DDB2: A Novel Regulator of NF-κB and Breast Tumor Invasion

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Abstract

The DNA repair protein damaged DNA-binding 2 (DDB2) has been implicated in promoting cell-cycle progression by regulating gene expression. DDB2 is selectively overexpressed in breast tumor cells that are noninvasive, but not in those that are invasive. We found that its overexpression in invasive human breast tumor cells limited their motility and invasiveness in vitro and blocked their ability to colonize lungs in vivo, defining a new function for DDB2 in malignant progression. DDB2 overexpression attenuated the activity of NF-κB and the expression of its target matrix metalloprotease 9 (MMP9). Mechanistic investigations indicated that DDB2 decreased NF-κB activity by upregulating expression of IκBα by binding the proximal promoter of this gene. This effect was causally linked to invasive capacity. Indeed, knockdown of DDB2-induced IκBα gene expression restored NF-κB activity and MMP9 expression, along with the invasive properties of breast tumor cells overexpressing DDB2. Taken together, our findings enlighten understanding of how breast cancer cells progress to an invasive phenotype and underscore potential clinical interest in DDB2 as a prognostic marker or therapeutic target in this setting. Cancer Res; 73(16); 5040–52. ©2013 AACR.

Introduction

Development of metastatic disease is the primary cause of mortality in patients with breast cancer. This is a multistep event, comprising invasion of mammary carcinoma cells into the adjacent tissues, entry of tumor cells in the systemic circulation, extravasation to distant organs, and finally metastatic colonization, mainly in lungs, liver, bones, and the central nervous system (1). Despite significant advances in diagnosing and treating breast cancer, one of the major clinical and scientific problems that remain unresolved is the prediction of breast tumor progression toward metastasis. Also, identification of new predictive markers of metastatic development seems relevant.

We described recently that the damaged DNA-binding 2 (DDB2) protein, which was originally identified as an accessory factor in nucleotide excision repair of UV-induced DNA damage, is involved in breast tumor growth. DDB2 is overexpressed in nonmetastatic breast tumor cells and plays a role in their proliferation by favoring G1–S transition entry and their progression through the S-phase of the cell cycle (2). We reported that DDB2 stimulates the proliferation of nonmetastatic breast tumor cells, at least in part by maintaining a low basal antioxidant enzyme was involved in the invasive ability of these cells through its control of cell migration, antioxidant enzyme was involved in the invasive ability of these cells through its control of cell migration, antioxidant enzyme was involved in the invasive ability of these cells through its control of cell migration, antioxidant enzyme was involved in the invasive ability of these cells through its control of cell migration, antioxidant enzyme was involved in the invasive ability of these cells through its control of cell migration.
to DNA as hetero- or homodimers that are composed of five possible proteins belonging to the Rel family (c-Rel, RelB, p65/RelA, p50, and p52) and are maintained in a latent form in the cytoplasm through interaction with the inhibitory IκB protein (5). NF-κB transcription factor is constitutively activated in metastatic breast tumor cells and plays a role in their migration and invasive abilities by regulating the expression of target genes (6, 7). However, the molecular mechanism involved in the constitutive activation of NF-κB is not yet clearly defined in breast cancer.

Materials and Methods

Plasmids and lentiviral particles

The pcDNA3(+) expression vector containing the DDB2 open reading frame cDNA was constructed as described previously (3). Lentiviral particles short hairpin RNA (shRNA) containing either three target-specific constructs that encode shRNA ensured by manufacturer for knockdown IκBα (sc-44265-V) gene expression, or nontargeting control sequences (sc-108080), and a puromycin resistance gene for selection of transduced cells, were purchased from Santa Cruz Biotechnology, Inc. The NF-κB transcriptional activity was assessed using a luciferase reporter plasmid under control of NF-κB-binding site (pGL3/NF-κB-Luc) from BD Biosciences. For animal studies, the DDB2-overexpressing and control MDA-MB231 cells were rendered fluorescent by transfection with the pWPT-GFP expression vector (Addgene). The 5′-flanking region (from −1224 to +288 nucleotide residues) of the human IκBα gene were amplified by PCR from genomic DNA using the following primer pair: forward 5′-TGAATTCAATGTCATGGCTTGGCAGGC-3′; reverse 5′-GGATCTCCTGCAGCTCCTTGACCAT-3′ and the iProof high-fidelity DNA polymerase according to the manufacturer's instructions (Bio-Rad). The 1,512-bp fragment of the IκBα promoter was ligated (fast ligation system; Promega) blunt end to the Smal-linearized promoterless pGL3-basic vector containing the firefly luciferase gene (Promega). The proper sense of the IκBα promoter into the plasmid was verified by the complete DNA sequence analysis (Beckman Coulter Genomics) and the resulting plasmid was designed pGL3/IκBα. One plasmid containing the reversed IκBα promoter was used as negative control and designed pGL3/IκBαRev Luc.

The DDB2-binding site was mutated according to the nucleotide changes in the mutant oligonucleotide Omut Mut 10. Plasmid carrying point mutations in the DDB2-binding site was obtained by high-fidelity PCR amplification using the iProof high-fidelity DNA polymerase, the pGL3/IκBαLuc as template (1 ng), and the following 5′-phosphorylated primers: forward 5′-TgCgTcCcTtTtGcCAGCAGCAGCAGCAG-3′ (nucleotides in lowercase indicate point mutations in the DDB2-binding site), and reverse 5′-AcTgtGgGgTTAcGgGgGgGgGc-3′. The PCR-amplified plasmid was circularized by ligation and the presence of point mutations in the DDB2-binding site was verified by DNA sequence analysis of the IκBα promoter (Beckman Coulter Genomics).

Cell culture, transfections, and infections

All human breast cancer cell lines (MCF-7, T47D, MDA-MB231, and SKBR3) were purchased from European Collection of Cell Cultures (Sigma) and authentication procedures were by PCR of short-tandem repeat sequences within chromosomal microsatellite DNA (STR-PCR). All cell lines were not passaged longer than 6 months after receipt and were cultured in RPMI-1640 medium without phenol red (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Sigma). Cells were transiently transfected with different luciferase reporter plasmids (1 μg), and the pSV40/β-gal plasmid (0.1 μg) expressing β-galactosidase (Invitrogen) as control for transfection efficiency, using JetPEI reagent (Ozyme), and harvested 24 hours after transfection. Cells were stably transfected with 4 μg of pcDNA3(+) expression vector containing the DDB2 open reading frame cDNA, using JetPEI reagent (Ozyme). One day later, the clones were selected with 400 μg/mL of G418 for 4 weeks. Resistant clones were isolated and then screened for DDB2 expression by reverse transcription PCR (RT-PCR) and Western blot analysis. One week before experiments, cells were placed into complete medium without G418. Transduction-ready lentiviral particles were used at the optimal conditions for efficient cell transduction and gene silencing with puromycin selection, and were determined for MDA-MB231 cells as 2 TU/cell. Two days later, the clones were selected with 1 μg/mL of puromycin for 7 days. Efficiency of IκBα knockdown was determined in resistant cells by RT-PCR and Western blot analyses.

Treatment of cell culture

For some experiments (migration and invasion assays), breast tumor cells were treated for 24 hours with 50 μM of SN50 or SN50 control peptide (Santa Cruz Biotechnology, Inc.).

Preparation of total, nuclear, and cytoplasmic extracts

Human breast cancer cell lines were lysed in a 10 mmol/L Tris–HCl buffer, pH 7.4, containing 5 mmol/L EDTA, 1% Triton X-100, and a protease inhibitor cocktail, at 4°C for 20 minutes. After centrifugation at 17,000 × g for 20 minutes at 4°C, the supernatant was collected as total protein extract. Nuclear and cytoplasmic extracts were prepared with nuclear extract kit according to the manufacturer's instructions (Active Motif). Protein concentrations were determined according to Lowry and colleagues (8) using bovine serum albumin (BSA) as a standard (Bio-Rad).

Antibodies, Western blot analysis, and immunofluorescence

Antibodies used for Western blot analysis and immunofluorescence at the optimized dilutions are explained in the Supplementary Methods. Western blot analysis and detection with chemiluminescent substrate were carried out, as described previously (4), with total (50 μg), cytoplasmic (20 μg), and nuclear (15 μg) proteins.

Breast tumor cells were cultured onto glass side for 5 days before confluence. Cells were fixed in 4% paraformaldehyde over 20 minutes at room temperature, then blocked and permeabilized with PBS-containing 2% BSA/0.2% Triton X-100. After exposure to the primary polyclonal antibodies diluted at 1:50 for 1 hour at room temperature and three washes in PBS, cells were incubated with the Alexa Fluor 594–conjugated secondary antibodies, diluted at 1:100 in PBS-containing BSA.
and Triton X-100 for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) diluted at 1:1,000 in PBS-containing BSA and Triton X-100. The cells were then mounted in antifading medium (FluorSave, Merck) and observed with an epifluorescence microscope Eclipse 80i with ×40 objective (Nikon). Images were collected with a digital camera (Nikon, DXM1200F) at the same time exposure between all cell types for the protein studied and monitored with Image Analysis Systems Lucia G (Laboratory Imaging s.r.o. software, version 4.81).

Gelatin zymography
The enzymatic activities of MMP2 and MMP9 were determined by gelatin zymography. Breast tumor cells were cultured under normal conditions and then in a serum-free medium for 24 hours with or without 5 μg/mL fibronectin, as described previously (9). SKBR3 cell medium was supplemented with 25 ng/mL of EGF. The conditioned medium was collected and equal aliquots were analyzed on gelatin substrate gels, as described previously (4). Gelatinase activities were visible as clear bands, indicative of proteolysis of the substrate protein.

Luciferase assay
The luciferase assays were conducted using the luciferase reporter gene assay system and β-galactosidase activity was detected using luminescent β-Gal detection assay (Roche). Relative luciferase activities were obtained by the ratio of luciferase/β-gal and expressed as mean ± SD from at least 3 independent experiments, each carried out in triplicate.

RT-PCR analysis
Gene expression was monitored by semiquantitative conducted as described elsewhere (3) and quantitative RT-PCR (qRT-PCR), using primers given in Supplementary Table S1. Total RNAs were isolated from the tumor cell lines using TRIzol RNA (Ozyme) for semiquantitative RT-PCR and the RNeasy Plus Universal Mini Kit (Qiagen) for qRT-PCR, according to the manufacturer’s instructions. One μg was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was conducted using 1 μL of diluted cDNA and PCR IQ SYBR Green Super Mix according to the manufacturer’s instructions (Bio-Rad) on an iCycler RT-PCR instrument (Opticon 2; Bio-Rad). The relative concentration of DDB2, IxBα, or MMP9 transcript was calculated using cycle threshold (Ct) values that were derived from a standard curve and normalized to the average Ct values of the Ubiquitin B (UBB) and ribosomal protein large P0 (RPLP0) internal controls.

Chromatin immunoprecipitation assay, electrophoretic mobility shift, and supershift assays
Chromatin isolation and enzymatic shearing were carried out using the ChIP-IT Express Kit (Active Motif) and immunoprecipitations with anti-DDB2, anti-p65 NF-κB, anti-histone modifications (acetylated on lysine 27 or dimethylated on lysine 4), anti-RNA polymerase II (polII) from Active Motif, and anti-CREB-binding protein (CBP)/p300 (Abcam) were conducted as described previously (3). Electrophoretic mobility shift assay (EMSA) and supershift assay for NF-κB and Sp1 were conducted with a 32P-labeled oligonucleotide representing the respective consensus site as probe, as described previously (10). EMSA and the supershift assay for DDB2 were conducted as detailed previously (3), with the oligonucleotides representing the region of the IκBα promoter where the binding of DDB2 was detected by chromatin immunoprecipitation (ChIP) assay (Supplementary Table S1).

In vivo invasion assay
All experiments were carried out with immunodeficient (nu/nu) mice (6-week-old) from Janvier Laboratories in accordance with National Animal Care Guidelines (European Commission directive 86/609/CEE; French decree no.87–848). In vivo invasive cells were evaluated after tumor cell injections as well as tumor xenografts. Mice were injected subcutaneously into both hind legs with 2 × 10⁶/flank either DDB2-over-expressing or Neo MDA-MB231 cells, respectively, which were rendered fluorescent after transfection with a GFP expression vector. When tumors reached about 1,000 mm³, mice were sacrificed and their lungs removed and sliced for fluorescence-activated cell sorting (FACS) and histologic analyses for invasion assay after cell injection. Some MDA-MB231 tumor xenografts were initiated by implantation of tumor fragments with the same size into the hind legs of each anesthetized mouse and were established in vivo during three sequential passages, as described previously (11). When tumors reached about 1,000 mm³ after the third passage, invasive cells were evaluated in the lungs by FACS and histologic analyses.

Infiltrated lungs (equal volume/weight between animals) were dissociated according to the protocol detailed in the Supplementary Data and cell suspensions were analyzed by FACS (FACScalibur; BD). For each analysis, the data were collected from 200,000 events and invasive cells for their GFP expression were quantified using CellQuest software (BD Biosciences; Clontech). For histologic analysis, several slices from each lung per mouse were cut and fixed in 4% buffered-formaldehyde for 16 hours and embedded in paraffin. Several sections (5 μm thick) were processed for in situ hybridization to detect specifically the genotype of human infiltrating breast tumor cells with the fragmented human genomic DNA, which was first digoxigenin-labeled by a random primer-labeling method, as described previously (12). Digoxigenin was detected using specific alkaline phosphatase–labeled anti-digoxigenin polyclonal antibodies from sheep (Roche) with a substrate solution containing 5-bromo-chloro-indolyl phosphate and nitroblue tetrazolium. After the color development, total numbers of human infiltrating cells, appearing purple in mouse tissue, were observed by microscopy (Axioskop 2; Leica) and counted in at least 8 sections per lung. Images were acquired using Axiovision 4.8 software.

Statistical analysis
The experiments were repeated at least three times. Results were expressed as mean ± SD or SEM, as indicated. All statistical analyses for migration, invasion, luciferase assays, qRT-PCR, and Western blot analyses were conducted using two-tailed Student t test (GraphPad Prism 5.0; GraphPad Software). For in vivo experiments with animals, statistical
analyses were conducted using the Mann–Whitney test (GraphPad Prism 5.0). In all cases, differences were considered to be statistically significant at a value of $P < 0.05$.

Results

DDB2 overexpression reduces migration and invasion in vitro of aggressive breast tumor cells

To examine the effect of DDB2 overexpression on migratory and invasive abilities of aggressive breast tumor cells, wild-type (WT) MDA-MB231 and SKBR3 cell lines were stably transfected with the pcDNA 3(+) DDB2 expression vector or the pcDNA 3(+) control vector (Neo). For each cell line, two clones expressing DDB2 (MDA or SKBR3 DDB2 cl1 and 2) were used for the subsequent studies (Fig. 1A). We next evaluated the motility of MDA-MB231 and SKBR3 cells expressing DDB2 in migration and invasion assays. Both migration and invasion were reduced between 50% and 65% according to the DDB2-expressing cell clones, as compared with control Neo cells (Fig. 1B–D). Similar results were obtained using wound-healing–induced migration assay (Supplementary Fig. S1). Inversely, knockdown of endogenous DDB2 in noninvasive T47D cell line induced migratory and invasive features (Supplementary Fig. S2). We also evaluated the secretion of MMP2 and MMP9, which are related to migration and invasion abilities of metastatic breast tumor cells (13). Zymographic analyses were conducted in the presence or absence of fibronectin, which has been reported to activate rapidly pro-MMP2 (72 kDa) and pro-MMP9 (92 kDa; ref. 9). Overexpression of DDB2 decreased significantly pro- and mature MMP9 (82 kDa) but not pro- and mature MMP2 (64 kDa) secretion in MDA-MB231 cells with or without fibronectin, as compared with control cells (Fig. 1E). These results were confirmed with HER2-positive SKBR3 cells cultured in the presence of EGF (Fig. 1E).

DDB2 overexpression reduces colonization of lungs in vivo by aggressive breast tumor cells

We next evaluated the invasive abilities in vivo by generating DDB2-overexpressing MDA-MB231 cells and control Neo cells stably expressing GFP. These cells were controlled by FACS for their GFP expression and exhibited similar in vitro properties as their parental counterparts (data not shown). Invasive abilities of GFP-positive cells were then evaluated after their subcutaneous injection into nude mice. The invasive cells quantified by FACS were reduced significantly by 7.5-fold in lungs of mice that received DDB2-overexpressing MDA-MB231 cells as compared with Neo cells (Fig. 2A and Supplementary Table S2). Invasive abilities of GFP-positive cells were then evaluated by control Neo or DDB2-overexpressing tumor xenografts, established after three sequential passages in mice (Fig. 2B and Supplementary Table S3). We confirmed that the quantified invasive cells were strongly and significantly reduced by 31.0-fold in lungs of mice with DDB2-overexpressing tumor xenografts as compared with control Neo tumors. Interestingly, the invasive cells from MDA-MB231 Neo tumor xenografts, quantified by FACS, were significantly increased by 4.0-fold in lungs of mice in contrast to the results from mice with MDA-MB231 Neo cell injections. These results were confirmed by histologic analysis where human breast cancer cells were detected by in situ hybridization using human fragmented and digoxigenin-labeled DNA as the probe and numbered by microscopic examination of different sections of lungs per mouse. We observed once again that the invasive cells were reduced significantly in lungs of mice injected or xenografted with DDB2-overexpressing MDA-MB231 cells or tumors, respectively, in contrast to those with Neo control cells or tumors (Fig. 2C and D). These in vitro and in vivo data indicate clearly that DDB2 plays a negative role in the migratory and invasive abilities of aggressive breast tumor cells. These results were supported by an inverse correlation between the DDB2 mRNA expression and the histologic SBR grade in a cohort of 92 samples from patients with breast carcinoma (Supplementary Fig. S3).

DDB2 overexpression is associated with a decrease in the constitutive NF-κB activity

As we observed a decrease in the secretion of MMP9 in aggressive DDB2-overexpressing tumor cells, and this gene is regulated by NF-κB (14, 15), we verified if DDB2 influenced the activity of this transcription factor. The transcriptional activity of NF-κB was evaluated using a luciferase reporter gene system under the control of a κB motif. Although NF-κB activity was increased by 2.0- to 4.6-fold in DDB2 shRNA-transfected T47D cells (Supplementary Fig. S2), it was decreased by 2.7- and 8.0-fold in DDB2-overexpressing MDA-MB231 and SKBR3 cells, respectively, in contrast to the respective control cells (Fig. 3A). This decrease in NF-κB activity was the consequence of a strong decrease in p65NF-κB nuclear translocation in DDB2-overexpressing cells, as seen by Western blotting with cytoplasmic and nuclear extracts as well as cytoimmunofluorescence (Fig. 3B and C). Only in DDB2-overexpressing SKBR3 cells was the p50NF-κB nuclear translocation decreased markedly. In addition, this decrease in NF-κB activity was also associated with a strong increase in the IκBα protein level in the cytoplasmic extract from DDB2-overexpressing cells, in contrast to control cells (Fig. 3C). This latter observation was the consequence of an increase in IκBα protein level in DDB2-overexpressing cells, in contrast to control Neo cells (Fig. 4).

DDB2 decreases NF-κB activity through its role in transcriptional IκBα gene expression

Given that overexpression of DDB2 increased IκBα protein, and decreased constitutive NF-κB activity as well as invasive abilities of aggressive breast tumor cells, we hypothesized that DDB2 could be involved in the regulation of the IκBα gene. Also, we verified the IκBα gene expression at the transcriptional level in MDA-MB231 cells by RT-PCR analysis. The IκBα mRNA level was increased in DDB2-overexpressing cells in contrast to control Neo cells (Fig. 5A and B). However, we observed a decrease in the MMP9 mRNA in DDB2-overexpressing cells according to the decrease in NF-κB activity, as compared with the control cells. Then, the occupancy of the IκBα promoter by DDB2 was investigated by ChIP experiments with MDA-MB231 cells using different sets of primers, which delineated four regions covering 1.4 kb of the proximal promoter of the IκBα gene (Fig. 5C and Supplementary Table S1).
The results showed that specific binding of DDB2 to region III (between -555 and -394 bp from initiation transcription site) only was observed in DDB2-overexpressing cells, in contrast to control Neo cells, indicating that the protein binds to the IkBa promoter gene (Fig. 5D). Interestingly, p65NF-kB was detected in region IV, containing a well-known NF-kB-binding site (16), in contrast to DDB2-overexpressing cells. We analyzed by ChIP if DDB2 binding to the IkBa proximal promoter was associated with an epigenetic process such as posttranslational histone modifications and recruitment of CBP/p300.
DDB2 Controls Breast Tumor Invasion via NF-κB

Figure 2. Overexpression of DDB2 reduces invasive abilities in lungs of aggressive breast tumor cells. Quantification by flow cytometry analysis of GFP-expressing and invading cells in lungs from nude mice injected (A) or xenografted (B) subcutaneously with GFP- and DDB2-overexpressing cells or established tumors, respectively. Data expressed as percentage of GFP-positive cells are mean ± SEM. Quantification by histologic analysis of invasive cells in lungs from mice injected (C) or xenografted (D) subcutaneously with DDB2-overexpressing cells or established tumors, respectively. Representative micrographs showing invasive breast tumor cells (in black) detected by in situ hybridization using human fragmented DNA as the probe. Bars represent 200 μm and black arrows indicate invasive MDA-MB231 cells and control cells.

Although no differences were observed in the acetylation as well as methylation of H3 histone to the lysine residues 27 and 4, respectively, between MDA-MB231 cells expressing or not expressing DDB2, the presence of the p300 protein was more detected for DDB2-overexpressing cells (Fig. 5E).

To identify precisely the sequence of the DDB2-binding site, a set of six oligonucleotides named O1 to O6 and corresponding to region III determined by ChIP assay were used for EMSA (Supplementary Table S1). Among them, only the O3 oligonucleotide formed a shifted DNA–protein complex in DDB2-overexpressing MDA cells compared with control cells (Fig. 6A), which was specific, as shown in competition experiment using different amounts of unlabeled O3 probe and a nonspecific NF-κB probe (Fig. 6B). The presence of DDB2 was detected on the O3 oligonucleotide by a marked decrease in its DNA-binding activity by supershift experiment using specific polyclonal antibodies (Fig. 6C). The shift formed with the O3 oligonucleotide was verified as nonspecific by competition using different amounts of unlabelled O3 probe (data not shown). A collection of mutant O3 oligonucleotides (Mut 1–14) were tested by EMSA to better characterize the DDB2-binding site. Only Mut 8–11 were unable to bind DDB2 (Fig. 6D). This result was confirmed by competition, showing that these mutant oligonucleotides were unable to compete with WT O3. Taken together, these data suggest that the TCCCCTTA (between −491 and −483 bp from initiation transcription site) sequence in the O3 oligonucleotide was important for DDB2 binding to the IκBα proximal promoter. The consequence of this DDB2 binding in the expression of the IκBα gene was studied using the luciferase reporter gene system under the control of the proximal IκBα promoter. Transfection reporter assays indicated that the transcription of the IκBα gene was increased significantly in DDB2-overexpressing MDA-MB231 cells, in contrast to neo cells (Fig. 6F). The functionality of the DDB2-binding site was tested by mutating it specifically in the

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pGL3/IκBα Luc construct. The resulting construct (pGL3/IκBα Mut10 Luc), containing two mutated residues and corresponding to the O3Mut10 sequence, was transfected into DDB2-overexpressing MDA-MB231 cells. The response to DDB2 in cells transfected with the mutant construct, was decreased dramatically (Fig. 6E). These results were confirmed with MCF-7 cells, which overexpress naturally DDB2 (Supplementary Fig. S4).

DDB2 reduces the invasive potential of aggressive breast tumor cells through its negative control of NF-κB activity

To evaluate whether DDB2 reduced the migratory and invasive abilities of aggressive breast tumor cells by inducing IκBα gene, this latter was knocked down in DDB2-overexpressing MDA-MB231 cells, using lentiviral shRNAs. Two DDB2-overexpressing cell clones transfected with shRNA IκBα were isolated (Fig. 7A), in which NF-κB activity, MMP9 expression, and migratory and invasive abilities were restored in part (Fig. 7B–E). No significant difference was observed between parental and shRNA control DDB2-overexpressing cells. These results support the negative effect of DDB2 on constitutive NF-κB activity by inducing IκBα gene expression to reduce the migratory and invasive potential of tumor cells. We confirmed that inhibition of NF-κB by treatment of control Neo cells with the cell-permeative peptide SN50 decreased invasive cell abilities (Supplementary Fig. S5).
It has also been reported that the constitutive activation for constitutive NF-κB in breast cancer cells and primary tumors, which was responsible for the constitutive activation of the NF-κB pathway in MDA-MB-231 and SKBR3 control cells fixed at 1 (mean ± SD from 3 separate experiments). Statistical significance (P < 0.05) between DDB2 overexpressing and Neo control cells. B, immunofluorescence showing the IκBα protein level in breast tumor cells. DAPI was used for nuclear staining (scale bar, 30 μm).

Discussion

We observed previously that DDB2 expression was inversely correlated with the invasive properties of breast tumor cells, and that a constitutive overexpression of DDB2 in nonmetastatic cells induced cell proliferation by activating the G1-S transition phase of the cell cycle (2). In the present study, our findings summarized in Fig. 7F provide for the first time strong evidence that DDB2 plays a role as a negative regulatory factor in the invasive abilities of breast tumor cells in vitro as well as in vivo. We showed that DDB2 plays this role via decreasing NF-κB activity, by its involvement in the regulation of the IκBα gene.

Several studies have reported that NF-κB also plays a key role in the induction of invasive abilities of cancer cells and is required for maintenance of the malignant phenotype, particularly for breast cancer cells. Inhibition of the NF-κB pathway abrogates generation of the Ras-dependent invasive phenotype acquired by breast cancer cells in vitro and prevents metastasis in a mouse model (17). In accordance with this study, inhibition of the constitutive or induced NF-κB pathway in MDA-MB-231 and HER2-positive SKBR3 cells, respectively, using the SN50 peptide (18), decreased migration and invasion of these cells (Supplementary Fig. S5). The NF-κB–dependent acquisition of invasive properties of breast cancer cells and tumors as well as of carcinogen-induced primary rat mammary tumors is often associated with an aberrant constitutive activation of this transcription factor (6, 19). Two molecular mechanisms have been identified to be related to the constitutive activation of NF-κB in breast cancer cells. An aberrant expression of IκB kinases and casein kinase II was observed in some breast cancer cells and primary tumors, which was responsible for constitutive NF-κB activation by controlling IκBα stability (20). It has also been reported that the constitutive activation of NF-κB could be due to an enhanced expression of NF-κB–inducing kinase involved in the activation of IκB kinases, resulting to an epigenetic alteration of its gene in the malignant basal-like breast cancer (21). About HER2-positive breast cancer cells, as the SKBR3 cell line, NF-κB is activated constitutively by the survival signaling pathway via PI3K/Akt and NIK/IKκB kinase, which is triggered by the binding of EGF to its overexpressed membrane receptor (22). This may explain the marked effect of DDB2 on NF-κB activity and the migration/invasion properties of EGF-sensitive SKBR3 cells. In the present study, we identified for the first time a new molecular mechanism corresponding to a lack of DDB2 expression in aggressive breast cancer cells, which leads to a low IκBα expression and a high constitutive NF-κB activity.

NF-κB plays a causal role in migration and invasion of tumor cells by regulating positively target genes. Among them, the gene encoding MMP9 is a well-known target gene of NF-κB, whose promoter contains a NF-κB site at −599 bp from the transcription start site (14). MMP9, a type IV collagenase, is partly responsible for the invasive phenotype of tumor cells, by degrading the basement membrane (7). In addition, MMP9 expression is correlated often with advanced tumor grade in breast cancer (23). The fact that MMP9 expression is reduced by the DDB2-dependent decrease in NF-κB activity could explain in part that DDB2 plays a negative role in the invasive properties of aggressive tumor cells. It is not excluded that DDB2 plays this role by maintaining a low expression of the MnSOD gene, via its binding to the proximal promoter, as we showed recently (3). MnSOD, which is constitutively overexpressed in highly invasive breast tumor cells, participates in the invasive properties of these cells by increasing the level of secreted MMP9 via the hydrogen peroxide produced from its enzymatic activity on the superoxide anion (4).

We identified that the involvement of DDB2 in the regulation of the IκBα gene is the molecular mechanism by which DDB2...
plays a negative role in the invasive abilities of aggressive breast tumor cells. Knockdown of IκBα gene expression induced by DDB2 restored constitutive NF-κB activity and invasive properties of aggressive breast tumor cells. The IκBα gene is the second newly identified target gene for DDB2. In contrast to the MnSOD gene, its expression is positively regulated in the presence of DDB2. The binding site of DDB2 is localized in the proximal IκBα gene promoter and differs from the one identified for the MnSOD gene (3). It is difficult to consider that the difference in the sequence of the binding site explains the distinct role of DDB2 in expression of IκBα and MnSOD genes. The regulation of IκBα gene expression is not well known, and from a footprinting analysis, only three κB sites and one Sp1 site in the proximal IκBα gene promoter have been characterized, all close to the TATA box and the initiation transcription site (Fig. 5). The κB1 and Sp1 sites have been shown to be required in an autoregulatory loop for full TNF-α–mediated induction of the IκBα gene in Jurkat T cells (16). Although no

Figure 5. DDB2 influences positively the transcription of IκBα gene and binds directly the proximal IκBα promoter. Total RNAs were isolated from MDA-MB231 overexpressing or not DDB2 and were reverse transcribed. Expression of IκBα, MMP9, and DDB2 was analyzed using semiquantitative (A) or qRT-PCR (B) RT-PCR. Their transcript levels were normalized to RPLP0 and UBB mRNA, and statistics were significant (P < 0.05) between DDB2-overexpressing MDA-MB231 cells and control cells for qRT-PCR. C, a schematic of the IκBα promoter highlighting the regions used in ChIP assay (primers are done in Supplementary Table S1) used to amplify the immunoprecipitated chromatin by PCR and to detect in vivo the interaction of DDB2 with the IκBα promoter. The initiation transcription site is indicated by a single arrowhead at position +1. Positions of NF-κB- and Sp1-binding sites are indicated by vertical arrows. D, ChIP assay showing the binding of DDB2 to the IκBα promoter in control (Neo) and DDB2-overexpressing MDA-MB231 cells. E, ChIP assay showing the presence of acetylated (AcH3K27) or dimethylated H3 histone (DiMeH3K4), and p300 at the region IV.
Figure 6. DDB2 induces IkBα promoter activity by binding to its specific DNA sequence. A, EMSA with 6 oligonucleotides (O1 to O6), corresponding to the region III of the IkBα promoter, and 32P-labeled incubated with nuclear proteins (2 μg) from control and DDB2-overexpressing MDA-MB231 cells (NS, nonspecific complex). EMSA for Sp1 or NF-κB was used as the nuclear protein loading control. B, the specificity of the DNA-binding activity on 32P-labeled O3 oligonucleotide was shown by EMSA using either an excess of specific and unlabeled O3 (S.C.) or with nonspecific oligonucleotide corresponding to the NF-κB consensus site (NS.C.) as competitor. C, supershift assay revealing the presence of DDB2 in nuclear proteins (2 μg) from DDB2-overexpressing MDA-MB231 cells. D, EMSA using nuclear proteins from DDB2-overexpressing cells and different 32P-labeled mutant O3 oligonucleotides with two nucleotide changes (Mut 1–14) as probes to determine the sequence of the DDB2-binding site. Competition was carried out with 50-fold molar excess of unlabeled mutant oligonucleotides. E, functional analysis of IkBα promoter in DDB2-overexpressing cells by transient transfection with luciferase reporter gene placed under the control of the WT IkBα promoter (pGL3/IκB Luc), the reversed IkBα promoter (pGL3/IκBRev Luc) as negative control, or mutated for DDB2-binding site (pGL3/IκB Mut10 Luc). Results from 3 independent experiments, each carried out in triplicate, are mean/SD of luciferase activity normalized to β-galactosidase activity and indicated as relative IkBα promoter activity. Statistical significance (P < 0.05), either between DDB2-overexpressing and control Neo cells, or pGL3/IκB Mut10 Luc and pGL3/IκB Luc.
Figure 7. Knockdown of IkBα expression suppresses the negative effect of DDB2 on the invasive abilities of aggressive breast tumor cells. IkBα expression was knocked down in DDB2-overexpressing MDA-MB231 cells (MDA DDB2 cl1), using lentiviral shRNAs, and two cell clones were isolated (MDA DDB2 cl1shIkBα1 and 2). Knockdown of IkBα expression was verified by RT-PCR (A) and Western blot analysis (B) in MDA DDB2 cl1shIkBα1 and 2, in contrast to MDA DDB2 cl1 transfected with control shRNA (MDA DDB2 cl1shcontrol 1 and 2). The result shown is representative of 3 separate experiments. C, consequence of the knockdown of IkBα expression on the NF-κB transcriptional activity after transient transfection of breast tumor cells with pGL3/NF-κB-Luc and luciferase assay. Results from 3 independent experiments carried out in triplicate are mean ± SD of luciferase activity normalized to β-galactosidase activity and indicated as relative NF-κB activity. D, cell migration and invasion assays through uncoated and Matrigel-coated membranes, respectively, were evaluated for control and MDA DDB2 cl1shIkBα cells. Data from 3 independent experiments (each in triplicate) were expressed as mean ± SEM of percentage of the shcontrol cells, defined as 100% of migrating or invading cells. E, representative phase contrast image of D; scale bar, 100 μm. F, a summarized model illustrating the molecular mechanism by which DDB2 reduces invasive properties of aggressive breast tumor cells. In all cases, differences were significant (P < 0.05) between the MDA DDB2 cl1shIkBα and shcontrol cells, and no significant difference was observed between all control cells.
Sp1 binding was detected on its site whatever the DDB2 expression (data not shown), we observed constitutive p65NF-κB binding to the xB1 site in control breast cancer cells. This was decreased in DDB2-overexpressing cells because of the decrease in the nuclear translocation of the p65NF-κB protein, suggesting that this latter is not involved in DDB2-dependent induction of the IκBα gene. This explains also why DDB2 maintains a low expression of the MnSOD gene, by decreasing NF-κB binding to a xB site located in intron 2, as we showed recently (24).

In an attempt to explain the role of DDB2 in the induction of the IκBα gene, we examined whether the DDB2-dependent increase in IκBα expression was associated with an epigenetic process such as posttranslational histone modification, as shown previously by us and others (3, 25). Both methylation and acetylation of histone H3 are known to be indicative of the active transcription of genes. The IκBα gene, which has a basal transcription level, is located into a region of chromatin opened continuously by basal histone modifications (http://genome.ucsc.edu/), explaining the presence on the proximal promoter of p65 NF-κB binding to the xB1 site and acetylated and dimethylated H3 histones to the lysine residue 27 and 4, respectively, in control cells. No changes were observed for histone modifications, regardless of the DDB2 expression. Only the p300 protein was detected more at region IV close to the transcription start site in DDB2-overexpressing cells than in control cells, which may explain the DDB2-dependent increase in IκBα expression. Indeed, the CBP/p300 family is well known to enhance transcription, not only by modifying chromatin structure through its histone acetyltransferase activity, but also by facilitating the assembly of activating transcription complexes at the promoter (26).

In addition, these proteins are able to interact with a variety of proteins, including DDB2, to increase their activity as coactivators of transcription (27).

The present findings bring a new understanding of how breast cancer cells progress toward an invasive phenotype, by decreasing the DDB2 expression. This protein represents a good candidate for predicting breast tumor progression toward a malignant phenotype, by its involvement in the control of invasive abilities of tumor cells, and by its expression inversely correlated with the histologic SBR grade tumors (Supplementary Fig. S3). Supporting this idea, a recent analysis in public database of gene expression arrays (free online software Kaplan–Meier Plotter; http://www.kmplot.com; ref. 28) reveals that the higher DDB2 expression in breast tumors is correlated with an improvement of the relapse-free, as well as overall and distant metastasis-free survival of patients. Finally, the molecular mechanism identified in our study may represent an interesting pathway for future development of new anticancer therapies, because NF-κB is already considered as a good therapeutic target (29).

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

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References
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