Fig. 2*B*). Moreover, using ELISA, the concentration of IL-8 protein secreted into the medium from the Tid1-suppressed cells was 3- to 4-fold higher than that of control cells (Fig. 2*C*), which concurred with the increased mRNA levels in these siTid1-transfected cells. These results indicated that Tid1 negatively regulates the *de novo* synthesis of IL-8 in breast cancer cells.

The increased motility of Tid1-depleted cells is caused by the increase in IL-8 production. IL-8-induced cell motility has been implicated in the metastatic phenotype of breast cancer in several studies (8, 9, 15, 16). We wondered whether the increased IL-8 production by Tid1-knockdown cells is the reason for the enhanced migration of MDA-MB2321 cancer cells. To test this, we suppressed IL-8 production or neutralized the activity of secreted IL-8 in Tid1-depleted MDA231 and later examined their migratory potential. IL-8 production in Tid1-depleted cells was blocked by cotransfecting siIL-8 and siTid1 oligos into MDA-MB231 cells (Fig. 3*A*), which led to the suppression of both the mRNA and protein level of IL-8 in the Tid1-depleted cells (Fig. 3*A* and *B*) and also reduced the cell motility of Tid1-depleted cells to that of control cells (Fig. 3*C*). The activity of IL-8 secreted by Tid1-depleted cells was inhibited by adding a neutralizing antibody against IL-8 to the culture medium, which led to the reversal of the enhancement of MDA-MB231 cell motility induced by Tid1 depletion (Fig. 3*D*). These data suggested that the increased production of IL-8 by Tid1 depletion causes the motility augmentation in breast cancer cells.

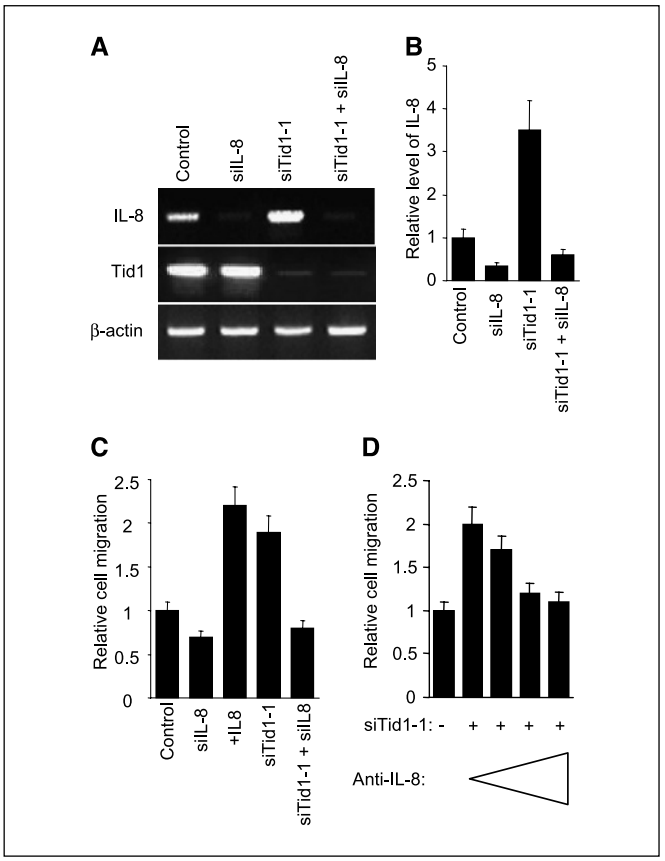


Figure 3. Increased IL-8 production leads to the up-regulation of cell motility in Tid1-depleted cancer cells. *A,* MDA-MB231 cells were transfected with siTid1-1 and/or siIL-8 oligos as indicated to silence the expression of *Tid1* and/or *IL8* genes, respectively. Two days after transfection, the mRNA levels of *Tid1* or *IL8* gene in these cells were analyzed by semiquantitative reverse transcription-PCR with Tid1- or IL-8-specific primers. *B,* MDA-MB231 cells were transfected as described in (*A*). One day after transfection, these cells were starved overnight and later their media was analyzed for IL-8 secretion by ELISA. *C,* the starved cells from (*B*) were used to examine their motility as described in Fig. 1*D*. IL-8 (1 ng/mL) was added to the upper chamber for the indicated experiments. The relative cell migration in these cells was normalized using cells transfected with control oligos whose value was taken as 1. *D,* MDA-MB231 cells were transfected as described in (*A*). Later, these cells were placed in a MilliCell still in the presence of the increasing concentrations of the neutralizing antibody and the motility of these cells was assessed as described in Fig. 1*D*.

Tid1 negatively modulates *de novo* synthesis of IL-8 through regulating NF-κB activity. For Tid1-depleted cells, the increase in IL-8 protein secretion is in accord with the increase in the steady-state level of IL-8 mRNA (Fig. 3*A* and *B*). Several studies showed that the steady-state level of IL-8 mRNA can be dynamically regulated by many factors at the transcriptional level (17). The responsive DNA sequence for IL-8 induction is located in the IL-8 promoter between −133 and −85, and has been shown to contain the binding sites for transcriptional factors, AP-1, NF-κB, and C/EBP1 (18). It has been shown that NF-κB, AP-1, and to a lesser extent, C/EBP binding sites play a crucial role in controlling IL-8 promoter activity (19, 20). These transcriptional factor binding sites in the IL-8 promoter have been individually or jointly mutated to keep them from binding their corresponding transcriptional factors, and the resultant mutated IL-8 promoters were placed separately in front of the luciferase reporter gene. These reporter constructs were individually transfected into Tid1-depleted cells to investigate the responsible *cis*-elements within the IL-8 promoter

for Tid1 depletion–induced IL-8 production (Fig. 4). Consistent with other studies (19, 20), we found that the transcriptional activity of the mutated promoters was significantly reduced compared with the wild-type construct whether transfected with Tid1 siRNA oligos or not (Fig. 4). Mutations within AP-1, C/EBP, and NF-κB binding sites decreased IL-8 promoter activity by about 50%, 30%, and 75%, respectively. However, only mutation of the NF-κB binding site abolished the Tid1-depletion-dependent up-regulation of IL-8 promoter activity (Fig. 4), indicating that Tid1 regulates the IL-8 promoter by modulating NF-κB activity. Furthermore, combined mutation of both the AP-1 and C/EBP sites reduced the activity of the IL-8 promoter to a level similar to NF-κB mutation alone, suggesting that the AP-1 and C/EBP sites are acting coordinately or are necessary for NF-κB activity. Together, these results suggested that regulation of the transcriptional activity of IL-8 induced by Tid1 suppression is dependent on the NF-κB pathway.

Tid1 inhibits FVIIa-induced NF-κB nuclear translocation and cell motility. FVIIa has been implicated in tumor metastasis, in part by its capacity to increase cell migration through induction of IL-8 secretion (7, 21). Because Tid1 negatively regulated the IL-8 production of cancer cells and their subsequent migration, it was of interest to test whether Tid1 could modulate FVIIa-induced migration of breast carcinoma cells. To test this, we increased the expression of cellular $Tid1_S$ in MDA-MB231 cells by infecting them with recombinant adenovirus encoding $Tid1_S$ (Ad-$Tid1_S$). A J-domain mutant of Tid1 (Ad-Tid1HQ) was used in this experiment to examine the involvement of the cochaperonic activity of Tid1 in this FVIIa-mediated cell activation; an empty virus, Ad-EV, was used as a control in these experiments. These cells were starved, treated with FVIIa or thrombin, and later analyzed for IL-8 secretion by ELISA (Fig. 5*B*). Consistent with previous reports (7), IL-8 secretion was increased by treatment with 10 nmol/L FVIIa, a concentration equivalent to that in plasma and IL-8 production was further increased by treatment with 50 nmol/L FVIIa, whereas thrombin has no effect on IL-8 secretion. As expected, increased expression of Tid1 in cancer cells significantly inhibited IL-8 secretion induced by FVIIa. In contrast, infection with Ad-Tid1HQ had no effect on the induction of IL-8 secretion by FVIIa, suggesting that the cochaperonic activity of Tid1 is required for blocking the FVIIa-induced IL-8 production in these cells. Next, we tested whether Tid1 can negatively regulate FVIIa-induced tumor cell motility. The migration of MDA-MB231 cells infected with Ad-$Tid1_S$, Ad-Tid1HQ, or Ad-EV was examined in a MilliCell with or without the presence of FVIIa in the top well. The higher level of cellular $Tid1_S$ (but not Tid1HQ) markedly blocked cell migration induced by FVIIa, which coincides with the ability of $Tid1_S$ (but not Tid1HQ) to block FVIIa-induced IL-8 production in MDA-MB231 cells. Because NF-κB is the dominant pathway for the induction of IL-8 by FVIIa (20) and because Tid1 negatively regulates NF-κB activity (Fig. 4), it is possible that Tid1 blocked FVIIa-induced IL-8 production by inhibiting NF-κB activity. In unstimulated cells, the NF-κB complex was located mainly in the cytoplasm and after FVIIa treatment, a portion of the NF-κB complex translocated into the nucleus (Fig. 5*D*). Increased cellular $Tid1_S$ significantly inhibited this FVIIa-induced NF-κB nuclear translocation in MDA-MB231 cells, as shown in Fig. 5*D*, indicating that Tid1 inhibits FVII-induced IL-8 production and cell migration by blocking NF-κB translocation to the nucleus. Together, these results suggested that Tid1 negatively regulates FVIIa-induced IL-8 expression and the consequent enhancement of cell motility of breast carcinoma cells by blocking activation of the NF-κB

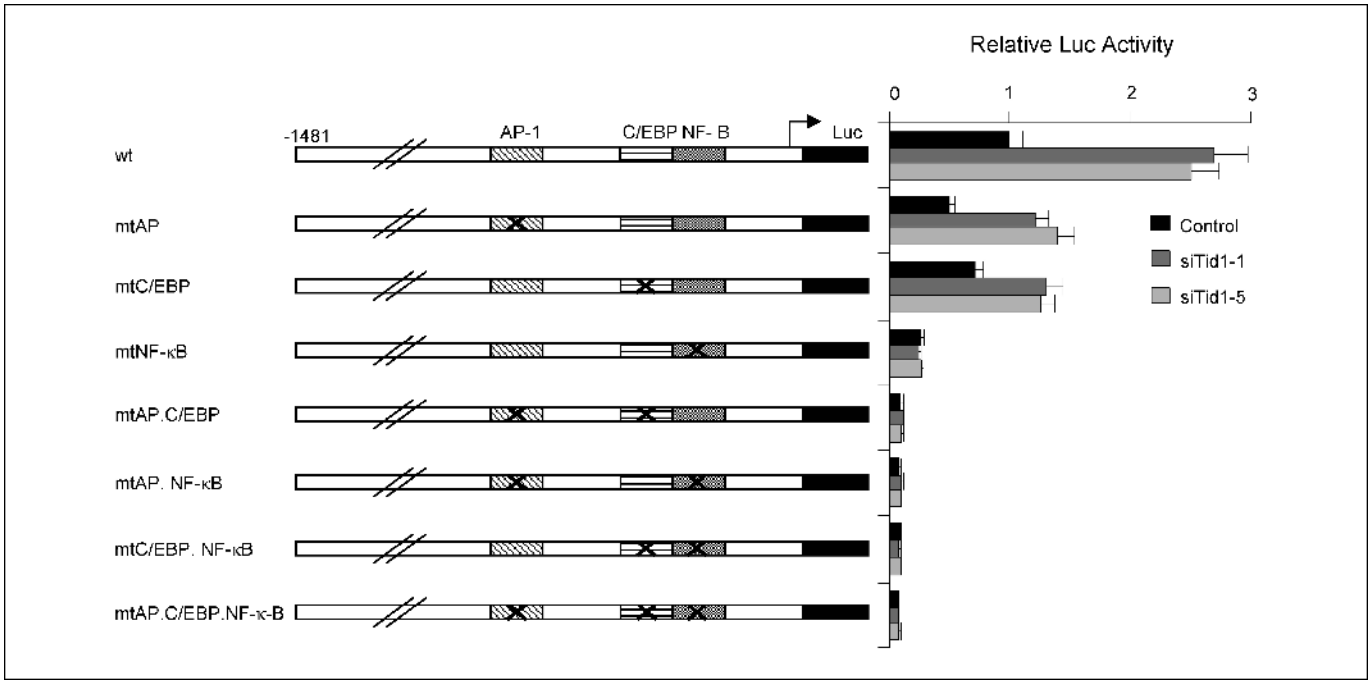


Figure 4. Tid1 depletion–induced *de novo* synthesis of *IL8* gene is dependent on NF-κB activity. *A,* MDA-MB231 cells were separately transfected with reporter plasmids which encode a luciferase gene driven by wild-type, or mutated IL-8 promoters harboring single, double, or triple mutations of AP-1, C/EBP, or NF-κB sites, along with siTid1 or siControl oligos as indicated. The pRL Renilla luciferase expression vector were also transfected into these cells for normalizing transfection efficiency. The cells were then starved overnight and assayed for luciferase activity. The activities of firefly and Renilla luciferase were quantified in the same sample using the dual-luciferase reporter assay (Promega Corporation). The luciferase activities were normalized against cells transfected with the control oligos and a reporter plasmid with the wild-type IL-8 promoter, whose value was taken as 1.

transcriptional factor. Interestingly, the fact that a functional J-domain in Tid1 is critical for this phenomenon indicates that the cochaperonic function of Tid1 to Hsp70 is required for Tid1-mediated negative regulation of NF-κB activation.

Tid1-depletion enhanced metastasis of mammary carcinoma cells. Because IL-8 has been strongly correlated with a metastatic phenotype (8, 9, 15, 16) and Tid1 depletion increased production of IL-8 in MDA-MB231, we tested whether reducing the cellular level of Tid1 can increase the metastatic potential of breast carcinoma cells. We first generated recombinant adenovirus producing shRNA specific for Tid1, Ad-shTid1, to prolong the period of Tid1-depletion in MDA-MB231 cells. The depletion of endogenous Tid1 in Ad-shTid1-infected MDA-MB231 cells can be maintained for >2 weeks (Fig. 6*A*). MDA-MB-231 cells infected with adenovirus- Tid1 shRNA or scramble (Sc) were injected into the tail vein of SCID mice. Four weeks after injection, lung metastases were visualized (Fig. 6*B*) and quantified by wet lung weight (Fig. 6*C*). We found that lung metastasis is significantly higher (~40%) in mice injected with Tid1-depleted cancer cells than in mice injected with cancer cells containing normal levels of Tid1. In addition, the individual tumor burden per lung was measured by quantitative reverse transcription-PCR using primers specific for the human housekeeping gene, *HPRT*, which detects human but not mouse HPRT mRNA (Fig. 6*D*). The expression level of human HPRT in lungs infiltrated with Tid1-depleted tumor cells are ~40% to 50% higher than that in lungs infiltrated with control cells (Fig. 6*D*). These data suggest that Tid1-depletion significantly increase the metastasis rate of breast carcinoma cells.

Discussion

The core IL-8 promoter contains NF-κB, AP-1, and C/EBP-binding sites. Our results showed that transcriptional activation of the IL-8 promoter induced by Tid1-knockdown is mostly dependent on the NF-κB pathway (Fig. 4). FVIIa-induced NF-κB nuclear translocation was also blocked by increased cellular Tid1

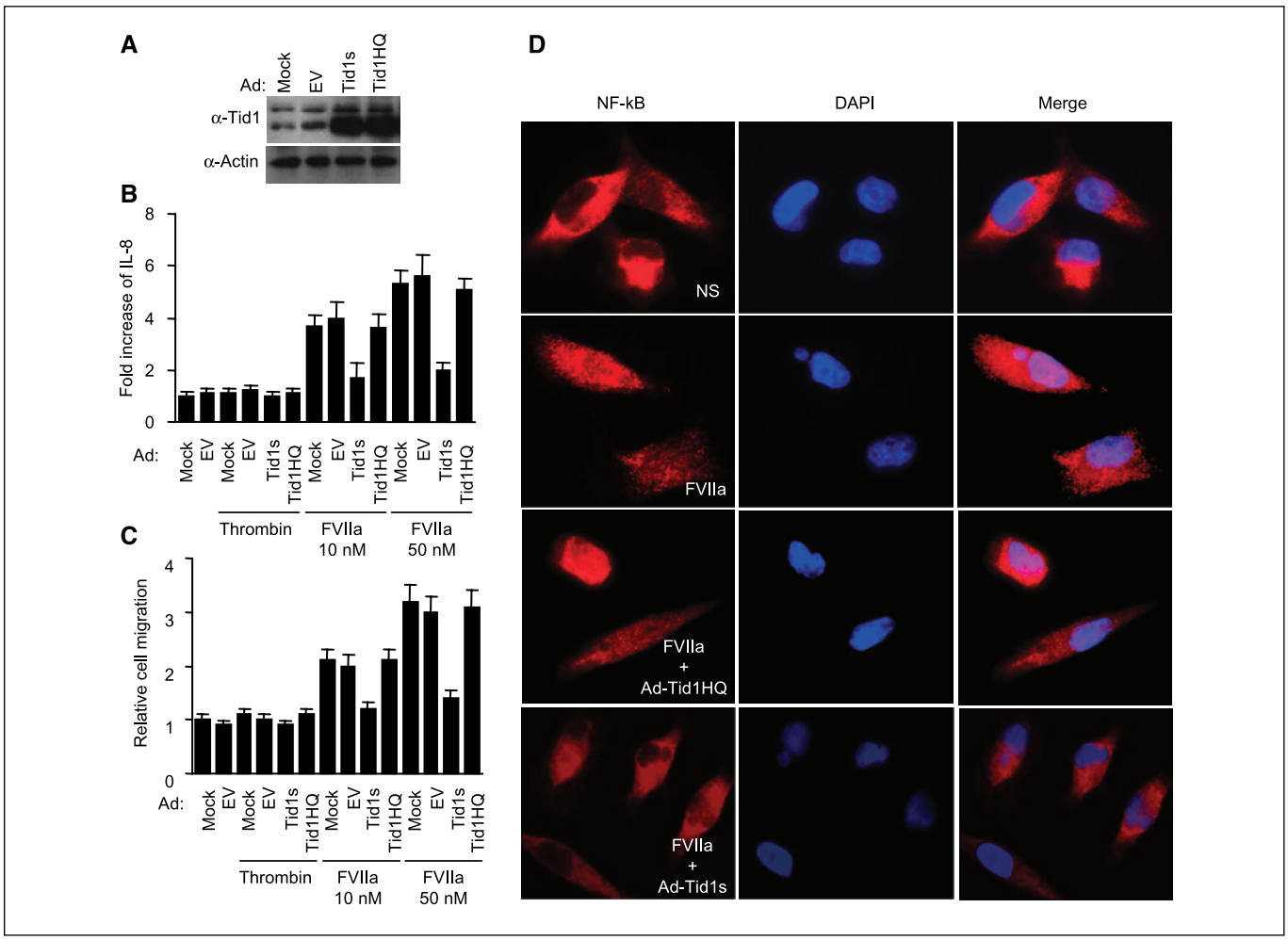


Figure 5. Tid1 inhibits FVIIa-induced IL-8 production and consequent up-regulation of cell motility of breast cancer cells. *A,* MDA-MB231 cells were infected with recombinant adenoviruses encoding $Tid1_S$ (Ad-$Tid1_S$), a J-domain mutant of $Tid1_S$ (Ad-Tid1HQ) or empty virus (Ad-EV). Two days after infection, cells were lysed and analyzed for Tid1 expression by an anti-Tid1 antibody in an immunoblot assay. *B,* MDA-MB231 cells were infected with recombinant adenoviruses as described in (*A*). After 24 hours, these cells were starved overnight and then treated with different concentrations of FVIIa (10 and 50 nmol/L) or thrombin (10 nmol/L) as indicated for 16 hours in 0.1% fetal bovine serum. IL-8 secretion by these cells was then evaluated by ELISA as described in Fig. 2*C*. *C,* the infected starved cells in (*B*) were placed in a MilliCell, and simultaneously, FVII (10 or 50 nmol/L) or thrombin (10 nmol/L) was added to the top chamber of the MilliCell for 6 hours at 37°C. The number of cells that migrated to the underside of the membrane was determined as described in Fig. 1*D*. *D,* the infected quiescent cells in (*B*) were stimulated with or without 50 mmol/L FVIIa for 2 hours at 37°C. Cells were then immunostained with an anti-p65 antibody (Santa Cruz Biotechnology) to detect the localization of NF-κB complex (*left*). Nuclei were visualized using 4′,6-diamidino-2-phenylindole (*middle*). Merged red and blue fluorescence (*right*).

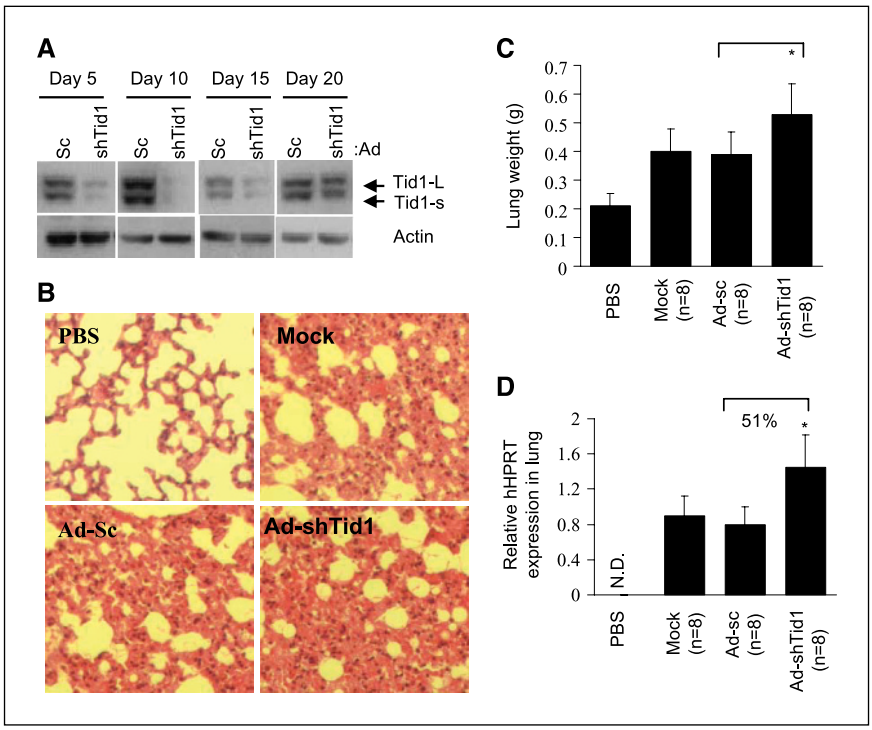


Figure 6. Tid1-depletion increases metastasis of breast carcinoma cells. *A,* MDA-MB231 cells were infected with recombinant adenoviruses encoding shTid1$_S$ (Ad-shTid1$_S$) or with Ad-scramble (Sc) as control. At the indicated day after infection, cells were lysed and analyzed for Tid1 expression by immunoblotting using an anti-Tid1 antibody. One day after infection, cells (1×10^6) infected with Ad-shTid1s, Ad-Sc, or nothing (mock) were collected and injected into the tail vain of CB17-SCID mice. *B,* lungs from these mice were isolated 4 weeks after injection and stained with H&E to check for tumor metastases. *C,* wet lung weights of mice. I.v. injection of MDA-MB231 cells infected with Ad-shTid1, Ad-sc, or nothing (mock). *D,* quantitation of metastases in the abovementioned lungs by quantitative reverse transcription-PCR analysis for human hHPRT mRNA. N.D., not detectable. *Columns,* mean; *bars,* ± SD. Percentages indicate relative enhancement compared with control (Ad-Sc treated) cells. *, $P < 0.05$, Student's *t* test.

(Fig. 5*E*). However, the precise NF-κB regulatory mechanism of Tid1 in breast cancer cells is not clear. In general, NF-κB is sequestered in the cytoplasm in an inactive complex with IκB proteins (22). NF-κB activation is initiated through phosphorylation of IκB by IκB kinases (IKKα/β and IKKγ/Nemo) followed by consequent degradation of the phosphorylated IκB, resulting in nuclear translocation of NF-κB (23, 24). Interestingly, it has been previously shown that Tid1 suppresses tumor necrosis factor-α and Bcl10-induced NF-κB activation by inhibiting IKKβ kinase activity (25). Furthermore, Hsp70, the protein in complex with Tid1, is known to suppress NF-κB activity by binding IKKγ (26, 27). In fact, interaction between Tid1 and Hsp70 was enhanced in the presence of ATP, implicating Tid1 as a regulatory cofactor of Hsp70 (28). Therefore, it is very likely that Tid1, in conjunction with Hsp70, negatively regulates NF-κB activity through modulating IKK activity, and it will be of interest to verify this hypothesis.

The role of IL-8 was observed in several studies in which its expression was strongly correlated with a metastatic phenotype. In breast cancer, IL-8 overexpression has been detected in tumors with highly invasive potential (29), and the expression of IL-8 has also been correlated with metastasis of breast cancer cells to lung after implantation in the mammary fat pad (8). Moreover, a highly metastatic breast cancer line was isolated in which IL-8 expression was significantly up-regulated compared with that of its less metastatic parental line (9). Increased IL-8 expression has also been shown to render nonmetastatic human melanoma cells metastatic (30). In clinical studies, IL-8 was overexpressed in breast tumor tissues compared with that of normal tissues (31). Moreover, the role of IL-8 in tumor development is not only in metastasis but also in promoting tumor cell proliferation and tumor-associated angiogenesis (32). As IL-8 is implicated in various aspects of tumor development and because Tid1 negatively regulates the *de novo* synthesis of IL-8 in tumor cells, it is reasonable to assume that in addition to Tid1's inhibitory effect on IL-8-induced cell migration, Tid1 may also block IL-8-mediated tumor cell growth, invasion, and metastasis, which are currently under investigation.

In Fig. 5, we showed that Tid1 inhibited FVIIa-induced IL-8 production and consequent cell migration of breast tumor cells. Previously, it has been shown that the tissue factor-FVIIa pathway regulated IL-8 expression (20) and that the tissue factor-FVIIa complex promoted melanoma metastasis, which is independent from its role in blood coagulation (33, 34). In breast cancer cells, treatment with an IL-8 neutralizing antibody blocked the cell migration induced by FVIIa, suggesting that the tissue factor-FVIIa pathway modulates the migratory potential of cancer cells through IL-8 production (7). As Tid1 blocks the IL-8 production of cancer cells and the consequent cell migration induced by FVIIa, it is very likely that Tid1 has a role in deterring tumor metastasis promoted by tissue factor-FVIIa pathway. Most recently, the tissue factor-FVIIa protease complex, independent of triggering coagulation, was shown to promote tumor-associated angiogenesis through protease-activated receptor-2 signaling in animals (11). Because NF-κB and the downstream effectors regulated by it have been linked to angiogenesis, and because Tid1 negatively regulates NF-κB activity, it is tempting to investigate whether Tid1 can attenuate the FVIIa-induced development of tumor vasculature.

In summary, we showed that tumor suppressor Tid1 negatively regulated cell motility and metastasis of breast carcinoma cells. This Tid1-dependent effect is, most likely, through the inhibitory role of Tid1 on activation of the transcriptional factor NF-κB, which is critical for the *de novo* synthesis of the *IL8* gene.

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