

Krüppel-like Factor 6 Is a Co-activator of NF- κ B That Mediates p65-dependent Transcription of Selected Downstream Genes*

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Background: Transcription factor NF- κ B is involved in various biological processes and regulated at multiple levels.

Results: KLF6 modulates NF- κ B-mediated transcription by promoting binding of p65 to promoters of downstream genes.

Conclusion: KLF6 is a co-activator of NF- κ B-mediated transcription of selected downstream genes.

Significance: Our study reveals a new regulatory mechanism for NF- κ B-mediated transcriptional activation in the nucleus.

The transcription factor NF- κ B plays a pivotal role in a broad range of physiological and pathological processes, including development, inflammation, and immunity. How NF- κ B integrates activating signals to expression of specific sets of target genes is of great interest. Here, we identified Krüppel-like factor 6 (KLF6) as a co-activator of NF- κ B after TNF α and IL-1 β stimulation. Overexpression of KLF6 enhanced TNF α - and IL-1 β -induced activation of NF- κ B and transcription of a subset of downstream genes, whereas knockdown of KLF6 had opposite effects. KLF6 interacted with p65 in the nucleus and bound to the promoters of target genes. Upon IL-1 β stimulation, KLF6 was recruited to promoters of a subset of NF- κ B target genes in a p65-dependent manner, which was in turn required for the optimal binding of p65 to the target gene promoters. Our findings thus identified KLF6 as a previously unknown but essential co-activator of NF- κ B and provided new insight into the molecular regulation of p65-dependent gene expression.

Nuclear factor κ B (NF- κ B)² is a family of structurally homologous transcription factors critically involved in various biological processes, including development, inflammation, and immunity (1). The mammalian NF- κ B family includes five proteins: p65 (RelA), RelB, c-Rel, p50, and p52, which bind to κ B sites of downstream gene promoters as homo- or hetero-dimers. All NF- κ B family members contain a Rel-homology domain (RHD) at the N terminus that mediates DNA binding and dimerization, whereas only p65, RelB, and c-Rel contain a transcriptional activation domains (TAD) at the C terminus that mediates transcription of downstream genes. NF- κ B is sequestered in the cytoplasm by inhibitor of κ B α (I κ B α) and

p100, two proteins which play primary roles in the canonical and noncanonical NF- κ B pathways, respectively (1, 2). p100 binds to and sequesters RelB in the cytoplasm in unstimulated cells and undergoes phosphorylation-dependent partial degradation upon certain stimulation, leading to formation of the noncanonical NF- κ B, RelB/p52 heterodimer that subsequently enters the nucleus to activate transcription of downstream genes. I κ B α is associated with the canonical NF- κ B, p65/p50 heterodimer in unstimulated cells, and treatment with various stimuli such as TNF α and IL-1 β results in phosphorylation and subsequent proteasome-mediated degradation of I κ B α , releasing the p65/p50 complex into nucleus (3, 4).

Although the key steps by which NF- κ B is activated in the cytoplasm have been proposed, and the importance of NF- κ B nuclear translocation is greatly appreciated, it remains less clear how NF- κ B functions in the nucleus to differentially regulate the transcription of thousands of genes in distinct types of cells upon various stimulations. In addition to the diversity of NF- κ B dimers that exert different DNA binding affinity and specificity, it has been suggested that various co-repressors and co-activators play critical roles in the process. Ribosomal protein S3 (RPS3) has been identified as a non-Rel subunit of p65 homodimer and p65/p50 heterodimer that regulates NF- κ B-mediated transcription of selected target genes (5). The evolutionarily conserved Akin proteins are localized in the nucleus and required for NF- κ B-mediated transcription of *IL-6*, *I κ B α* , *IP-10*, but not *KC* or *I κ B δ* in MEFs upon stimulation with IL-1 β or LPS, though the exact mechanisms of which are unknown (6). The deacetylase SIRT6 modulates the transcription of NF- κ B-dependent genes that are involved in organismal life span, while Nurr1 recruits the CoREST corepressor to clear the promoter-binding p65, resulting in transcriptional repression in microglia and astrocytes (7, 8). A few transcription factors including Sp1, AP-1, IRFs, STAT3, and CEBP/ β are also reported to synergize with NF- κ B to regulate transcription of downstream genes (9). Whether and how other proteins are involved in nuclear NF- κ B-mediated transcription is of great interest.

The Krüppel-like factors (KLFs) are transcriptional regulators that regulate diverse processes including development, homeostasis, tumorigenesis, and cell malignancy. Seventeen

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² The abbreviations used are: NF- κ B, nuclear factor κ B; KLF6, Krüppel-like factor 6; TNF α , tumor necrosis factor α ; IL-1 β , interleukin 1 β ; TAD, transcriptional activation domains; I κ B α , inhibitor of κ B α ; SVs, splicing variants; ChIP, chromatin immunoprecipitation; ZNFs, zinc finger domains; RARP1, polymerase 1.

KLF family members have been characterized in the human genome so far (10). The C termini of KLFs are highly conserved and consist of 81 amino acid residues characterized with the C₂H₂ zinc finger, which binds to “GC-box” or “CACCC-box” motifs in the promoters and/or mediates protein-protein interactions. The N-terminal regions of KLFs are much more variable and mediate transcriptional activation or repression (11). For example, KLF1 interacts with and is acetylated by the coactivators p300 and CBP, which alters the ability of KLF1 to interact with SWI/SNF to regulate tissue-specific expression of β -globin (12), while KLF3 and KLF8 interact with the co-repressor protein C-terminal-binding protein (CtBP) to repress transcription (13). However, little is known about the roles of KLFs in regulation of NF- κ B-mediated transcription.

In this study, we identified KLF6 as a co-activator of NF- κ B essential for NF- κ B-mediated transcription of selected downstream genes after TNF α - and IL-1 β stimulation. KLF6 interacts with p65 in the nucleus and binds to the promoters of p65 target genes, which is required for the optimal binding of p65 to the promoters and subsequent transcription of a subset of downstream genes, including *MCPI*, *CXCL2*, and *IL-8*. Our findings thus uncovered a new regulatory mechanism for NF- κ B-mediated transcriptional activation in the nucleus.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human TNF α and IL-1 β (Peprotech); mouse monoclonal antibodies against Flag (Sigma), HA (Covance), and β -actin (Sigma); rabbit anti-p65 (Santa Cruz Biotechnology) and rabbit anti-LMN1 (ProteinTech) were purchased from the indicated manufacturers. Rabbit polyclonal serum against KLF6 was made against KLF6 (aa1–200) of human origin and the anti-KLF6 IgG was purified by affinity chromatography. Mouse anti-I κ B α was previously described (14).

Constructs—NF- κ B, ISRE, and IRF1 promoter luciferase reporter plasmids, mammalian expression plasmids for Flag-tagged I κ B α , TRADD, MyD88, TRAF2, TRAF6, TAK1, TAB1, and IKK β were previously described (14–16). Mammalian cDNA expression plasmids were purchased from Origene Inc. Flag-p65, Flag-p50, Flag- or HA-tagged KLF6, and its mutants and isoforms were constructed by standard molecular biology techniques.

Transfection and Reporter Gene Assays—HEK293 cells (1×10^5) were seeded on 24-well plates and transfected on the following days by standard calcium phosphate precipitation method. In the same experiment, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.01 μ g of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega), and the firefly luciferase activities were normalized based on *Renilla* luciferase activities.

RNAi Experiments—Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper. RetroRNAi plasmid (Oligoengine Inc.). The following sequences were targeted for human KLF6: KLF6-RNAi #1: CAGGAAAGTT-TACACCAAAA; KLF6-RNAi #2: CTTTAACGGCTGCAGGAAA.

Coimmunoprecipitation and Western Blot Analysis, Retrovirus-mediated Construction of KLF6-RNAi Stable Cell Line—These experiments were performed as described (17–19).

Quantitative Real-time PCR (qPCR)—Total RNA was isolated from cells using Trizol reagent (TAKARA) and subjected to qPCR analysis to measure expression of mRNA. The mRNA levels of specific genes were normalized to *GAPDH* mRNA. Gene-specific primer sequences were as following: MCP1: 5'-AGAATCACCAGCAGCAAGTGTCC-3', 5'-TCCTGAACCCACTTCTGCTTGG-3'; CXCL2: 5'-CAAGAACATCCAAAGTGTGA-3', 5'-CCATTCTTGAGTGTGGCTAT-3'; IL8: 5'-GAGAGTGATTGAGAGTGGACCAC-3', 5'-CACAACCCTCTGCACCCAGTTT-3'; I κ B α : 5'-GTCCTTGGGTGCTGATGT-3', 5'-GAGAATAGCCCTGGTAGGTAA-3'; GAPDH: 5'-GAGTCAACGGATTGGTTCGT-3', 5'-GACAAGCTTCCCCTTCTCAG-3'.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed as previously described (20, 21). Immunoprecipitation was performed with 1 μ g of p65 antibody (C-20) or 5 μ g of KLF6 antibody, and the immune complexes were absorbed with protein A beads blocked with bovine serum albumin and salmon sperm DNA (Millipore). Gene-specific primer sequences were as following: MCP1: 5'-GACCCCGGGAGGAATGAAGAAA-3', 5'-CAGAGGGGCTATGGGGAAAATGA-3'; IL8: 5'-TGATAAGGAACAAATAGGAAGTG-3', 5'-GTGTGCTCTGCTGTCTCTGA-3'; CXCL2: 5'-CTCGCAGGCGGTTATCTCGGTATC-3', 5'-GGGGTTCGGGGCACTCACG-3'; I κ B α : 5'-TAGCAGAGGACGAAGCCAGT-3', 5'-TGGCTGGGGATTCTCTG-3'.

Apoptosis Assays—HCT116 cells were treated with TNF α (100 ng/ml) together with Smac mimetic (100 nM) (Xiaodong Wang, NIBS, Beijing) (22) for 6 or 12 h followed by staining with annexin V-FITC in PBS for 15 min and propidium iodide (PI) for 3 min. The cells were washed and subjected to flow cytometry using a FACS caliber flow cytometer (XDP, Beckman Counter).

RESULTS

Overexpression of KLF6 Potentiates TNF α - and IL-1 β -induced NF- κ B Activation—To identify additional regulators involved in TNF α - or IL-1 β -induced activation of NF- κ B, we screened ~15,000 independent human cDNA clones by NF- κ B reporter assays in HEK293 cells and found that KLF6 (clone 61A3), a tumor suppressor in multiple types of cancers (4, 23), markedly potentiated TNF α - and IL-1 β -induced NF- κ B activation (Fig. 1A). KLF6 belongs to the KLF family consisting of 17 members in humans. Interestingly, of the ten KLFs that were examined in our screening, only KLF6 substantially increased TNF α - and IL-1 β -triggered activation of NF- κ B (Fig. 1B). KLF6 is ubiquitously expressed in mammalian tissues and its splicing variants KLF6-SV1, KLF6-SV2, and KLF6-SV3 have been shown to function in different pathways (10). We thus made Flag-tagged expression plasmids encoding human KLF6 or its splicing variants independently of the clone 61A3 and examined their ability to activate NF- κ B by reporter assays. However, only the full-length KLF6 potentiated TNF α - and IL-1 β -induced NF- κ B activation in a dose-dependent manner in HEK293, HeLa, and HCT116 cells (Fig. 1, C–E). In addition,

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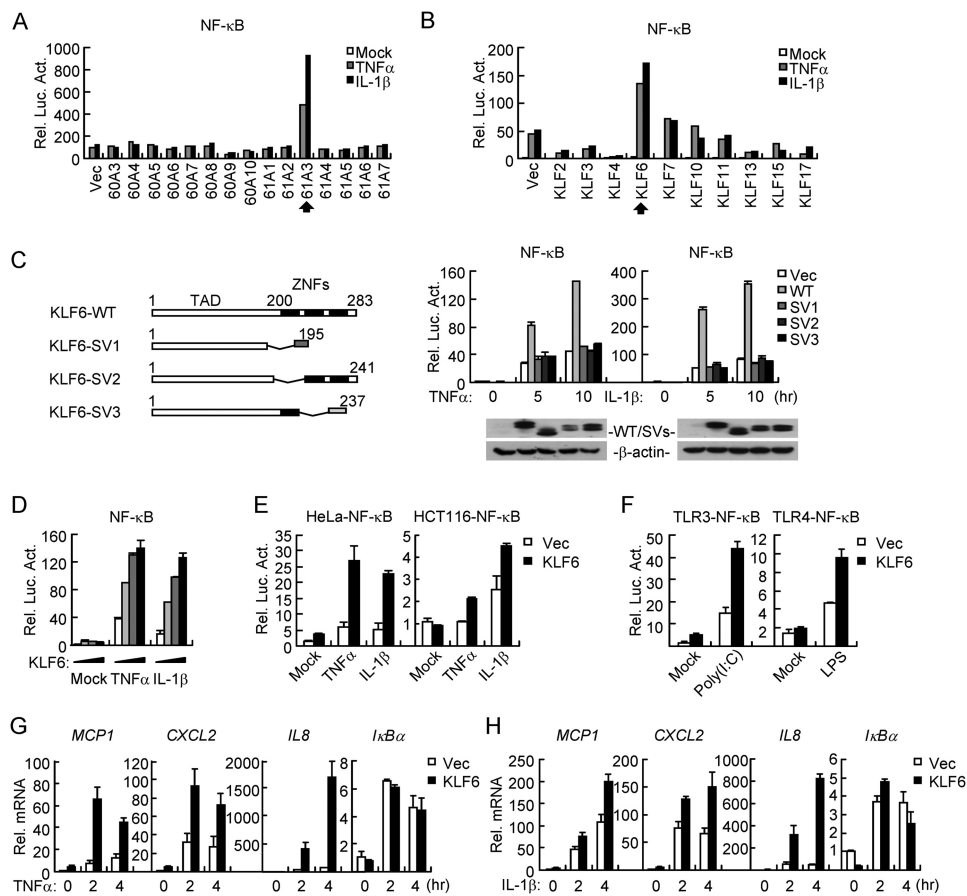


FIGURE 1. Overexpression of KLF6 potentiates TNF α - and IL-1 β -induced NF- κ B activation. *A*, identification of KLF6 (61A3) as a positive regulator of TNF α - and IL-1 β -induced NF- κ B activation. Human and mouse expression cDNA clones (Origene Inc.) (0.05 μ g) were individually transfected into HEK293 cells (5×10^4) together with an NF- κ B luciferase reporter (0.02 μ g). Twenty hours after transfection, cells were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) or left untreated for 10 h before reporter assays were performed. *B*, KLF6 but not other members of the KLF family potentiated TNF α - and IL-1 β -induced NF- κ B activation. Experiments were performed as in *A* except that indicated plasmids were used. *C*, KLF6 but not its splicing variants potentiated TNF α - and IL-1 β -induced NF- κ B activation. HEK293 cells are transfected with KLF6-WT and KLF6 SV1/2/3 expression plasmid (0.05 μ g) together with an NF- κ B luciferase reporter (0.02 μ g). Twenty hours after transfection, cells were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) or left untreated for 5 or 10 h before reporter assays were performed. (WT: wild-type; SV1: splicing variant 1; SV2: splicing variant 2; SV3: splicing variant 3). *D*, overexpression of KLF6 potentiates TNF α - and IL-1 β -induced NF- κ B activation in a dose-dependent manner. HEK293 cells (1×10^5) were transfected with Flag-KLF6 expression plasmid (0, 0.01, 0.02, or 0.05 μ g) together with an NF- κ B luciferase reporter (0.02 μ g). Twenty hours after transfection, cells were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) or left untreated for 10 h before reporter assays were performed. *E*, overexpression of KLF6 potentiates TNF α - and IL-1 β -induced activation of NF- κ B in HeLa and HCT116 cells. HeLa or HCT116 cells (5×10^5) were transfected with NF- κ B luciferase reporter (0.04 μ g) and Flag-KLF6 plasmid (0.05 μ g) by lipofatamine 2000. Twenty hours after transfection, cells were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) or left untreated for 10 h before reporter assays were performed. *F*, overexpression of KLF6 potentiates poly(I:C)- or LPS-induced activation of NF- κ B in TLR3-293 or TLR4-293 cells. TLR3/4-293 cells were treated as in *E* except cells are stimulated with poly(I:C) (10 μ g/ml) or LPS (1 μ g/ml) for 6 h. *G* and *H*, overexpression of KLF6 potentiates TNF α - and IL-1 β -induced expression of *MCP1*, *CXCL2*, and *IL8*. HEK293 cells (4×10^5) were transfected with an empty control vector or Flag-KLF6 plasmid (0.5 μ g). Twenty hours after transfection, cells were treated with TNF α (20 ng/ml) (*G*) or IL-1 β (10 ng/ml) (*H*) or left untreated for the indicated time points before qPCR was performed.

overexpression of KLF6 also potentiated poly(I:C)-triggered TLR3-mediated and LPS-induced TLR4-mediated NF- κ B activation (Fig. 1*F*). Results from quantitative real-time PCR (qPCR) experiments indicated that KLF6 promoted TNF α - and IL-1 β -induced transcription of NF- κ B downstream genes *MCP1*, *CXCL2*, and *IL8* but not *I κ B α* (Fig. 1, *G* and *H*). These data suggest that KLF6 regulates NF- κ B-mediated transcription of a subset of downstream genes after TNF α and IL-1 β stimulation.

Knockdown of KLF6 Impairs TNF α - and IL-1 β -triggered NF- κ B Activation—We next determined the effect of KLF6 knockdown on TNF α - and IL-1 β -induced NF- κ B activation by reporter assays. We constructed two human KLF6-RNAi plasmids, both of which could markedly inhibit expression of transfected KLF6 in HEK293 cells or endogenous KLF6 in HCT116 cells (Fig. 2*A*). In reporter assays, knockdown of KLF6 inhibited

TNF α - and IL-1 β -induced NF- κ B activation in HEK293, HeLa, and HCT116 cells, and the degrees of inhibition were correlated with the efficiencies of knockdown of KLF6 by each RNAi plasmids (Fig. 2, *B* and *D*). We selected KLF6-RNAi-#1 plasmid for additional experiments described below, and similar results were obtained with KLF6-RNAi-#2. We found that knockdown of KLF6 also inhibited poly(I:C)- or LPS-induced NF- κ B activation in TLR3 or TLR4-293 cell lines (Fig. 2*C*). Results from qPCR experiments suggested that knockdown of KLF6 inhibited TNF α - and IL-1 β -induced expression of NF- κ B downstream genes *MCP1*, *CXCL2*, and *IL8* but not *I κ B α* (Fig. 2, *E* and *F*), indicating that KLF6 mediates the transcription of a subset of downstream genes of NF- κ B. However, neither overexpression nor knockdown of KLF6 had noticeable effects on IFN γ -induced IRF1 promoter activation (data not shown). Taken together, these data suggest that KLF6 plays a crucial role in the

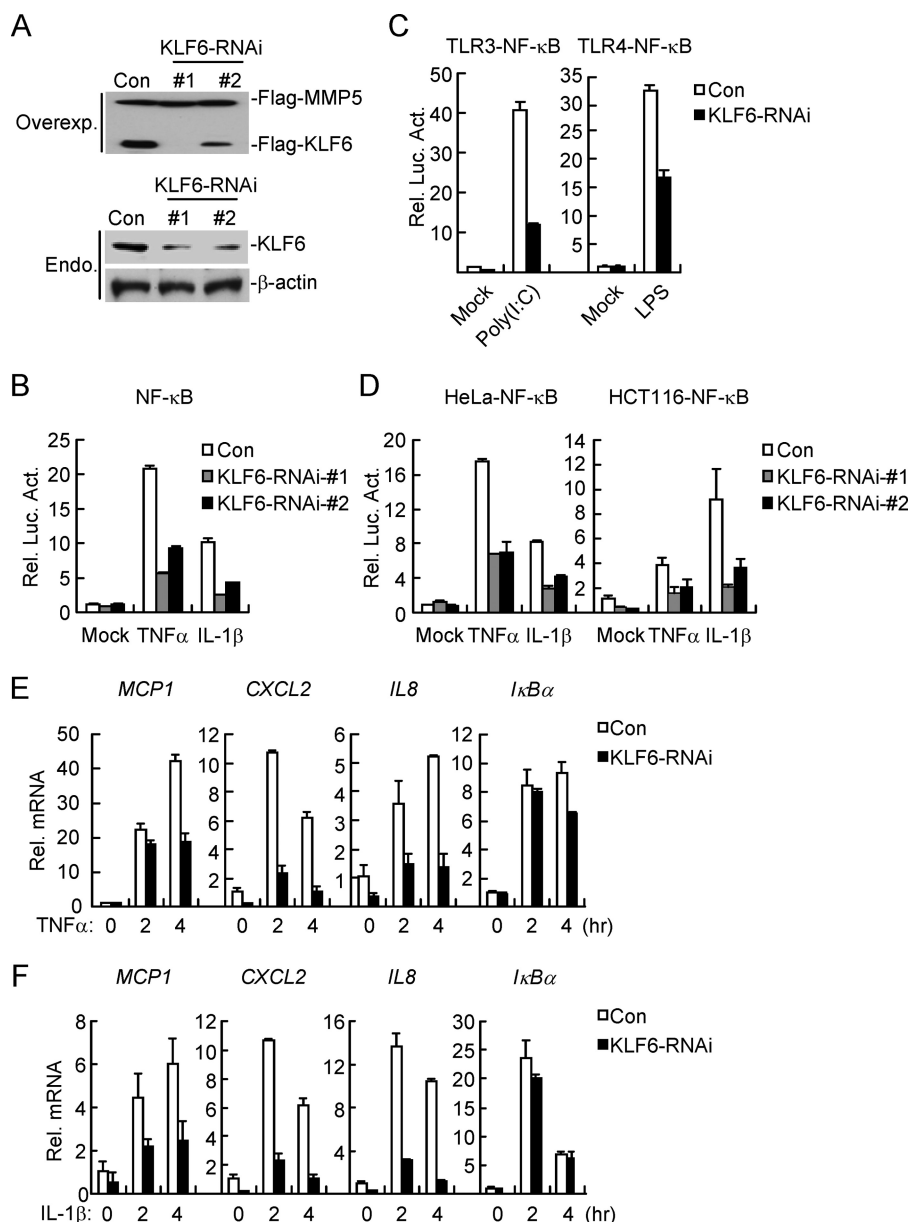


FIGURE 2. Knockdown of KLF6 impairs TNF α - and IL-1 β -triggered NF- κ B activation. *A*, efficiencies of KLF6-RNAi plasmids on KLF6 expression. In the upper panels, HEK293 cells (4×10^5) were transfected with expression plasmids for Flag-KLF6 (0.2 μ g), Flag-MMP5 (0.05 μ g) and the indicated RNAi plasmids (2 μ g each). Twenty-four hours after transfection, cell lysates were analyzed by immunoblots with anti-Flag. In the lower panels, HCT116 cells (1×10^6) were transfected with a control or the indicated KLF6-RNAi plasmids (10 μ g each). Thirty-six hours later, cells were lysed, and the cell lysates were analyzed by immunoblots with anti-KLF6 or anti- β -actin. *B*, knockdown of KLF6 inhibits TNF α - and IL-1 β -induced NF- κ B activation. HEK293 cells (1×10^5) were transfected with the indicated KLF6-RNAi (0.5 μ g each) and NF- κ B luciferase reporter (0.02 μ g) plasmids. Thirty-six hours after transfection, cells were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) or left untreated for 10 h before luciferase assays were performed. *C*, knockdown of KLF6 potentiates poly(I:C)- or LPS-induced activation of NF- κ B in TLR3–293 or TLR4–293 cells. TLR3/4–293 cells (5×10^5) were transfected with the indicated KLF6-RNAi (1 μ g each) and NF- κ B luciferase reporter (0.02 μ g) plasmids by lipofectamine 2000. Thirty-six hours after transfection, cells were treated with poly(I:C) (10 μ g/ml) or LPS (1 μ g/ml) for 6 h before luciferase assays were performed. *D*, knockdown of KLF6 inhibits TNF α - and IL-1 β -induced activation of NF- κ B in HeLa and HCT116 cells. HeLa and HCT116 cells were treated as in *C* except cells are stimulated for TNF α (20 ng/ml) or IL-1 β (10 ng/ml) for 10 h. *E* and *F*, knockdown of KLF6 inhibits TNF α - and IL-1 β -induced expression of MCP1, CXCL2, and IL8. HEK293 cells (4×10^5) were transfected with a control or KLF6-RNAi plasmid (3 μ g). Thirty-six hours after transfection, cells were left untreated or treated with TNF α (20 ng/ml) (*E*) or IL-1 β (10 ng/ml) (*F*) for the indicated time points before qPCR was performed.

regulation of TNF α - and IL-1 β -induced NF- κ B-mediated transcription of a subset of downstream genes.

KLF6 Is Dispensable for TNF α - and IL-1 β -triggered Nuclear Translocation of NF- κ B—Various molecules have been reported to regulate TNF α - and IL-1 β -triggered NF- κ B activation, including TRADD, MyD88, TRAF2, TRAF6, TAK1/TABs, IKK β , and p65. To determine the molecular order of KLF6 in regulating NF- κ B activation, we transfected plasmids encoding these mole-

cules in the presence or absence of KLF6 and performed reporter assays. Results indicated that KLF6 enhanced NF- κ B activation mediated by p65- and its upstream molecules (Fig. 3A). Conversely, knockdown of KLF6 had the opposite effects (Fig. 3B), suggesting that KLF6 functions at the level of or downstream of p65. To further confirm this conclusion, we established HCT116 cell lines stably transfected with a control or KLF6 RNAi plasmid and examined TNF α - or IL-1 β -induced phos-

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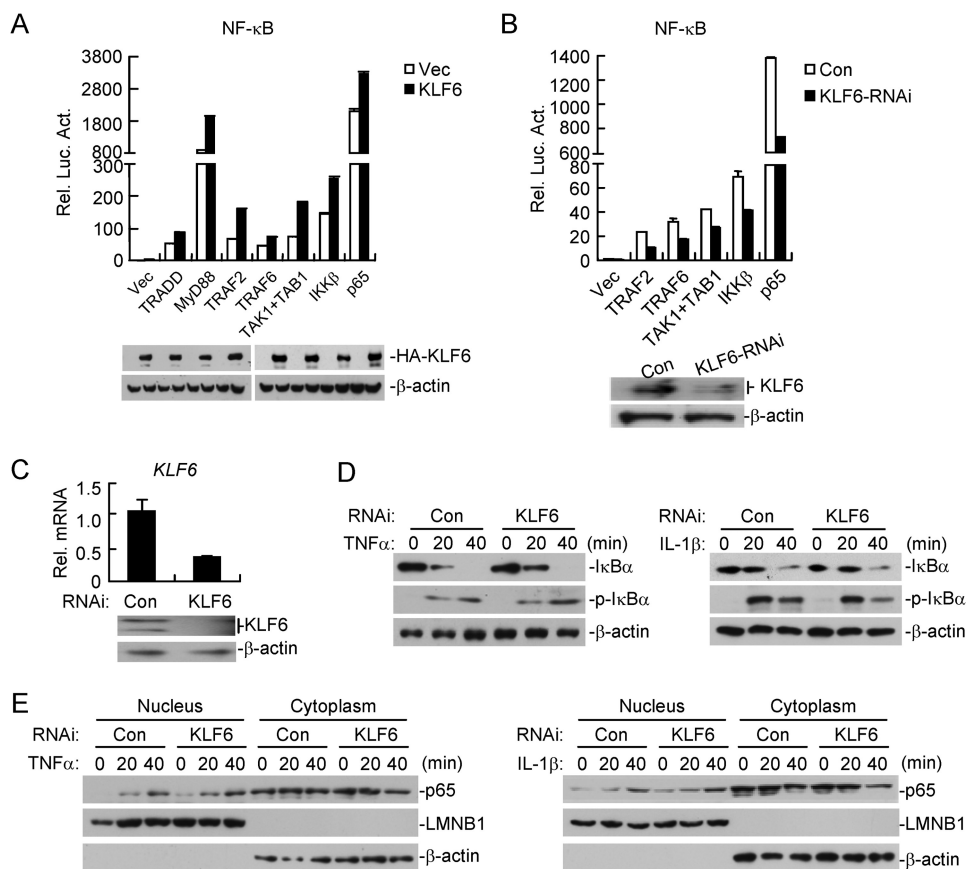


FIGURE 3. KLF6 is dispensable for TNF α - and IL-1 β -triggered nuclear translocation of NF- κ B. *A*, overexpression of KLF6 potentiates TRADD-, MyD88-, TRAF2-, TRAF6-, TAK1/TAB1-, IKK β -, or p65-mediated NF- κ B activation. HEK293 cells (1×10^5) were transfected with an empty control vector or HA-tagged KLF6 expression plasmid (0.05 μ g) and the indicated plasmids (0.1 μ g each). Reporter assays were performed 20 h after transfection. The lysates were analyzed by immunoblots with anti-HA or anti- β -actin. *B*, knockdown of KLF6 inhibits TAK1/TAB1-, TRAF2-, TRAF6-, IKK β -, or p65-mediated NF- κ B activation. KLF6-RNAi or control cell line, which was constructed in 293 cells (1×10^5) was transfected with the indicated plasmids (0.1 μ g each) and NF- κ B reporter plasmid (0.02 μ g). Reporter assays were performed 20 h after transfection. The efficiency of the knockdown of KLF6 was analyzed by immunoblots. *C*, construction of a KLF6-RNAi stable cell line. HCT116 cells were stably transfected with control RNAi or KLF6-RNAi by retroviral-mediated gene transfer. The cells (4×10^5) were analyzed by qPCR or immunoblots. *D*, knockdown of KLF6 has no marked effects on TNF α - or IL-1 β -triggered phosphorylation and degradation of I κ B α . The cells (2×10^5) were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) for the indicated time points before immunoblot analysis was performed. *E*, knockdown of KLF6 has no marked effects on p65 translocation. Cells in *C* (2×10^5) were treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) for the indicated time points. The cytoplasm extracts were prepared in 1 ml of homogenization buffer, whereas the nuclear extracts were prepared in 0.2 ml of nuclear lysis buffer. Equal volumes of the cytoplasm and nuclear extracts were loaded for immunoblot analysis with the indicated antibodies.

phorylation and degradation of I κ B α , two hallmarks of canonical NF- κ B activation. As shown in Fig. 3*D*, knockdown of KLF6 had minimal effects on TNF α - or IL-1 β -triggered phosphorylation and degradation of I κ B α . In addition, TNF α - or IL-1 β -induced nuclear translocation of p65 was comparable between the cells stably transfected with a control or KLF6-RNAi plasmid (Fig. 3*E*). These data collectively suggest that KLF6 is dispensable for NF- κ B nuclear translocation upon TNF α or IL-1 β stimulation.

KLF6 Interacts with p65 in the Nucleus—We next examined whether KLF6 interacts with canonical NF- κ B protein p65 or p50. In transient transfection and coimmunoprecipitation experiments, overexpression of KLF6 interacted with p65 constitutively but not with p50 and I κ B α (Fig. 4*A*). Results from domain mapping experiments indicated that the N-terminal transcription activation domain of KLF6(aa1–200) was required for their interaction (Fig. 4*B*). The endogenous KLF6 interacted with p65 weakly without stimulation, and this association was enhanced upon TNF α or IL-1 β stimulation (Fig. 4*C*). It has been reported that KLF6 shuttles between the cyto-

plasm and nucleus (24). We thus performed cellular fractionation assays to isolate the cytosol and nuclear fractions and examined whether KLF6 interacts with p65 in the nucleus or cytoplasm by coimmunoprecipitation. Consistent with previous studies, KLF6 was found both in the nucleus and the cytoplasm. However, KLF6 only interacted with p65 in the nucleus, and this interaction could be increased following TNF α or IL-1 β treatment (Fig. 5*A*). As a negative feedback regulator, I κ B α is synthesized at later times after TNF α or IL-1 β stimulation and drives p65/p50 from the nucleus back to the cytoplasm. Interestingly, co-expression of I κ B α but not p50 dose-dependently disrupted KLF6-p65 interaction, indicating that I κ B α sequesters p65 in the cytoplasm to prevent interaction of p65 with KLF6 (Fig. 5*B*). Because TNF α - or IL-1 β -induced expression and degradation of I κ B α was not altered by overexpression or knockdown of KLF6 (Figs. 1, *G* and *H* and 3*D*), we suspected that KLF6 inhibited I κ B α -mediated retention of p65 in the cytoplasm by associating with p65 in the nucleus, resulting in retention of p65 in the nucleus for continued transcriptional activation. To test this possibility, we examined I κ B α -

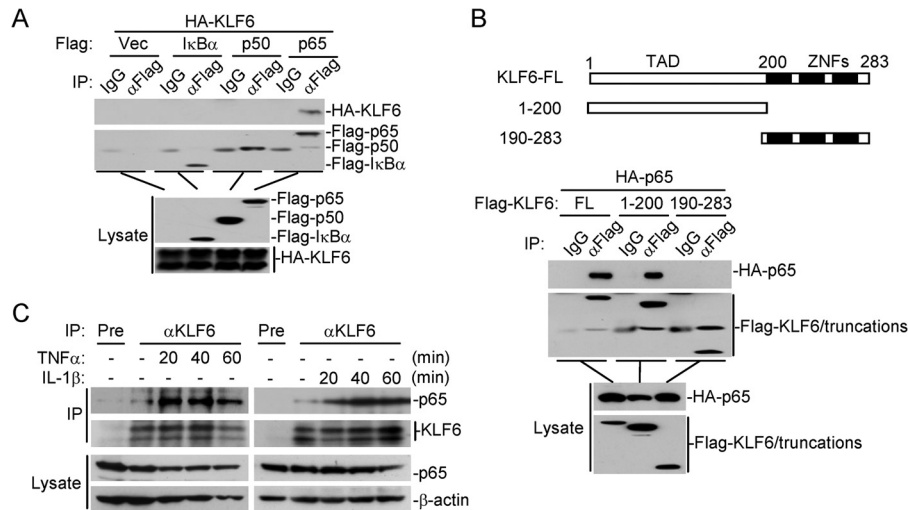


FIGURE 4. KLF6 interacts with p65. *A*, KLF6 interacts with p65 in mammalian overexpression system. HEK293 cells (3×10^6) were transfected with the indicated plasmids ($5 \mu\text{g}$ each) for 24 h. Coimmunoprecipitation and immunoblots were performed with the indicated antibodies. *B*, transcription activation domain of KLF6 is required for its interaction with p65. HEK293 cells (2×10^6) were transfected with HA-p65 ($1 \mu\text{g}$) and Flag-KLF6 or its truncation mutants ($5 \mu\text{g}$ each) for 20 h before coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. KLF6-FL: KLF6-full length. *C*, endogenous KLF6 interacts with p65. HEK293 cells (3×10^7) were left untreated or treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) for the indicated time points. Endogenous coimmunoprecipitation and immunoblots were performed with the indicated antibodies.

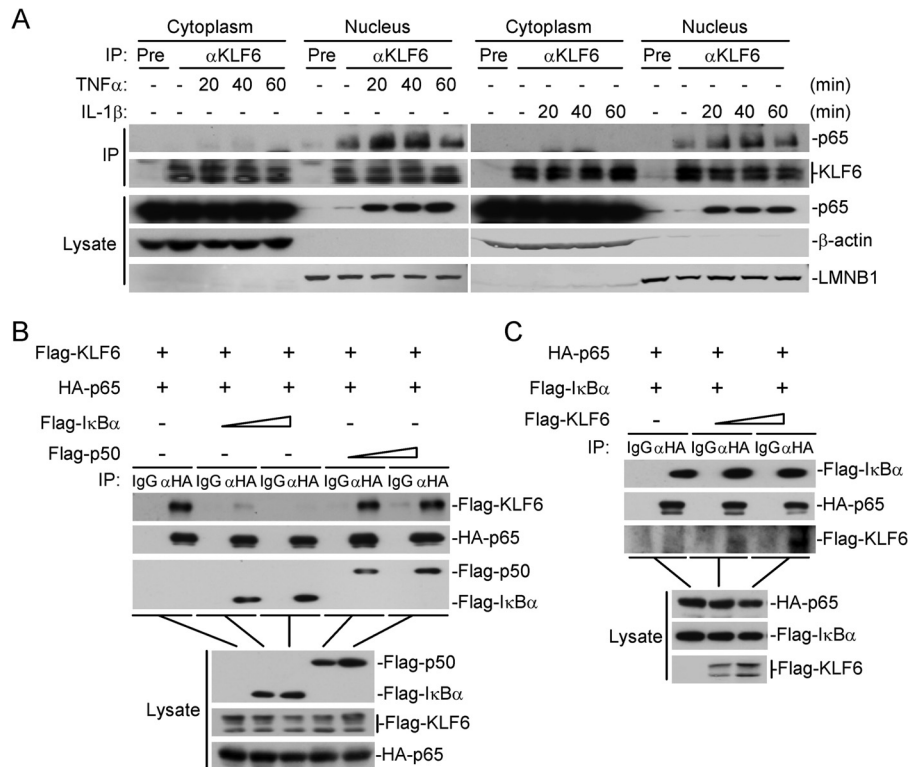


FIGURE 5. KLF6 interacts with p65 in the nucleus. *A*, KLF6 interacts with p65 in the nucleus. HEK293 cells (5×10^7) were left untreated or treated with TNF α (20 ng/ml) or IL-1 β (10 ng/ml) for the indicated time points. Cells fractionation was performed, and the cytoplasmic and nuclear proteins were prepared in 1 ml of homogenization buffer or nuclear extraction buffer, respectively, following by coimmunoprecipitation and immunoblot analysis. *B*, IκB α but not p50 disrupts the p65-KLF6 interaction in a dose-dependent manner. HEK293 cells (3×10^6) were transfected with the indicated plasmids together with IκB α (1 or $2 \mu\text{g}$) or p50 (1 or $2 \mu\text{g}$) expression plasmids. Coimmunoprecipitation and immunoblots were performed with the indicated antibodies. *C*, KLF6 does not affect p65-IκB α interaction. HEK293 cells (3×10^6) were transfected with the indicated plasmids together with KLF6-expression plasmids (4 or $8 \mu\text{g}$). Coimmunoprecipitation and immunoblots were performed with the indicated antibodies.

p65 interaction in the presence or absence of KLF6. As shown in Fig. 5C, however, IκB α -p65 interaction remained unchanged when KLF6 was co-expressed, indicating that KLF6 does not interfere IκB α -p65 interaction and possibly regulates p65 transcriptional activity in the nucleus.

KLF6 Binds to the Promoters of a Subset of p65 Target Genes— Because KLF6 interacted with p65 in the nucleus and was required for TNF α - and IL-1 β -induced expression of a subset of downstream genes, we hypothesized that KLF6 might be recruited to the promoters of these genes to promote p65-de-

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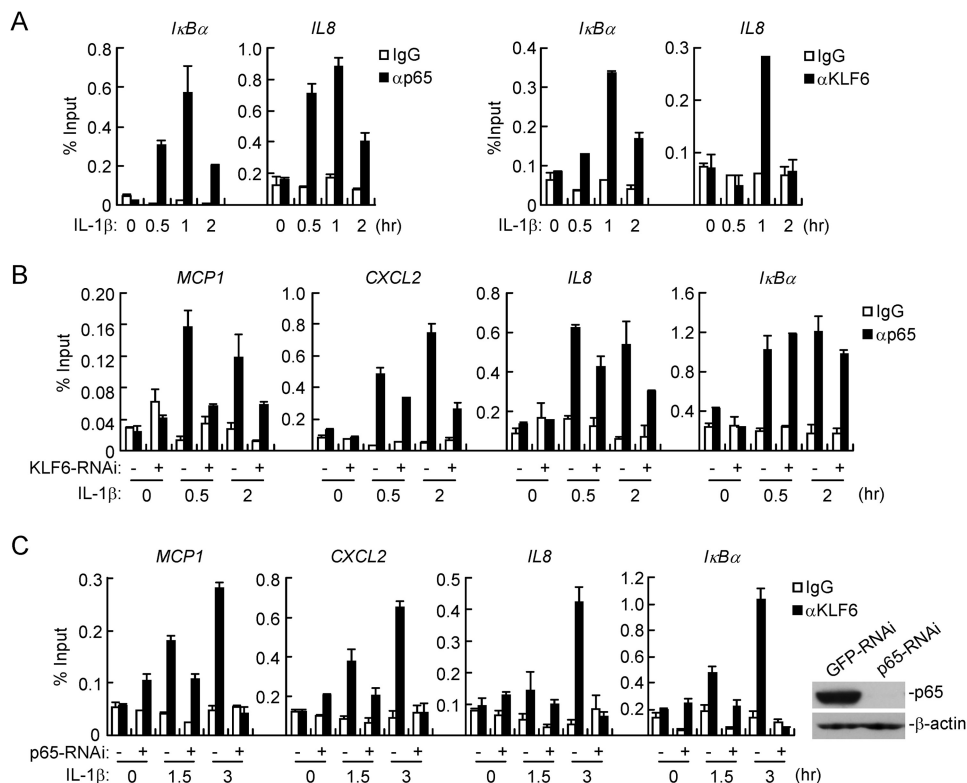


FIGURE 6. KLF6 binds to promoters of a subset of p65 target genes. *A*, KLF6 and p65 bind to the promoters of *IκBα* and *IL8* genes after IL-1 β stimulation. HCT116 cells (5×10^6) were left untreated or treated with IL-1 β (10 ng/ml) for the indicated time points, and then ChIP assays were performed with the indicated antibodies. Binding of KLF6 and p65 to the indicated promoters was determined by qPCR with primers specific for the promoters of the indicated genes. *B*, knockdown of KLF6 diminishes binding of p65 to promoters of *MCP1*, *CXCL2*, and *IL8* but not *IκBα* genes. KLF6-RNAi or control RNAi stable cell lines (5×10^6) were left untreated or treated with IL-1 β (10 ng/ml) for the indicated time points. ChIP assays were performed with the indicated antibodies. Binding of p65 to the indicated promoters was determined by qPCR. *C*, knockdown of p65 impairs DNA binding of KLF6 to promoters of *MCP1*, *CXCL2*, *IL8*, and *IκBα* genes. HCT116 cells (5×10^6) stably transfected with control or KLF6-RNAi plasmids were left untreated or treated with IL-1 β (10 ng/ml) for the indicated time points. ChIP assays were performed with the indicated antibodies. Binding of KLF6 to the indicated promoters was determined by qPCR. Knockdown of p65 was measured by immunoblot analysis.

pendent transcription. We focused on KLF6-mediated regulation of NF- κ B target genes after IL-1 β stimulation, as TNF α stimulation similarly induced NF- κ B activation and KLF6-p65 interaction as IL-1 β . Using ChIP assays, we found that IL-1 β stimulation induce binding of p65 and KLF6 to the promoters of *IκBα* and *IL8*, all of which are NF- κ B target genes downstream of TNF α and IL-1 β (Fig. 6A). Interestingly, p65 bound to the promoters of *MCP1*, *CXCL2*, and *IL8* genes following IL-1 β stimulation, which was diminished in cells stably transfected with KLF6-RNAi (Fig. 6B). In addition, IL-1 β -induced binding of KLF6 to the promoters of *MCP1*, *CXCL2* and *IL8* genes was impaired by knockdown of p65 (Fig. 6C). It should be noted that knockdown of KLF6 did not affect binding of p65 to the promoter of *IκBα* gene whereas knockdown of p65 impaired binding of KLF6 to the promoter of *IκBα* gene. These data suggest that KLF6 and p65 are mutually required for their binding to a subset of NF- κ B target genes downstream of IL-1 β .

The DNA Binding Activity of KLF6 Is Essential to Promote Binding of p65 to the Promoters of Downstream Genes—KLF6 consists of an N-terminal transcriptional activation domain (TAD) and three C-terminal zinc finger domains (ZNFs), which are responsible for its transcriptional and DNA binding activities, respectively (11). Although the N-terminal TAD interacted with p65 and the C-terminal ZNFs bound to the promoters of *IκBα* or *CXCL2* (Figs. 4B and 7A), neither of these two

domains alone potentiated IL-1 β -induced activation of NF- κ B in reporter assays (Fig. 7B), indicating that the intact KLF6-p65 complex formation is critical for mediating p65-driven expression of target genes. Previously, it has been demonstrated that the Cys²⁶⁵→Tyr²⁶⁵ (C265Y) mutation of KLF6 impairs its association with DNA or proteins (25). In our experiments, we found that KLF6(C265Y) could not enhance NF- κ B activation upon IL-1 β stimulation or potentiate IL-1 β -triggered induction of *MCP1*, *CXCL2*, or *IL8* (Fig. 7C&D), although KLF6(C265Y) was fully capable of interacting with p65 (Fig. 7E). Consistent with these observations, KLF6(C265Y) lost the binding activity to the promoters of *IκBα* or *IL8* genes upon IL-1 β stimulation (Fig. 7F). In addition, KLF6(C265Y) failed to promote binding of p65 to the promoters of *MCP1*, *CXCL2*, or *IL8* genes (Fig. 7G). These data together suggest that the ZNFs-mediated DNA binding activity of KLF6 is required for optimal binding of p65 to the promoters of target genes.

KLF6 Regulates TNF α -triggered Apoptosis—We next examined the physical relevance of KLF6 in regulating TNF α -mediated cellular effects. As shown in Fig. 8A, TNF and Smac mimetic-induced apoptosis of HCT116 cells was substantially enhanced by knockdown of KLF6 or p65. Consistent with these observations, the cleavage of PARP1 was potentiated in KLF6-RNAi compared with control cells (Fig. 8B). These data suggest

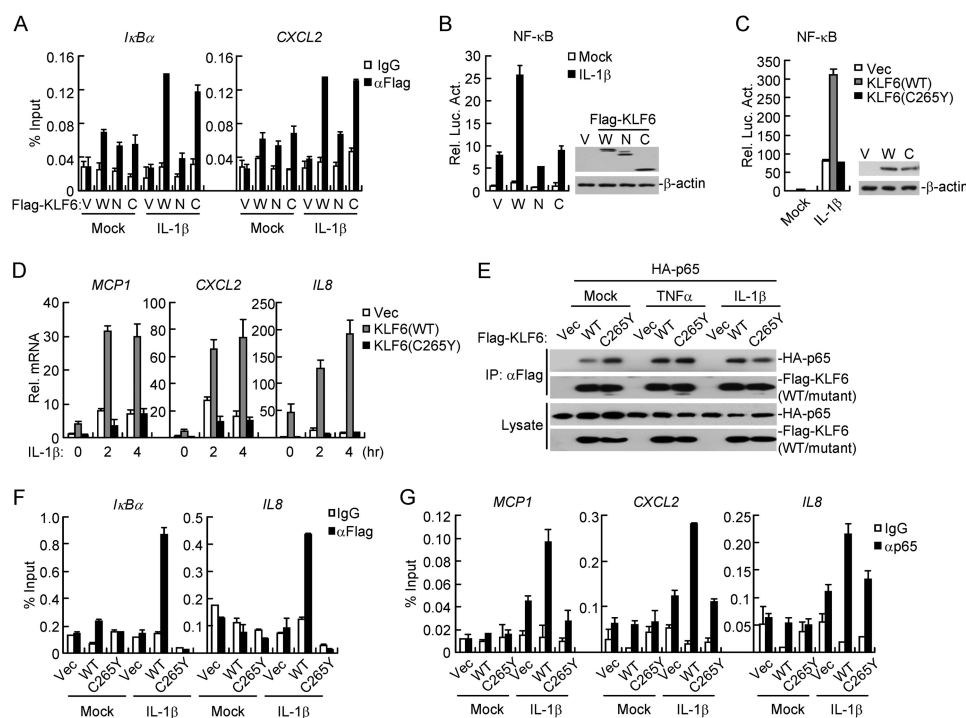


FIGURE 7. The DNA binding activity of KLF6 is essential to promote p65 binding to the promoters. A, KLF6-WT and its ZNFs but not its TAD could bind to the promoters of *I κ B α* and *CXCL2*. HEK293 cells (3×10^6) were transfected with indicated plasmids ($5 \mu\text{g}$ each). Twenty hours after transfection, cells were treated with IL-1 β (10 ng/ml) for 30 min. ChIP assays were performed with the indicated antibodies. The binding of Flag-tagged KLF6 or its truncation mutants with indicated promoters were determined by qPCR analysis. V: Vector, W: wild-type of KLF6, N: KLF6(aa1–200), and C: KLF6(aa190–283). B, neither the N terminus nor C terminus of KLF6 potentiates IL-1 β -induced NF- κ B activation. HEK293 cells (1×10^5) were transfected with the NF- κ B luciferase reporter (20 ng) and Flag-KLF6 or its truncation mutants (20 ng each). Twenty hours after transfection, cells were untreated or treated with IL-1 β (10 ng/ml) for 10 h before reporter assays were performed. C, wild-type KLF6 but not the KLF6(C265Y) potentiates IL-1 β -induced NF- κ B activation. HEK293 cells (1×10^5) were transfected with an NF- κ B luciferase reporter (0.02 μg) and the indicated plasmids (0.02 μg each). Twenty hours after transfection, cells were untreated or treated with IL-1 β (10 ng/ml) for 10 h before reporter assays were performed. The results were analyzed by immunoblots with the indicated antibodies. D, wild-type KLF6 but not KLF6(C265Y) mutant potentiates IL-1 β -induced transcription of *MCP1*, *CXCL2*, and *IL8* genes. HEK293 cells (4×10^5) stably transfected with control vector, Flag-KLF6 or Flag-KLF6(C265Y) were untreated or treated with IL-1 β (10 ng/ml) for the indicated time points before qPCR analysis was performed. E, KLF6(C265Y) interacts with p65. HEK293 cells (2×10^6) were transfected with HA-tagged p65 (1 μg) and Flag-tagged KLF6 or its C265Y mutant (5 μg each) for 20 h before coimmunoprecipitation and immunoblot analysis was performed with the indicated antibodies. F, wild-type KLF6 but not KLF6(C265Y) mutant could bind to promoters *I κ B α* and *IL8* genes after IL-1 β stimulation. HEK293 cells (4×10^5) stably transfected with control vector, Flag-KLF6, or Flag-KLF6(C265Y) were stimulated with IL-1 β (10 ng/ml) for 30 min. ChIP assays were performed with the indicated antibodies and binding of KLF6 or KLF6(C265Y) to the cognate sites was determined by qPCR. G, overexpression of wild-type KLF6 but not KLF6(C265Y) potentiates IL-1 β -induced DNA binding of p65 to promoters of *MCP1*, *CXCL2*, and *IL8* genes. The experiments were performed as in F except that the p65 antibody was used.

that KLF6 plays a role in antagonizing apoptosis induced by TNF α and Smac mimetic.

DISCUSSION

The transcription factor NF- κ B is involved in various physiological and pathological processes, including development, inflammation, immunity, and cancers. In this study, we screened a pool of 15,000 independent human and mouse expression clones for potential factors involved in TNF α - or IL-1 β -induced activation of NF- κ B. This effort identified KLF6, as a positive regulator of TNF α - and IL-1 β -induced NF- κ B activation. Overexpression of KLF6 potentiated TNF α - and IL-1 β -induced expression of a subset of NF- κ B target genes, while knockdown of KLF6 had opposite effects. In contrast, neither overexpression nor knockdown of KLF6 had any effects on IFN γ -induced IRF1 activation. These data suggest that KLF6 selectively regulates TNF α - or IL-1 β -induced NF- κ B activation.

Because knockdown of KLF6 had minimal effects on TNF α - or IL-1 β -induced phosphorylation and degradation of *I κ B α* or

nuclear translocation of p65, we reasoned that KLF6 might function at the level of or downstream of p65 in the nucleus. This notion was supported by the observations that KLF6 interacted with p65 in the nucleus and was required for optimal binding of p65 to the promoters of a subset of downstream genes. KLF6 contains a TAD domain in its N terminus and three ZNF domains in its C terminus, which are required for its interaction with p65 and the binding to the promoters of p65 target genes, respectively. In addition, the KLF6(C265Y) mutant, which lost DNA binding activity but retained the p65-interacting ability, failed to potentiate IL-1 β -induced expression of downstream genes and binding of p65 to the promoters. Our study also demonstrated that p65 was indispensable for optimal binding of KLF6 to the promoters of target genes, as knockdown of p65 diminished IL-1 β -induced binding of KLF6 to the promoters of *MCP1*, *CXCL2*, *IL8*, and *I κ B α* genes. A simple interpretation for these observations is that KLF6 binds to the promoters of p65 target genes and interacts with p65 through its respective C-terminal and N-terminal domains, which anchors p65 on the promoters to enhance the transcrip-

KLF6 Modulates NF- κ B Activity in the Nucleus

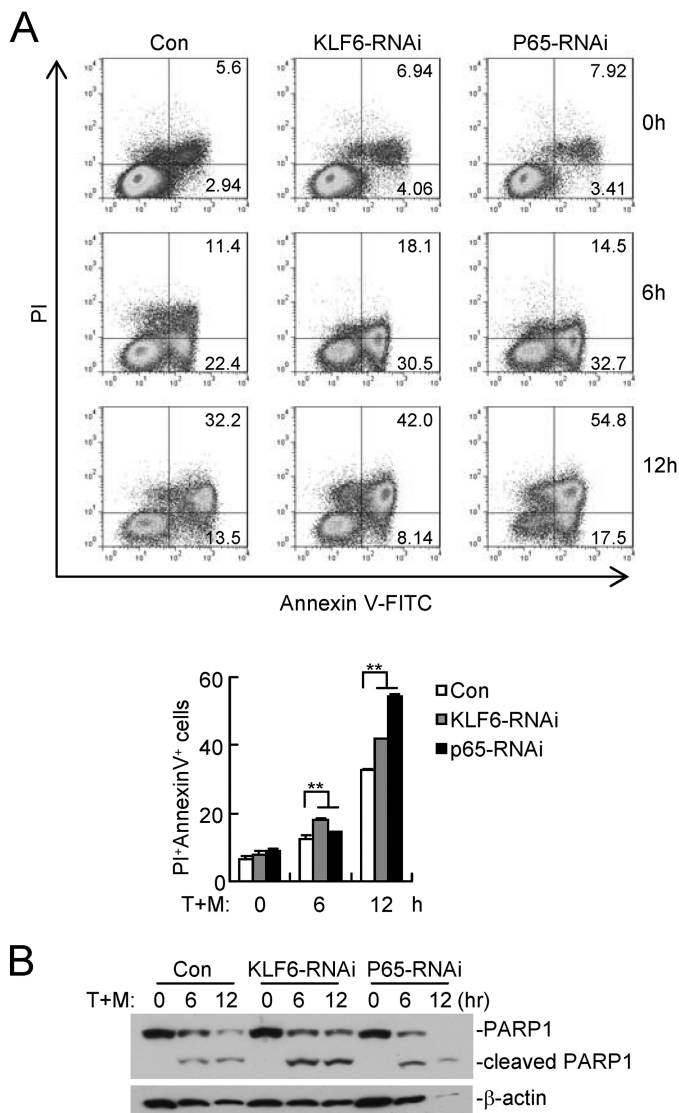


FIGURE 8. KLF6 antagonizes TNF α -induced apoptosis. A, knockdown of KLF6 or p65 enhanced TNF α and Smac mimetic-induced apoptosis. HCT116 cells stably transfected with the indicated RNAi plasmids were untreated or treated with TNF α (100 μ g/ml) and Smac mimetic (100 nM) for 6 or 12 h. The apoptotic cells were stained with annexin V-FITC and PI followed by flow cytometry analysis (*upper panel*). The flow cytometry data are also presented quantitatively (*lower histogram*). The graphs show mean \pm S.D., $n = 3$, **, $p < 0.01$; T+M: TNF α +Smac mimetic. B, knockdown of KLF6 or p65-enhanced TNF α and Smac mimetic-induced cleavages of PARP1. Experiments were performed as in A except that cells were lysed and analyzed by immunoblots with the indicated antibodies.

tion of downstream genes, and that the binding of p65 to the promoter facilitates binding of KLF6 to the same promoters (discussed below).

The p65/p50 heterodimer binds to the so-called κ B site 5'-GGGRNYYCC-3' (where R is a purine, Y is a pyrimidine, and N is an unspecified base), whereas the KLF family proteins recognize the "CACCC-box" that represents the reverse complement sequence for "GGGRN" from the κ B site, indicating that KLF6 and p65 may bind to the same DNA sequences on the promoters of target genes. It has been reported that NF- κ B binding to the κ B sites is transient and quite dynamic, which allows efficient but proper transcription of the target genes. TNF α or IL-1 β stimulation induces formation of KLF6 and p65

complex and collaborative binding of these two proteins to the promoters of downstream genes. When one factor is disassociated from the promoters because of the high on/off binding rate, the other would bind the same site, thereby increasing the chance with which KLF6 or p65 binds to the target promoters and induces the transcription of target genes. This explains why KLF6 and p65 are mutually required for promoter binding ability for a subset of downstream genes.

It should be noted that not all p65 target genes, *I κ B α* for instance, were regulated by KLF6; although KLF6 bound to the promoter of the *I κ B α* gene after TNF α or IL-1 β stimulation, indicating that other proteins may be involved in the process. Interestingly, knockdown of KLF6 also enhanced apoptosis triggered by TNF α and Smac mimetic, which could be due to impaired KLF6-mediated expression of p65-dependent genes. High throughput ChIP-sequencing may be used for a thorough identification of KLF6/p65-binding promoters in the future. Nonetheless, our study has clearly demonstrated that KLF6 is a co-activator of p65 for transcription of selected downstream genes after TNF α or IL-1 β stimulation.

Although much progress has been made to characterize the activation of NF- κ B upon various stimulations, how NF- κ B in limited forms of dimers functions to differentially induce transcription of thousands of downstream genes remains largely unclear. Our identification of KLF6 as a co-activator of p65 has thus uncovered a previously unknown mechanism by which NF- κ B target genes are regulated in a gene-specific manner. Our findings thus identified KLF6 as a previously unknown but essential co-activators of NF- κ B and provided new insight into the molecular regulation of p65-dependent gene expression.

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