FEBS Letters 582 (2008) 3193–3200

# Selective repression of YKL-40 by NF-κB in glioma cell lines involves recruitment of histone deacetylase-1 and -2

Krishna P. Bhat[a,b,*], Christopher E. Pelloski[a,b,c], Yujian Zhang[a,d], Se Hoon Kim[a,b,f], Clarissa deLaCruz[a,b], Michael Rehli[e], Kenneth D. Aldape[a,b,*]

[a] *Brain Tumor Center, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[b] *Department of Pathology, Unit 85, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[c] *Department of Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[d] *Department of Neurosurgery, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[e] *Department of Hematology and Oncology, University of Regensburg, Regensburg 93042, Germany*
[f] *Department of Pathology, College of Medicine, Yonsei University, Seoul, Republic of Korea*

Received 9 June 2008; revised 29 July 2008; accepted 9 August 2008

Available online 15 August 2008

Edited by Ned Mantei

**Abstract** **Here we show that in contrast to other cancer types, tumor necrosis factor (TNF)-α suppresses YKL-40 expression in glioma cell lines in a nuclear factor κB (NF-κB) dependent manner. Even though TNF-α causes recruitment of p65 and p50 subunits of NF-κB to the YKL-40 promoter in all cell types, recruitment of histone deacetylases (HDAC)-1 and -2, and a consequent deacetylation of histone H3 at the YKL-40 promoter occurs only in glioma cells. Importantly, using chromatin immunoprecipitation assays in frozen glioblastoma multiforme tissues, we show that YKL-40 levels decrease consistent with HDAC1 recruitment despite high levels of nuclear p-p65. This study presents a paradigm for NF-κB regulation of one of its targets in a strict cell type specific manner.**
**Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.**

*Keywords:* YKL-40; NF-κB; Glioma; HDAC

## 1. Introduction

YKL-40 (also known as chitinase 3-like 1 or human cartilage glycoprotein 39) is a glycoprotein that belongs to the family 18 of chitinases, though it does not have glycolytic properties [1]. Purified YKL-40 has been shown to induce proliferation in a variety of cell lines, stimulate endothelial cell migration, and protect cells against inflammatory damage [2–5]. Using expression profiling, we previously found that expression of the YKL-40 gene was strongly associated with poor outcome in glioblastoma multiforme (GBM) [6,7]. In addition, our previous studies on immortalized human astrocytes stably transfected with YKL-40 found that YKL-40 conferred radiation resistance and increased invasion across a chemotactic gradient in vitro [6,7]. In recent years, YKL-40 has garnered much attention as a serum biomarker for multiple diseases, including cancer, arthritis, and asthma [8–10]. However, little is known about how YKL-40 functions as well as its transcriptional regulation. Therefore, we sought to learn more about the biological regulation of this protein, specifically in GBM.

Recently, it was shown that the YKL-40 promoter sequence contains consensus binding sites for several known transcription factors, and specific binding of the nuclear PU.1, Sp1, Sp3, USF, AML-1, and C/EBP proteins was confirmed using gel shift assays [11]. Recklies et al. found that inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, induced sustained levels of YKL-40 in chondrocytes via nuclear factor κB (NF-κB) signaling [12].

NF-κB complexes are composed of homo- or heterodimers formed from members of the RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) multigene family. These genes mediate the transcriptional activity of a number of target genes involved in oncogenesis, apoptosis, and inflammation [13,14]. p65, a well-characterized member of the NF-κB family, is found in most unstimulated cells in an inactive form, sequestered in the cytoplasm by inhibitory κBs (IκBs). In response to treatment with typical (TNF-α, IL-1β) or atypical (ultraviolet [UV]-B radiation, chemotherapeutic agents) activators, IκB is phosphorylated, ubiquitinated, and ultimately degraded by the proteosome, resulting in the release of the p65 complex to the nucleus [13,14]. The p65 and p50 heterodimers then bind to the DNA promoter of target genes and mediate transcriptional activation [13,14]. However, studies have found that NF-κB can also cause transcriptional repression of select genes, such as antiapoptotic factors, by direct recruitment of corepressors, such as histone deacetylases (HDAC)-1 and -2 [15,16].

In this study, we show that NF-κB, specifically its p65 and p50 subunits, act as a negative regulators of YKL-40 in glioma cell lines. Using chromatin immunoprecipitation (ChIP) assays, we further show that TNF-α treatment recruits HDAC1 and HDAC2 in glioma cells, leading to deacetylation of histone H3 at the YKL-40 promoter.

*Corresponding authors. Address: Department of Pathology, Unit 85, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA. Fax: +1 713 745 1105.
*E-mail address:* kbhat@mdanderson.org (K.P. Bhat).

*Abbreviations:* ChIP, chromatin immunoprecipitation; EDTA, ethylenediamine tetraacetic acid; EMSA, electrophoretic mobility shift assay; GBM, glioblastoma multiforme; HDAC, histone deacetylase; IκB, inhibitory κB; IL, interleukin; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TSA, trichostatin A

0014-5793/$34.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2008.08.010

## 2. Materials and methods

### 2.1. Cell culture, plasmids, and transfection

We obtained the SNB-75 glioma cell line from the Division of Cancer Treatment and Diagnosis at the National Cancer Institute (Frederick, MD) and the U87 (glioma) BT549 (breast), SW1353 (chondrosarcoma), and THP-1 (leukemia) cell lines from American Type Culture Collection (Manassas, VA). All cells were maintained in Dulbecco's modified Eagle medium/F-12 (50:50 mixture) supplemented with 10% fetal bovine serum, 10 units/ml penicillin G sodium, and 10 μg/ml streptomycin sulfate. We purchased the IκB-SR adenovirus from Vector Biolabs (Philadelphia, PA). The −1300 to −1 bp region of the YKL-40 promoter was cloned into PGL-3 luciferase vector (Promega, WI) as previously described [11]. The −751 and −800 mutant vectors were created using site directed mutagenesis with following primers: −751-S-mut, 5′-AAGCATTCTTGAGCTCTTCC-CTGTCTTTCC-3′; −751-AS-mut, 5′-GGAAAGACAGGGAAGAG-CTCAAGAATGCTT-3′; −800-S-mut, 5′-CTCTCTTTATGAGCTC-TTCAAAACAGAAGC-3′; −800-AS-mut, 5′-GCTTCTGTTTTGA-AGAGCTCATAAAGAGAG-3′. For transient transfection studies, cells were split 24 h prior into triplicate 60 mm dishes followed by transfection with 1 μg of indicated luciferase constructs. A β-actin *Renilla* luciferase construct was co-transfected for normalizing luciferase activity. Twenty-four hours after transfection, cells were treated with TNF-α (100 ng/ml) or trichostatin A (TSA) (100 nM) for an additional 24 h. Cells were lysed and analyzed for luciferase activity using Dual luciferase reporter system (Promega, WI).

### 2.2. Real time reverse transcriptase-polymerase chain reaction (RT-PCR) for mRNA detection

We detected mRNA using a one-step RT-PCR method with reagents purchased from Applied Biosystems (Foster City, CA). Briefly, 0.3–0.5 μg total RNA was reverse transcribed using MuLvRT (2.5 units/μl). To amplify the cDNA, we used primers and Taqman probes for YKL-40 (Hs00609691_m1) and β-actin (Hs99999903_m1) in conjunction with 1× Taqman Universal PCR Master Mix (Applied Biosystems). RNase inhibitor (0.4 units/μl; Roche Applied Science, Indianapolis, IN) was included in every reaction. Reaction mixtures were incubated at 48 °C for 30 min, followed by 10 min at 95 °C for 1 cycle and then 15 s at 95 °C and 1 min at 60 °C for 40 cycles. We measured the fluorescent signal using the ABI Prism 7700 Sequence Detector (Applied Biosystems), and we calculated the relative level of fold changes in YKL-40 expression using the absolute $\Delta\Delta C_T$ method.

### 2.3. Western blot analyses

We performed Western blot analyses according to standard protocols to determine YKL-40 expression in all the cell lines. YKL-40 antibodies were obtained from Quidel Corporation (San Diego, CA) and β-actin control antibodies were obtained from Lab Vision (Fremont, CA).

### 2.4. Electrophoretic mobility shift assay (EMSA)

SNB-75 cells were treated with TNF-α for the appropriate amount of time and nuclear extracts were prepared for EMSA according to standard protocols. Briefly, $^{32}P$ end-labeled wild-type DNA probes to the two consensus sites (−751-sense: 5′-AAGCATTCTTGGGA-ATTTCCCTGTCTTTCC-3′; −751-antisense: 5′-GGAAAGACAGG-GAAATTCCCAAGAATGCTT-3′; −800-sense: 5′-CTCTCTTTAT-GGGAATTTCAAAACAGAAGC-3′; −800-antisense: 5′-GCTTCT-GTTTTGAAATTCCCATAAAGAGAG-3′) were incubated with 10 μg of nuclear extract in a 10-μl reaction volume containing 75 mM NaCl, 15 mM Tris–HCl (pH 7.5), 1.5 mM ethylenediamine tetraacetic acid (EDTA), 1.5 mM dithiothreitol, 25% glycerol, 20 μg/ml bovine serum albumin, and 1 μg poly(dI-dC). The reaction mixture was incubated on ice for 40 min then at 25 °C for 20 min; it was then applied to a 4% non-denatured polyacrylamide gel containing 0.25× TBE (22.5 mM Tris, 22.5 mM borate, 0.5 mM EDTA [pH 8.0]) buffer. For competition assays, we added a 50- to 250-fold molar excess of unlabeled wild-type or mutant (−751-mut-sense: 5′-AGCATTCTT-GAGCTCTTCCCTGTCTTTCC-3′; −751-mut-antisense: 5′-GGAA-AGACAGGGAAGAGCTCAAGAATGCTT-3′) oligodeoxyribonucleotides to the binding reaction. For antibody supershift assays, 2 μl of polyclonal antibody against p65 (H-286) or p50 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated for 45 min on ice before the probe was added. After electrophoresis, the gel was dried for 1 h at 80 °C and exposed on Kodak film (Rochester, NY) at −80 °C.

### 2.5. ChIP assay

We performed ChIP assays as described previously [17]. Briefly, cells were plated in a 150-mm dish 24 h and then treated for 1 h with TNF-α. The proteins were cross-linked by incubation with 1% formaldehyde, and the cells were then washed with phosphate-buffered saline (PBS)-containing protease inhibitors, pelleted, and treated with sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl [pH 8.1]) for 10 min. We performed sonication six to eight times for 10 s each at a constant duty cycle with an output of 3, and we incubated the cells on ice after every sonication. The debris was then pelleted, and the supernatant was diluted to 1/10 concentration with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl [pH 8.1], 167 mM NaCl). The proteins were precleared with 50 μl of 1:1 protein A-Sepharose beads in TE buffer (22.5 mM Tris and 0.4 mM EDTA [pH 8.0]); 200 μl of the mixture was reserved as input, and the remaining 800 μl was incubated with 5 μg of antibodies and 2 μg of sheared salmon sperm DNA (Stratagene, La Jolla, CA) overnight at 4 °C. The protein–DNA–antibody complex was precipitated using 1:1 protein A-Sepharose beads and 2 μg of salmon sperm DNA at 4 °C for 2 h. The beads were pelleted and washed once each with high-salt wash buffer, low-salt wash buffer, and 1× TE. The DNA–protein complex was obtained by extracting the beads with 50 μl of extraction buffer (1% SDS, 0.1 M $NaHCO_3$) three times. We reversed the cross-linking of the DNA protein complex by incubating it at 65 °C for 16 h. The DNA was extracted with a QIAquick PCR purification kit (QIAGEN, Valencia, CA). For ChIP assays using frozen tissues, each frozen section was histologically assessed for tumor by a neuropathologist (K.D.A.) and used only if at least 90% of the tissue was determined to be tumor. Tumor tissue was dissected and quickly ground in liquid nitrogen. Tissues were washed four times with a PBS-containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride to remove any blood. We prepared tissue lysates using a method similar to that used for preparing adherent cells 50 μl of packed cell volume was then used for each ChIP assay. We purchased antibodies against p65 and p50 from Santa Cruz Biotechnology, antibodies against HDAC1 and HDAC2 from Cell Signaling Technology (Danvers, MA), and antibodies against acetylhistone-H3 from Upstate Biotechnologies (Billerica, MA). PCR was performed using primers that amplified both NF-κB consensus sites (YKL-NF-F [forward]: 5′-CGAGCTTGCAAAAGATCCTCTC-3′; YKL-NF-R [reverse]: 5′-GAAGGAAAGCAAAGAGCCTGAAA-3′), the −751 consensus site only (−751-F [forward]: 5′-GGGA-ATTTCCCTGTCTTTCC-3′; −751-R [reverse]: 5′-AGGCTCAG-CATTGCCCTGC-3′), or the −800 consensus site only (−800-F [forward]: GAGAGGGGCTGTATCATCAGGCT-3′; −800-R [reverse]: 5′-GCCCCGGTGCTATTTTGC-3′). The IκB promoter was amplified using published primer sets [16].

### 2.6. Immunohistochemical analysis

We performed immunohistochemical (IHC) analyses using 5 μmol/l sections that were cut from paraffin blocks, deparaffinized, hydrated through an ethanol series. After microwave antigen retrieval, antibodies against phosphorylated p65 (p-p65; 1:100; Cell Signaling Technology) or YKL-40 (1:1,000; Quidel) were incubated with the slides overnight at 4 °C. Staining was performed using the DAKO Envision kit according to the manufacturer's instructions (DAKO, Carpinteria, CA). p-p65 and YKL-40 staining were scored as strongly positive, weakly positive, or negative. The staining for YKL-40 was described previously [6]. A neuropathologist (K.D.A.) identified blocks with sufficient tumor available for analysis of each case. In all cases, scoring was based on the most positive area present in the tumor.

### 2.7. Statistical analyses

Student's *t*-test was performed on all real time PCR data to evaluate fold changes. *P* values of $<0.05$ were considered statistically significant. Fisher's exact test was done to identify positive or negative relationships between expression levels of p-p65 and YKL-40.

## 3. Results

### 3.1. TNF-α modulates YKL-40 levels in a cell type-specific manner via NF-κB signaling

We analyzed the promoter region of YKL-40 using the Genomatix (Ann Arbor, MI) software tool and identified a consensus sequence for NF-κB at −800 upstream of the transcription start site in addition to the previously reported −751 site [12]. Since cytokines have been shown to activate YKL-40 in chondrocytes [12], we evaluated the effects of TNF-α treatment, a known inducer of the NF-κB signaling pathway, in glioma cell lines. TNF-α treatment of SNB-75 and U87 cells, which consti-

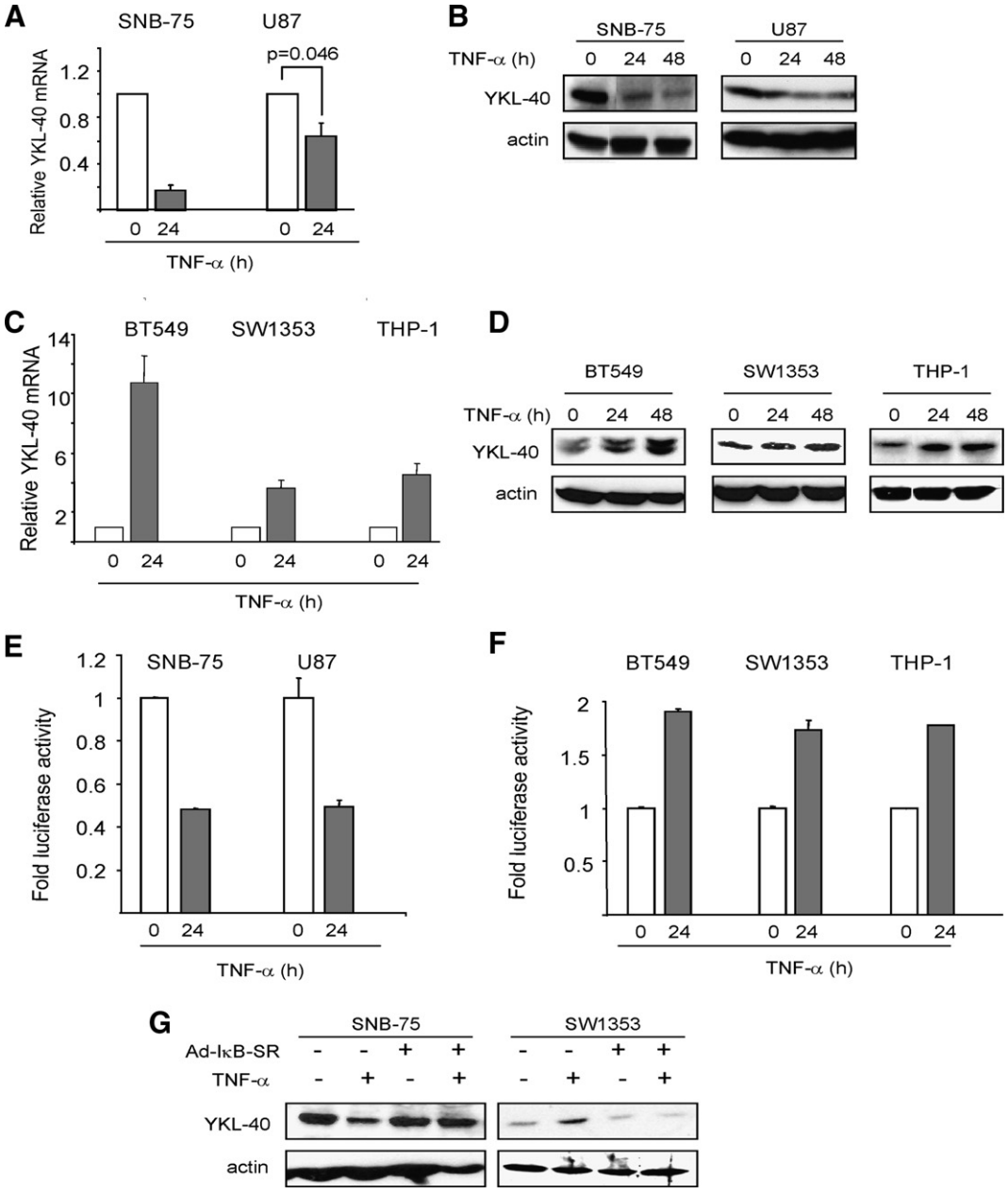


Fig. 1. TNF-α modulates YKL-40 levels in a cell type specific manner via NF-κB signaling. (A) Cells were treated with TNF-α (20 ng/ml) for 24 h and total RNA was isolated, converted to cDNA and analyzed for mRNA expression of YKL-40 using real time PCR. Results were normalized to β-actin levels. The fold change for the control group was set at one and compared to TNF treatment groups. (B) SNB-75 and U87 cells were treated with TNF-α for the indicated time points and lysed. Fifty micrograms protein was loaded onto 10% SDS–poly acrylamide gel and subject to Western blotting. (C) Cells were treated with TNF-α (20 ng/ml) for 24 h and total RNA was isolated, converted to cDNA and analyzed for mRNA expression of YKL-40 and results normalized to β-actin levels as described previously. The fold change for the untreated group was set at one and compared to TNF treatment groups after 24 h. (D) Western analyses of cells as indicated upon treatment with TNF-α for the indicated time points. Actin antibody was used as loading control and YKL-40 expression was compared. (E) and (F) SNB-75, U87, BT549, SW1353, and THP-1 cells were transiently transfected with YKL-40 promoter construct and luciferase activity was measured after treatment with TNF-α for 24 h. A *Renilla* luciferase plasmid driven by the β-actin promoter was used as control to normalize luciferase activity. Bar graphs indicate average of triplicate measurements and luciferase values without TNF-α treatment were set to one and compared. (G) SNB-75 and SW1353 cells were infected with adenovirus expressing stable mutant form of IκB-alpha (S32/36) at an MOI of approximately 5 for 24 h followed by TNF-α treatment for additional 24 h. Cells that were not pre-treated with IκB-SR were mock infected with a GFP expressing adenovirus. At the end of treatment period, cells were lysed and analyzed for expression of YKL-40 and actin by Western blotting.

tutively express YKL-40, surprisingly resulted in the downregulation of endogenous YKL-40 mRNA (Fig. 1A) and protein in a time-dependent manner (Fig. 1B). However, TNF-α treatment of the same cell lines caused a parallel increase in the expression of Cox-2, a downstream target of NF-κB (data not shown). To address these seemingly contradictory findings, we examined whether TNF-α treatment repressed YKL-40 in additional cancer cell lines (BT549, SW1353, and THP-1) that expressed varying levels of YKL-40. All three cell lines showed induction of YKL-40 in response to TNF-α treatment at both the mRNA and protein level (Fig. 1C and D). Both activation and repression of YKL-40 occurred as early as 6 h after TNF-α treatment and could be sustained up to 72 h (data not shown). We next asked if the activation or repression of YKL-40 by TNF-α could be recapitulated at the promoter level using transient transfection assays. A −1300 to −1 bp promoter region of YKL-40 cloned into PGL-3 luciferase vector was transfected into the indicated cell lines followed by TNF-α treatment for 24 h. As expected, treatment of glioma cell lines with TNF-α caused a significant repression of the promoter activity (Fig. 1E), whereas activation was observed in all non-glioma cell lines upon TNF-α treatment (Fig. 1F).

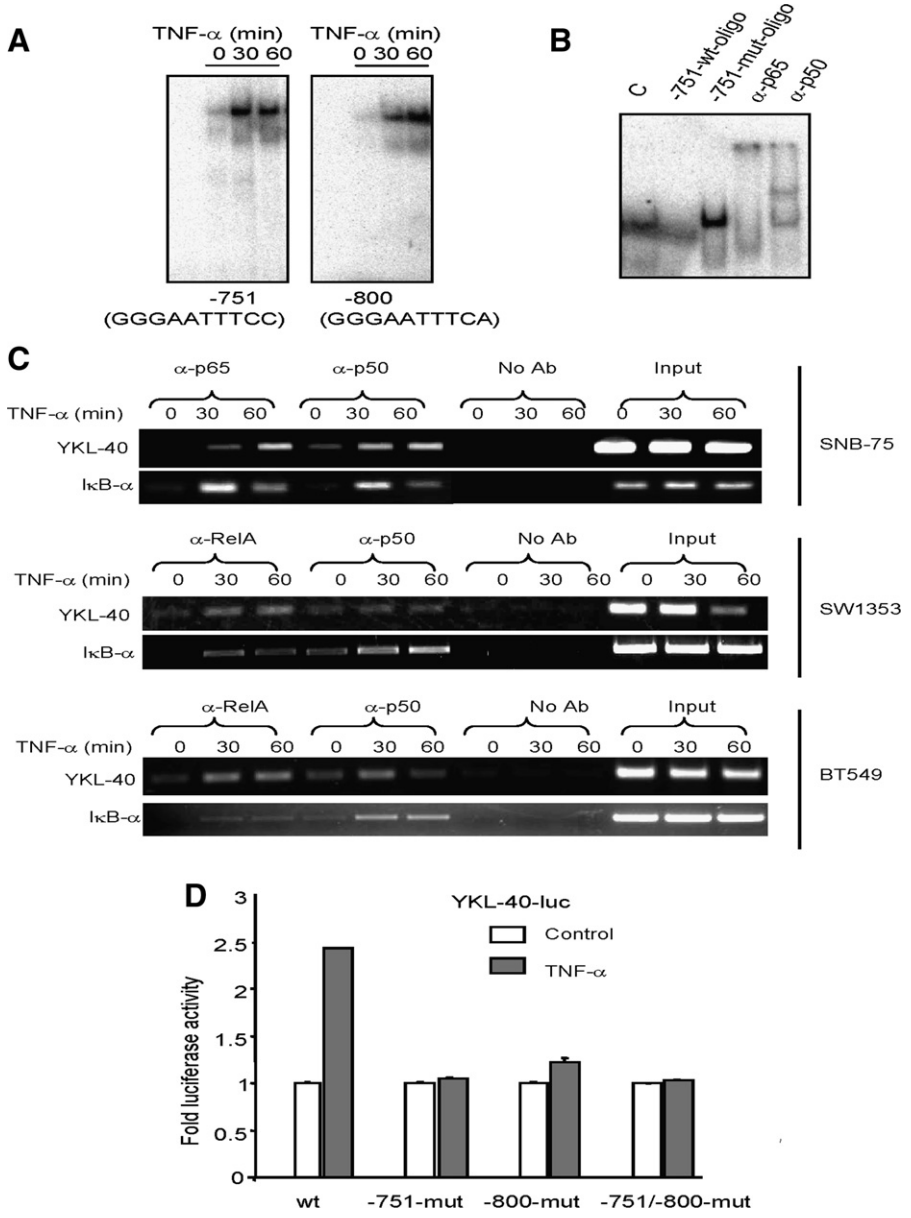


Fig. 2. p65/p50 are actively recruited to the YKL-40 promoter in response to TNF-α. (A) Nuclear extracts were prepared from SNB-75 cells that were either untreated or treated with TNF-α for the indicated time points. Gel shift analyses was performed after incubating with $^{32}$P-labeled oligos representing either −751 and −800 κB consensus and complexes were analyzed as described. (B) Competition using cold wild type or mutant −751 κb consensus oligos or supershift assays using antibodies against p65 or p50 were performed as indicated. (C) SNB-75, SW1353 or BT549 cells were stimulated with TNF-α for the indicated time points and ChIP assays were performed using antibodies directed to p65 or p50. Binding to the IκB or YKL-40 promoters was detected by PCR. (D) NIH3T3 cells were transfected with the indicated reporter plasmids for 24 h followed by treatment with TNF-α for additional 8 h. Cells were then lysed and analyzed for luciferase activity as described. Bar graphs represent mean values of triplicate measurements.

To examine whether the alteration of YKL-40 levels by TNF-α was primarily mediated by NF-κB, we transfected cells with a stable mutant form of IκB-α (S32/36) that retains inactive NF-κB in the cell cytoplasm. Under these conditions, TNF-α induced repression and activation of YKL-40 was abolished (Fig. 1G). These results indicate that TNF-α differentially modulates YKL-40 in a cell type-dependent manner by means of NF-κB signaling.

### 3.2. p65 and p50 are actively recruited to the YKL-40 promoter in response to TNF-α

Since p65 and p50 are ubiquitous NF-κB subunits that are primarily activated in response to TNF-α, we next studied whether p65 and p50 mediated repression of YKL-40. We performed EMSA with double-stranded oligonucleotides corresponding to the putative NF-κB consensus site sequences (−751 and −800) using nuclear extracts of the SNB-75 cells. TNF-α treatment induced the formation of strong DNA–protein complexes at both consensus sites, which exhibited similar kinetics (Fig. 2A). In both cases, complex formation was disrupted by the addition of an excess amount of unlabeled wild-type oligonucleotides but not by oligonucleotides with a mutated $5' \rightarrow 3'$ core sequence (Fig. 2B; data not shown for −800). To further characterize the proteins in the EMSA complexes, we performed supershift assays with antibodies specific for p65 and p50. We observed a supershift with p50 in particular, whereas the p65 antibody simply interfered with complex formation, indicating a possible p65/p50 heterodimer (Fig. 2B).

To examine the in vivo recruitment of NF-κB proteins to the YKL-40 promoter in untreated versus TNF-α-treated cells, we performed ChIP assays using p65- and p50-specific antibodies. The primers were designed to amplify both NF-κB consensus sites (−751 and −800) on the YKL-40 promoter. Primers specific to the IκB promoter were used as positive controls. p65 and p50 were both recruited to the YKL-40 promoter as early as 30 min, similar to the recruitment by the IκB promoter (Fig. 2C). We did not find any significant differences in the kinetics or the extent of p65/p50 bound to the YKL-40 promoter in all cell lines tested.

Having established that both NF-κB consensus sites were occupied in response to TNF-α treatment in both cell types, we then tested the functional relevance of these sites in altering YKL-40 expression. We performed reporter assays using the YKL-40 promoter construct transfected in NIH 3T3 cells. Treatment with TNF-α induced luciferase activity that was

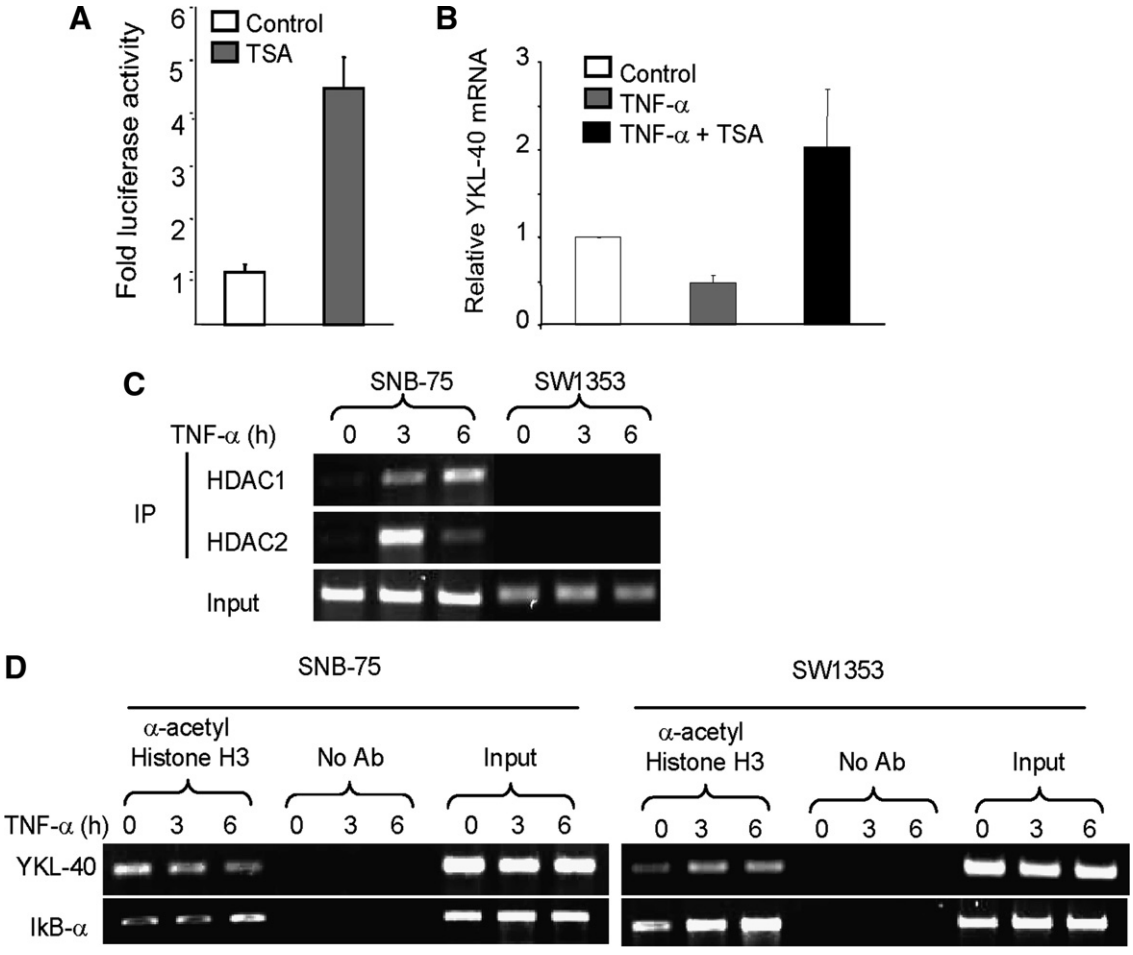


Fig. 3. HDAC1 and HDAC2 are recruited to the YKL-40 promoter during repression mediated by TNF-α. (A) SNB75 cells were transfected with YKL-40 promoter construct and luciferase activity was measured after treatment with TSA for 24 h. (B) SNB-75 cells were treated with either TNF-α alone (20 ng/ml) or in combination with TSA (20 nM) as indicated for 24 h and analyzed by real time RT-PCR for YKL-40 mRNA expression after normalizing to β-actin. The control YKL-40 levels were set at one and compared to treated samples. (C) SNB-75 cells were treated with TNF-α for the indicated time points and analyzed by ChIP assay using antibodies directed against HDAC1, HDAC2 or acetyl-histone-H3 (D).

attenuated when either or both of the consensus sites were mutated (Fig. 2D), indicating that p65 and p50 formed a heterodimeric complex at the YKL-40 promoter and that both consensus sites were occupied by NF-κB upon TNF-α treatment.

### 3.3. HDAC1 and HDAC2 are recruited to the YKL-40 promoter during repression

Because HDACs have been shown to be recruited to the NF-κB targets that are repressed upon treatment with various activators of this pathway [16,18–20], we examined the role of HDACs in the NF-κB-mediated repression of YKL-40. SNB-75 cells were transiently transfected with the YKL-40 promoter construct and treated with TSA, a known inhibitor of HDAC activity. We observed a dramatic induction of promoter activity indicating that inhibiting HDAC activity is sufficient to induce YKL-40 expression (Fig. 3A). Further, repression of YKL-40 mRNA in SNB-75 cells was countered, as well as induced, by prior treatment with TSA (Fig. 3B). Next, we studied whether HDACs were recruited to the YKL-40 promoter region upon TNF-α treatment. As shown in Fig. 3C, HDAC-1 and -2 were strongly recruited to the YKL-40 promoter in SNB-75 cells; however, we did not observe HDAC recruitment in SW1353 cells that previously showed activation of YKL-40.

If HDAC recruitment seen above was of functional significance, we expected that histone acetylation around the YKL-40 promoter chromatin would be reduced. To determine if this was the case, we performed ChIP assays directed against the histones using an acetyl-histone-H3 antibody. TNF-α treatment was associated with a striking decrease in acetylated histones at the YKL-40 promoter in SNB-75 cells, while SW1353 cells showed increased acetylated histone H3 (Fig. 3D). In contrast, because IκB-α is primarily induced by NF-κB, we noticed a concordant increase in acetyl-histone-H3 associated with the IκB promoter in both cell types (Fig. 3D).

To extend our in vitro findings further, we examined the regulation of YKL-40 by NF-κB in primary cancer tissues. We evaluated 100 cases of human GBM by IHC. We stained the tumor sections with p-p65 (Ser276) rather than with unphos-

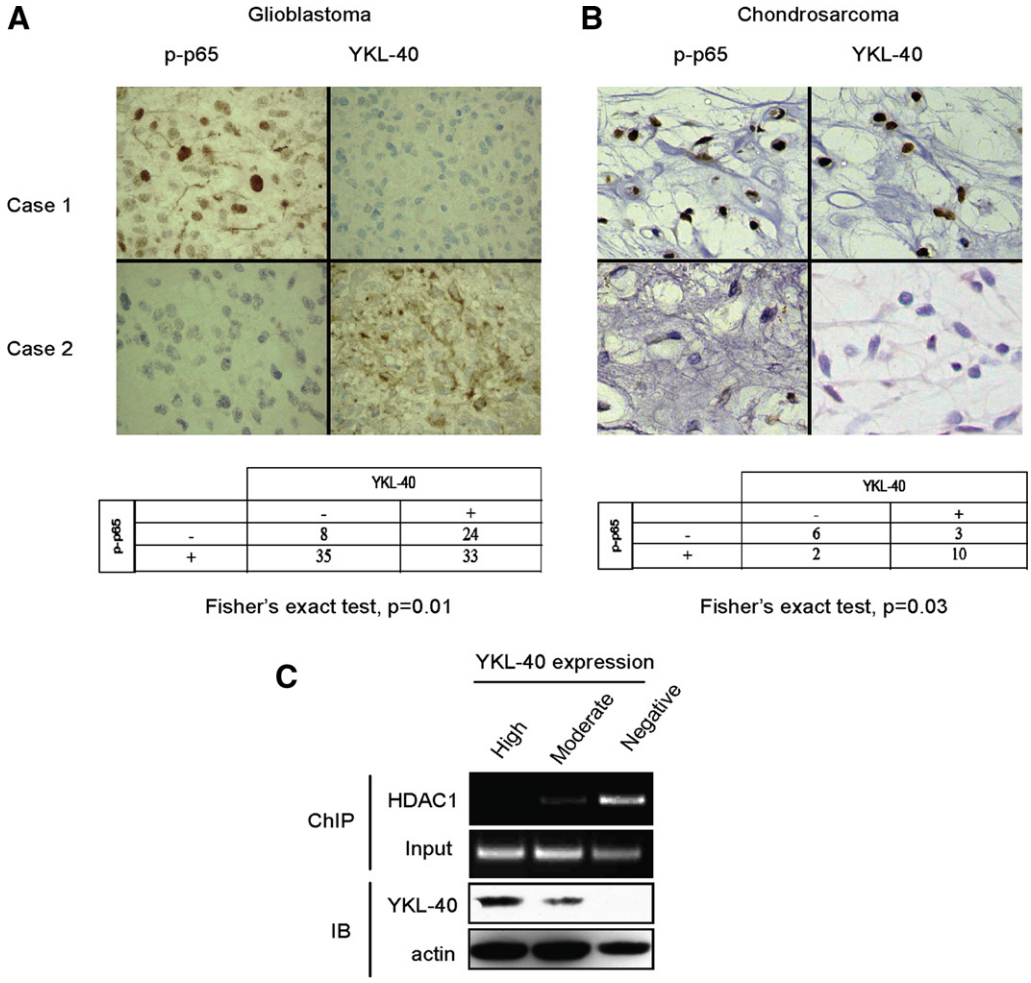


Fig. 4. (A) Representative immunohistochemical staining of GBM samples for YKL-40 or p-p65 expression. Case 1 shows positive staining for p-p65 and negative staining for YKL-40, whereas case 2 represents negative staining for p-p65 and positive staining for YKL-40. A table summarizing the number of positive and negative cases is shown below and a Fisher's exact test indicates negative correlation between the two proteins in GBM ($P = 0.01$). (B) Immunohistochemical staining of human chondrosarcoma samples for YKL-40 or p-p65 expression. The upper panel of case 1 shows positive staining for both proteins, whereas lower panel indicates negative staining. A table summarizing the number of positive and negative cases is shown below and a Fisher's exact test indicates significant positive correlation between the two proteins in chondrosarcoma ($P = 0.03$) (C). Three frozen GBM tissues were either analyzed by ChIP assay using HDAC1 antibody (top two panels) or Western blotting (bottom two panels).

phorylated p65, because nuclear localization of the phosphorylated protein represents the transcriptionally active form of p65 [21] (Fig. 4A). YKL-40 showed cytoplasmic staining in about 50% of GBM samples (Fig. 4A). Statistical analyses revealed a significant negative correlation between YKL-40 and nuclear p-p65 expression in GBM tissues (Fig. 4A, Fisher's exact test, $P = 0.01$). We next compared the expression of YKL-40 and p-p65 in chondrosarcoma specimens and observed a significant positive correlation between these two proteins (Fig. 4B; Fisher's exact test, $P = 0.03$).

Finally, we examined whether HDACs played a significant role in regulating YKL-40 levels in GBM tumors by performing ChIP analyses on frozen GBM specimens. We selected three cases of GBM that had high p-p65 expression but various levels of YKL-40 expression. Tumors were carefully separated from neighboring normal or necrotic tissue and lysates were subjected to either ChIP or Western blot analyses. As shown in Fig. 4C, the amount of HDAC1 recruited to the YKL-40 promoter increased with a corresponding decrease in YKL-40 protein levels. Taken together, our data strongly suggest that HDACs play a critical role in altering levels of YKL-40 in response to NF-κB in glioma cells.

## 4. Discussion

We found that YKL-40, an NF-κB target, is both activated and repressed in response to TNF-α and that either response could be attenuated by cotransfection with a non-degradable mutant form of IkB-α. Both negative and positive regulation of YKL-40 involve p65/p50 occupancy of the YKL-40 promoter. However, our response from GBM specimens suggest that in vivo, HDACs are recruited to the YKL-40 promoter and YKL-40 expression is consequently repressed.

Although TNF-α primarily mediates activation of NF-κB target genes by triggering IkB-α degradation and inducing nuclear translocation of NF-κB, previous studies have shown that TNF-α and other activators of NF-κB can indeed induce transcriptional repression of some NF-κB target genes. For example, TNF-α negatively regulates *COL1A2* expression by means of JNK1 signaling [22]. Other targets, such as *MIS*, were shown to be downregulated by TNF-α in coordination with transcriptional regulators, such as SF-1 [23]. Similarly, TNF-α repressed EAAT-2 by recruiting N-myc to the NF-κB promoter [24]. Other studies have found that atypical activators of NF-κB, such as UV, and chemotherapeutic agents but not TNF-α repress global antiapoptotic gene expression, exemplifying the complexity of the NF-κB signaling pathway [16].

We tested other signals that activate NF-κB, including IL-1β, doxorubicin, and daunorubicin, and found that YKL-40 was repressed by all these stimuli in glioma cells in a NF-κB-dependent manner, ruling out a signal-specific effect (data not shown). Also, since phosphorylation of the S276 and S536 residues of p65 favors recruitment of coactivator complexes and is important for p65 transcriptional activation function [25,26], we compared glial versus non-glial tumors and did not find any significant differences in the phosphorylation states upon treatment with TNF-α (data not shown). Also, other targets of NF-κb such as Bcl-xL, IκBα and Cox-2 were induced in a similar fashion in both cell types. Moreover, our ChIP analyses using GBM specimens revealed that HDAC1 recruitment was the major determinant of the YKL-40 level, regardless of the p-p65 level (Fig. 4C). However, the possible role of modification of other amino acids on p65, such as phosphorylation at Thr-505 or acetylation of lysine residues 122, 123, and 310, which facilitates corepressor functions, needs to be explored [25,26].

Our data show that chromatin remodeling of the YKL-40 promoter in glioma cell lines specifically accounts for these differential effects for the following reasons. First, using reporter assays with the YKL-40 promoter (1.3 kb) in a neutral environment, we found that YKL-40 activation is a primary response to TNF-α treatment (Fig. 2D). Second, treatment with TSA alone was sufficient to increase basal YKL-40 promoter activity in glioma cells indicating that HDACs repress the YKL-40 promoter in the absence of NF-κB signaling in these cell types (Fig. 3A). Third, TNF-α treatment does not repress other NF-κB target genes in glioma cells that we tested (data not shown), and finally we have identified multiple binding sites for neural specific transcriptional factor MYT1 as well as Oct-1 in the YKL-40 promoter and both these factors have been reported to recruit HDACs via interaction with Sin-3b and SMRT, respectively [27,28]. Alternatively, glial-specific factors that bind to long-distance enhancers further upstream of the YKL-40 promoter may play a role in modulating YKL-40 expression, similar to that seen with *MCP-1* [29]. Therefore, in-depth biochemical analyses of upstream enhancer regions should be done and the influence of MYT1 and Oct-1 on chromatin folding and YKL-40 expression needs to be explored.

The apparent paradoxical relationship between NF-κB activation and YKL-40 expression observed in glioma may have an adverse impact on the potential future use of HDAC inhibitors as a mode of therapy against this disease. YKL-40 is overexpressed in approximately 70% of GBM cases, and several studies have demonstrated a role for YKL-40 in the cellular and clinical aggressiveness of GBM. Earlier observations that HDAC inhibitors suppress the inducibility of NF-κB [30] coupled with our data showing that NF-κB suppresses YKL-40, would suggest that GBM carries an inherent mechanism of resistance to such a therapeutic strategy against this devastating disease.

As more physiological targets of YKL-40 are uncovered, we will be able to assess the impact of NF-κB on YKL-40 function. Given that most solid tumors exhibit necrosis accompanied by inflammation during which both NF-κB and YKL-40 are induced, the interplay between these proteins should be explored under these conditions.

*Acknowledgements:* This work was supported by a Grant from the American Brain Tumor Association (to K.P. Bhat), and an Odyssey Fellowship sponsored by the Theodore N. Law Award for scientific achievement (to K.P. Bhat).

## References

[1] Houston, D.R., Recklies, A.D., Krupa, J.C. and van Aalten, D.M. (2003) Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. J. Biol. Chem. 278, 30206–30212.

[2] De Ceuninck, F., Gaufillier, S., Bonnaud, A., Sabatini, M., Lesur, C. and Pastoureau, P. (2001) YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. Biochem. Biophys. Res. Commun. 285, 926–931.

[3] Recklies, A.D., White, C. and Ling, H. (2002) The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem. J. 365, 119–126.
[4] Malinda, K.M., Ponce, L., Kleinman, H.K., Shackelton, L.M. and Millis, A.J. (1999) Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. Exp. Cell Res. 250, 168–173.
[5] Ling, H. and Recklies, A.D. (2004) The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha. Biochem. J. 380, 651–659.
[6] Nigro, J.M. et al. (2005) Integrated array-comparative genomic hybridization and expression array profiles identify clinically relevant molecular subtypes of glioblastoma. Cancer Res. 65, 1678–1686.
[7] Pelloski, C.E. et al. (2006) Prognostic associations of activated mitogen-activated protein kinase and Akt pathways in glioblastoma. Clin. Cancer Res. 12, 3935–3941.
[8] Johansen, J.S. (2006) Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan. Med. Bull. 53, 172–209.
[9] Johansen, J.S., Jensen, B.V., Roslind, A., Nielsen, D. and Price, P.A. (2006) Serum YKL-40, a new prognostic biomarker in cancer patients? Cancer Epidemiol. Biomarkers Prev. 15, 194–202.
[10] Chupp, G.L. et al. (2007) A chitinase-like protein in the lung and circulation of patients with severe asthma. N. Engl. J. Med. 357, 2016–2027.
[11] Rehli, M., Niller, H.H., Ammon, C., Langmann, S., Schwarzfischer, L., Andreesen, R. and Krause, S.W. (2003) Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. J. Biol. Chem. 278, 44058–44067.
[12] Recklies, A.D., Ling, H., White, C. and Bernier, S.M. (2005) Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. J. Biol. Chem. 280, 41213–41221.
[13] Hayden, M.S. and Ghosh, S. (2008) Shared principles in NF-kappaB signaling. Cell 132, 344–362.
[14] Campbell, K.J. and Perkins, N.D. (2006) Regulation of NF-kappaB function. Biochem. Soc. Symp., 165–180.
[15] Campbell, K.J. and Perkins, N.D. (2004) Reprogramming RelA. Cell Cycle 3, 869–872.
[16] Campbell, K.J., Rocha, S. and Perkins, N.D. (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. Mol. Cell 13, 853–865.
[17] Lambert, J.R. and Nordeen, S.K. (2001) Analysis of steroid hormone-induced histone acetylation by chromatin immunoprecipitation assay. Methods Mol. Biol. 176, 273–281.
[18] Zhong, H., May, M.J., Jimi, E. and Ghosh, S. (2002) The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell 9, 625–636.
[19] Ashburner, B.P., Westerheide, S.D. and Baldwin Jr., A.S. (2001) The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol. Cell. Biol. 21, 7065–7077.
[20] Williams, S.A., Chen, L.F., Kwon, H., Ruiz-Jarabo, C.M., Verdin, E. and Greene, W.C. (2006) NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J. 25, 139–149.
[21] Chen, L.F. and Greene, W.C. (2004) Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. 5, 392–401.
[22] Verrecchia, F., Wagner, E.F. and Mauviel, A. (2002) Distinct involvement of the Jun-N-terminal kinase and NF-kappaB pathways in the repression of the human COL1A2 gene by TNF-alpha. EMBO Rep. 3, 1069–1074.
[23] Hong, C.Y., Park, J.H., Seo, K.H., Kim, J.M., Im, S.Y., Lee, J.W., Choi, H.S. and Lee, K. (2003) Expression of MIS in the testis is downregulated by tumor necrosis factor alpha through the negative regulation of SF-1 transactivation by NF-kappa B. Mol. Cell. Biol. 23, 6000–6012.
[24] Sitcheran, R., Gupta, P., Fisher, P.B. and Baldwin, A.S. (2005) Positive and negative regulation of EAAT2 by NF-kappaB: a role for N-myc in TNFalpha-controlled repression. EMBO J. 24, 510–520.
[25] Perkins, N.D. (2006) Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene 25, 6717–6730.
[26] Perkins, N.D. and Gilmore, T.D. (2006) Good cop, bad cop: the different faces of NF-kappaB. Cell Death Differ. 13, 759–772.
[27] Hitomi, T., Matsuzaki, Y., Yasuda, S., Kawanaka, M., Yogosawa, S., Koyama, M., Tantin, D. and Sakai, T. (2007) Oct-1 is involved in the transcriptional repression of the p15(INK4b) gene. FEBS Lett. 581, 1087–1092.
[28] Romm, E., Nielsen, J.A., Kim, J.G. and Hudson, L.D. (2005) Myt1 family recruits histone deacetylase to regulate neural transcription. J. Neurochem. 93, 1444–1453.
[29] Teferedegne, B., Green, M.R., Guo, Z. and Boss, J.M. (2006) Mechanism of action of a distal NF-kappaB-dependent enhancer. Mol. Cell. Biol. 26, 5759–5770.
[30] Imre, G., Gekeler, V., Leja, A., Beckers, T. and Boehm, M. (2006) Histone deacetylase inhibitors suppress the inducibility of nuclear factor-kappaB by tumor necrosis factor-alpha receptor-1 down-regulation. Cancer Res. 66, 5409–5418.