Transcriptional activation of USP16 gene expression by NFkB signaling

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Abstract

Ubiquitin Specific Peptidase 16 (USP16) has been reported to contribute to somatic stem-cell defects in Down syndrome. However, how this gene being regulated is largely unknown. To study the mechanism underlying USP16 gene expression, USP16 gene promoter was cloned and analyzed by luciferase assay. We identified that the 5′ flanking region (~1856 bp ~ +468 bp) of the human USP16 gene contained the functional promoter to control its transcription. Three bona fide NFkB binding sites were found in USP16 promoter. We showed that p65 overexpression enhanced endogenous USP16 mRNA level. Furthermore, LPS and TNFα, strong activators of the NFkB pathway, upregulated the USP16 transcription. Our data demonstrate that USP16 gene expression is tightly regulated at transcription level. NFkB signaling regulates the human USP16 gene expression through three cis-acting elements. The results provide novel insights into a potential role of dysregulation of USP16 expression in Alzheimer's dementia in Down Syndrome.

Keywords: Down syndrome, USP16, NFkB, Transcriptional regulation, Promotor

Introduction

Down syndrome (DS) is a complex developmental disorder caused by genetic defects, leading to intellectual and developmental disabilities. It is a result of complete or partial trisomy of chromosome 21 [1, 2]. Individuals with DS therefore have three copies of 161 known protein-encoding genes. The phenotypes of DS are believed to be related with abnormal gene expression and functions due to the extra copy of the genes on chromosome 21 [3, 4], and the DS patients invariably develop Alzheimer's disease (AD)-related neuropathology [5–10]. Although a few candidate genes have been linked to the spectrum of disorders associated with DS [11–16], it is unclear how trisomy of specific genes contributes to the disease.

The human USP16 gene is mapped on chromosome 21 and triplicated in DS. USP16 gene contains 3 mRNA transcripts which share the same start codon, stop codon, and translation frame. USP16 has been reported to contribute to the somatic stem-cell defects in DS and reduce the self-renewal of multiple somatic stem cells [17], suggesting that some of the pathological features associated with DS may result from a stem-cell imbalance due to overexpression of USP16. It was first identified as a histone H2A-specific deubiquitinase that regulates cell cycle progression and gene expression in human cells [18]. This deubiquitinating enzyme, USP16, removes the ubiquitin protein from H2A-K119, and upregulates the transcription of the Ink4a locus [17]. The Ink4a locus encodes the p16^{Ink4a} and the p19^{Arf} genes, which are important members participating in self-renewal and senescence pathways. It was reported that USP16 was upregulated in response to DNA damage, and the upregulation of its expression was HECT and RCC1-like domain-containing protein 2 (HERC2)-dependent [19]. Furthermore, USP16 was shown to regulate embryonic stem cell gene expression and hematopoietic stem cell function [20, 21]. A recent study reported that USP16 was involved in cancer, and its downregulation promoted hepatocellular carcinoma cells growth [22]. The converging lines of evidence shed light on USP16’s functions, but the transcriptional regulation of USP16 gene is largely unknown.
NFκB signaling pathway plays an important role in the gene regulation [23–25] and is associated with inflammation [26], oxidative stress [27], and apoptosis [28]. The mammalian NFκB family consists of five members, including NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB, and C-Rel [29]. These members form various homo- or heterodimeric complexes. Activation of NFκB is tightly controlled by an inhibitory subunit, known as the inhibitor of NFκB (IκB). IκB binds to NFκB dimers to block their nuclear localization sequences, thus NFκB dimers are retained within the cytoplasm [30]. Once cells are stimulated by activators, such as tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS) [31], IκB is phosphorylated by IκB kinase (IKK) complex, making itself being degraded by ubiquitin-proteasome pathway [32]. Then NFκB dimers are released and translocated into the nucleus, where they regulate the transcription of the NFκB target genes [29].

Previous studies have shown that NFκB plays essential roles in cell cycle progression [33], senescence [34], DNA damage repair [35], maintenance of stem cells pluripotency [36] and cancer. In the present study, we aim to elucidate how USP16 gene expression is regulated and the role of NFκB in USP16 gene regulation. We cloned and functionally analyzed the human NFκB gene promoter region. We showed that the USP16 gene promoter contained functional cis-acting NFκB binding sites. By using EMSA, we identified three bona fide binding sites, through which NFκB signaling regulates USP16 gene transcription. p65 overexpression was shown to increase the endogenous USP16 mRNA level and the activators of the NFκB pathway, including LPS and TNFα, also upregulated the USP16 transcription. By knocking out p65 in mice embryonic fibroblasts, the effects of TNFα on upregulating USP16 transcription was abolished.

**Cell culture, luciferase assays, and transfection**

Human embryonic kidney 293 (HEK293) (RRID:CVCL_0045) and human neuroblastoma SH-SY5Y cell lines (RRID:CVCL_0019) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco). Wildtype (WT) mouse embryonic fibroblasts (MEFs) and p65 knockout (KO) MEFs were maintained in DMEM supplemented with 15% FBS, β-mercaptoethanol, and ESGRO (LIF) [24]. All cells were maintained in a 37 °C incubator containing 5% CO2. For luciferase assays, pCMV-Rluc (Promega) was co-transfected with pUSP16-related promoter plasmids as a control to normalize the transfection efficiency. Specifically, 270 ng pUSP16-related promoter plasmids and 30 ng pCMV-Rluc were cotransfected into each well of a 48 well-plate by using 0.9 μl Lipofectamine™ 2000 reagent (Invitrogen). Cells were harvested 24 h after transfection and lysed with 60 μl 1 × passive lysis buffer (Promega) per well. Activities of the Firefly and Renilla luciferases from the same sample were sequentilly assayed by a luminometer (GloMax 20/20) following the protocol of the dual-luciferase reporter assay system (Promega, E1910). The Firefly luciferase activity was normalized by the Renilla luciferase activity and the results reflected the relative promoter activity. For RNA extraction analysis, 4 μg plasmid DNA was transfected by 12 μl Lipofectamine™-2000 reagent per well of a 6 well-plate.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed as previously described [38]. To obtain NFκB-enriched nuclear extract, HEK293 cells were transfected with the p65 expression plasmid (pMTF-p65) for 24 h. Nuclear protein was extracted by using NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer’s instructions. Five oligonucleotides probes were labeled with IR700 dye (Bioneer Corporation) and annealed with corresponding anti-sense oligonucleotides to generate double-stranded probes at a final concentration of 0.01 pmol/μl. Among them, USP16 3x NFκB contained three NFκB cis-acting elements, including NFκB2, NFκB3, and NFκB4. USP16-NFκB1, USP16-NFκB2, USP16-NFκB3, and USP16-NFκB4 oligonucleotide had a corresponding sequence. For competition experiments, 2 μl of nuclear extract was incubated with 0.01 pmol/μl of labeled probes and 100× (1 pmol/μl) unlabeled competition probes for 20 min at room temperature. For the supershift assay, monoclonal anti-NFκB p65 antibody (Cell Signaling, 8424 s) was added. The reaction mixtures were separated on a 4% Tris-glycine-EDTA gel for 70 min at 70V in darkness. The gel was scanned using LI-COR Odyssey (LI-COR Biosciences) at a wavelength of 700 nm.

**Materials and methods**

**Primers and plasmids construction**

The 5’ flanking region of the human USP16 gene was amplified by polymerase chain reaction (PCR) from human genomic DNA. The primers were designed with restriction enzymes sites compatible with multi-cloning sites of vector pGL4.10 (Promega). The pGL4.10 vector lacks eukaryotic promoter and enhancer sequences upstream of a reporter luciferase gene. We first cloned the longest 2324 bp (~ 1856 bp + 468 bp) promoter region into pGL4.10 at the Xhol and HindIII sites to generate pLUSP16-A. Then, promoter deletion assays were conducted as previously described [25, 37]. Briefly, a series of deletion fragments were amplified by using pLUSP16-A as the template and sub-cloned into pGL4.10 at proper restriction enzymes sites. All used primers were listed in Additional file 1.
nm. The sequences of the oligonucleotides were listed in Additional file 1.

**LPS and TNFα treatment**

LPS (Sigma, L4516) was reconstituted in DMEM and further diluted to a final concentration of 50 ng/ml, while TNFα (Sigma, H8916) was reconstituted in sterile phosphate buffered saline (PBS) containing 0.1% endotoxin-free recombinant human serum albumin at a final concentration of 10 ng/ml. For qRT-PCR, HEK293 and SH-SY5Y cells were exposed to LPS and TNFα at the proper concentration for 24 h and then lysed for RNA extraction. For RT-PCR, MEF WT and p65 KO cells were treated with 5 ng/ml TNFα for 24 h.

**qRT-PCR**

Total RNA was extracted from cells using TRI reagent (Bio-Tek, RP1202), and quantified with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). PrimeScript™ RT reagent Kit (Takara, RR037A) was used to synthesize the first-strand cDNA from an equal amount of various RNA samples according to the manufacturer’s instructions. qRT-PCR was performed by using SYBR® Premix Ex Taq™ II (Takara, RR820A) and the PCR program included one initial denaturation step at 95 °C for 3 min, 39 cycles of 95 °C for 10 s, 58 °C for 30s, and 72 °C for 30 s (Bio-Rad CFX96). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Primers used in this assay were listed in Additional file 1. Amplification efficiency of those primers were checked by standard curve method, with an E value around 100% (R² > 0.998). The relative expression of mRNA was calculated with the 2^(-ΔΔCt) method. Each sample was triplicated. qRT-PCR data were analyzed and converted to relative fold changes. Additionally, total RNA was extracted from MEF WT or p65 KO cells by TRIzol reagent (Invitrogen). Thermoscript™ SuperScript IV first-strand synthesis system (Invitrogen) was applied to amplify the first-strand cDNA by using 1.5 μg of total RNA as the template and then the newly synthesized cDNA was used as the template to perform PCR by Taq DNA polymerase. A pair of primers to amplify a 150 bp region of mice USP16 gene was as follows: forward, 5’-ctgccgaaggctggctggagcagc, and reverse, 5’-cggctgctgtagctgctgtaag. Additionally, a pair of primers for amplifying a 205 bp fragment of mouse GAPDH gene coding sequence was as follows: forward, 5’-ggatttggtcgtgtagtgctt, and reverse, 5’-ggaagatggtgatgggatt. All samples were analyzed on 2.5% agarose gels.

**Statistical analyses**

Three or more independent experiments were performed. All results were presented as mean ± the standard error of the mean (SEM) and 2-tailed Student’s t test was used to analyze the difference between two groups. One-way Analysis of variance (ANOVA) was applied to analyze the data in Fig. 2 and multiple comparison tests were conducted by post-hoc Turkey’s method. Statistical analysis in Fig. 3 and Fig. 5e-f was performed by two-way ANOVA followed by post-hoc Turkey’s multiple comparisons test. p < 0.05 was considered as statistically significant.

**Results**

**Cloning of the human USP16 promoter**

The human USP16 gene is mapped on chromosome 21, at 21q21.3. It has three mRNA transcripts which share the same start codon, stop codon, and translation frame. Transcript 1 and 3 encode the same isoform a and transcript 2 encodes isofrom b. Transcripts 3 is the longest one which contains all the 19 exons, while the other two lack Exon 2. The only difference between transcript 1 and transcript 2 is that the later is shorter and lacks the first exon “CAG” in Exon 7 (Fig. 1a). Human genomic DNA were extracted from HEK293 cells and a 2324 bp 5' flanking region of the USP16 gene was amplified by PCR. The human USP16 gene has a complex transcriptional machinery as suggested by the results from a computer-based transcription factor binding site search using Genomatix and TFSearc. The human USP16 gene promoter was shown to contain several putative regulatory elements, such as MAF and AP1 related factors (AP1R), NFκB, hypoxia-inducible factor (HIF), nuclear factor of activated T-cells (NFAT), cAMP-responsive element binding proteins (CREB), and Ying Yang 1 (YY1) (Fig. 1b). There are 4 putative NFκB binding sites spanning a wide region of USP16 gene promoter in our predication results and NFκB signaling pathway is an essential player in the inflammatory response, which has been recognized as a factor to facilitate the pathogenesis of DS and AD phenotypes in DS patients [39]. Therefore, we mainly focused on NFκB signaling pathway in the following experiments.

**Functional analysis of the human USP16 gene promoter**

To determine the functional promoter region of the human USP16 gene, we cloned a 2324 bp 5’ flanking region of the USP16 gene into a promoter-lacking vector pGL4.10 to generate pUSP16-A (~1856 bp to +468 bp) plasmid. The luciferase activities of cells transfected with this plasmid largely rely on the presence of a functional promoter upstream of a luciferase gene. The plasmid pUSP16-A was transfected into HEK293 cells, and luciferase activity was measured by a GloMax 20/20 Luminometer to examine its promoter activity. Compared with cells transfected with an empty vector pGL4.10, pUSP16-A showed a significant increment of luciferase activity (84.48 ± 4.55RLU, p < 0.0001) (Fig. 2c). This result indicates
that the 2324 bp fragment contains the functional promoter region of the human USP16 gene.

To identify the regulatory elements in the USP16 promoter region, a series of deletion fragments within pUSP16-A was generated (Fig. 2a). The luciferase assays of these deletion plasmids were performed. The results indicated that pUSP16-B \((-1325 \text{ bp} \sim +468 \text{ bp}, 75.91 \pm 4.40 \text{ RLU}; p > 0.9999)\), pUSP16-C \((-653 \text{ bp} \sim +468 \text{ bp}, 50.98 \pm 4.74 \text{ RLU}; p = 0.1065)\), and pUSP16-D \((-200 \text{ bp} \sim +468 \text{ bp}, 55.21 \pm 3.57 \text{ RLU}; p = 0.2514)\) have no significant changes of luciferase activities when compared with pUSP16-A \((-1856 \text{ bp} \sim +468 \text{ bp}, 84.48 \pm 4.55 \text{ RLU})\). A series of 3’end deletion plasmids was constructed and transfected into HEK293 cells. Luciferase activities of these plasmids showed that a 41 bp deletion (pUSP16-E, \(-200 \text{ bp} \sim +427 \text{ bp}, 172.13 \pm 7.16 \text{ RLU}\)) significantly reduced promoter activity \((p < 0.0001)\), indicating that there are negative regulatory elements located in this region. A further 20 bp deletion (pUSP16-F, \(-200 \text{ bp} \sim +407 \text{ bp}, 95.36 \pm 5.98 \text{ RLU}\)) did not show significant changes to that of pUSP16-E \((-200 \text{ bp} \sim +427 \text{ bp})\) \((p < 0.0001)\). However, a further 20 bp deletion (pUSP16-I, \(-200 \text{ bp} \sim +130 \text{ bp}, 266.32 \pm 7.80 \text{ RLU}\)) from pUSP16-H \((-200 \text{ bp} \sim +150 \text{ bp})\) largely enhanced promoter activity \((p < 0.0001)\), indicating that the 20bp region contains negative regulatory elements.

Further deletion analysis found that a 20 bp deletion from pUSP16-I \((-200 \text{ bp} \sim +130 \text{ bp}, 266.32 \pm 7.80 \text{ RLU})\) to pUSP16-J \((-200 \text{ bp} \sim +110 \text{ bp}, 201.22 \pm 12.10 \text{ RLU}; p < 0.0001)\) and a 12 bp deletion from pUSP16-J \((-200 \text{ bp} \sim +110 \text{ bp})\) to pUSP16-N \((-200 \text{ bp} \sim +98 \text{ bp}, 100.74 \pm 6.89 \text{ RLU})\) significantly reduced promoter activity \((p < 0.0001)\).
2.94RLU, $p < 0.0001$) substantially reduced promotor activity, while a 48 bp deletion from pUSP16-N ($−200$ bp~ + 98 bp) to pUSP16-O ($−200$ bp~ + 40 bp, $73.13 \pm 3.51$RLU) did not significantly change the promotor activity ($p = 0.3357$). However, a further 19 bp deletion from pUSP16-O ($−200$ bp~ + 40 bp) to pUSP16-P ($−200$ bp~ + 21 bp, $1.74 \pm 0.07$RLU) almost abolished promotor activity ($p < 0.0001$). These results showed that +40 bp was the proper $3'$ boundary of the USP16 promotor region. Since we found pUSP16-J ($−200$ bp ~ + 110 bp, $201.22 \pm 12.10$RLU) still had a high promotor activity compared with the empty vector pGL4.10 ($p < 0.0001$), pUSP16-J was chosen to investigate the effect of 5'end on promotor activity. A series of 5'end deletion plasmids was constructed and transfected into HEK293 cells. Luciferase activity showed that a 101 bp deletion (pUSP16-K, $−99$ bp~ + 110 bp, $332.87 \pm 17.59$RLU) from pUSP16-J ($−200$ bp~ + 110 bp) increased USP16 promotor activity ($p < 0.0001$), while a 47 bp deletion (pUSP16-L, $−52$ bp~ + 110 bp, $199.65 \pm 7.69$RLU) from pUSP16-K ($−99$ bp~ + 110 bp) decreased USP16 promotor activity ($p < 0.0001$) and a further 52 bp deletion (pUSP16-M, +1 bp~ + 110 bp, $109.72 \pm 3.88$RLU) from pUSP16-L ($−52$ bp~ + 110 bp) reduced USP16 promotor activity ($p < 0.0001$). Taken together, our data illustrate that the promoter region from +1 bp to +40 bp has the minimal promotor activity required for basal transcription and various cis-acting regulatory elements are located in the 5' flanking region of USP16 gene.

NFκB upregulates the human USP16 gene promotor activities

Computer-based transcription factor binding site analysis revealed four putative NFκB cis-acting elements in the 1793 bp ($−1325$ bp ~ + 468 bp) promoter region of the human USP16 gene (Fig. 1b). To determine whether

Fig. 2 Deletion analysis of the human USP16 gene promotor. a Schematic diagram of the human USP16 deletion promotor constructs in pGL4.10 vector. Arrow shows the direction of transcription. The numbers represent the start and end points for each construct. b The deletion plasmids were confirmed by restriction enzyme digestion, and the digested samples were analyzed on a 1.0% agarose gel. The vector size is 4.2 kb; USP16 promotor fragment size ranges from 0.11 to 2.3 kb. The sequences of the inserts were further confirmed by sequencing. c The promotor plasmids were co-transfected with pCMV-Luc into HEK293 cells. After 24 h transfection, the cells were harvested and luciferase activity was measured with a luminometer and presented in relative luciferase units (RLU). The pCMV-Luc luciferase activity was used to normalize for transfection efficiency. The values represent means ± SEM. n = 3, ***$p < 0.001$ by one-way ANOVA test followed by post-hoc Turkey’s test. Comparisons were made between all USP16 promotor reporter plasmids and the empty pGL4.10 as a negative control.
NFκB signaling regulates *USP16* gene transcription by interacting with these putative NFκB cis-acting elements, the effects of NFκB overexpression on the promoter activity of the 1793 bp region were examined. Four human *USP16* promoter deletion constructs, p*USP16*-N1, −N2, −N3, and −N4, were cloned into pGL4.10 vector, with sequential elimination of one upstream putative NFκB-binding element (Fig. 3a, b). We used a dual-luciferase reporter assay to examine the *USP16* promoter activity in HEK293 cells overexpressed with NFκB p65 expression plasmid (pMTF-p65) or empty vector (pMTF). Compared with empty vector pMTF, NFκB p65 expression plasmid co-transfecting with p*USP16*-N1, p*USP16*-N2, p*USP16*-N3, and p*USP16*-N4 resulted in enhanced luciferase activities to about 2.69, 2.51, 2.24, and 2.04 folds, respectively (Fig. 3d). To further confirm the involvement of p65 in regulating *USP16* gene promoter, two more deletion constructs, p*USP16*-N5 and -N6, were generated. Both p*USP16*-N5 and -N6 plasmids do not contain any putative NFκB-binding element (Fig. 3a, b). Unexpectedly, the promoter activity of p*USP16*-N5 was still significantly increased to 8.67 folds in HEK cells (p < 0.0001) and to 1.85 folds in SH-SY5Y cells (p = 0.0005) by p65 overexpression compared with the empty vector transfection. A further deletion fragment, p*USP16*-N6, was completely abolished the effect of p65 overexpression on affecting its promoter activity (Fig. 3e, f), suggesting a non-canonical NFκB binding site sequence located in p*USP16*-N5 was indirectly affected by p65 [40]. Taken together, these results demonstrate that NFκB p65 up-regulates human *USP16* promoter activity.

**USP16** promoter activity is regulated through three NFκB binding elements

To investigate which putative NFκB binding elements of *USP16* interact with NFκB p65, we performed EMSA to determine whether four putative NFκB cis-acting elements physically bind to NFκB p65. We synthesized NFκB consensus oligonucleotides end-labeled with IR700 dye as probes and four oligonucleotides (*USP16* NFκB 1–4) containing each NFκB cis-acting element as competitors. Labelled NFκB consensus probes were visualized as a heavy band on the bottom of the gel (Fig. 4b, lane 1), and a shift band was formed after the addition of the p65-enriched nuclear extracts (Fig. 4b, lane 2), suggesting DNA-protein complex formation. Moreover, the shift band was abolished when NFκB consensus, 100 × *USP16* NFκB2, or 200 × *USP16* NFκB2 (Fig. 4b; lanes 3, 5, 6) was applied, but not for NFκB mutant or *USP16* NFκB2 mutant (Fig. 4b; lanes 4, 7). Addition of anti-NFκB p65 antibody resulted in a slower migrating super shifted band (Fig. 4b, lane 8), confirming the existence of NFκB in the complex formation. Similar results were seen in *USP16* NFκB3 group (Fig. 4c) and *USP16* NFκB4 group (Fig. 4d), but not in *USP16* NFκB1 group (Fig. 4a). The data suggests that the second, third and the fourth NFκB-binding elements in *USP16* gene are able to interact with NFκB p65.

To further confirm these results, we performed additional EMSA with the three NFκB cis-acting elements (*USP16* NFκB2,3,4) end-labeled with IR700 dye as probes. Each probe contained a single putative NFκB cis-acting site. A shifted protein-DNA complex band was detected after incubating *USP16* NFκB2 probe with NFκB-enriched nuclear extract (Fig. 4e, lane 2). This shifted band was abolished by addition of 100 × *USP16* NFκB2 or NFκB consensus oligonucleotide (Fig. 4e; lanes 3, 5), but not *USP16* NFκB2 mutant or NFκB mutant (Fig. 4e; lanes 4, 6). Similar results were also obtained for *USP16* NFκB3 (Fig. 4f), *USP16* NFκB4 (Fig. 4g). In addition, we synthesized a single probe (*USP16* 3x NFκB) containing three NFκB cis-acting elements and gained similar results (Fig. 4h). The data demonstrated that the second, third, and the fourth NFκB binding elements in *USP16* gene interact with NFκB p65.

**Activation of NFκB signaling increases the human *USP16* gene transcription**

To examine whether NFκB affects the endogenous gene transcription, qRT-PCR was conducted to measure the endogenous *USP16* mRNA level. Overexpression of NFκB markedly upregulated endogenous mRNA level of the *USP16* gene compared to the control group (1.52-fold, p < 0.0001) (Fig. 5a). LPS and TNFa are strong activators of NFκB signaling pathway. To explore whether NFκB binding elements in the human *USP16* promoter mediate the inflammatory effect on *USP16* transcription, we examined *USP16* mRNA levels after LPS and TNFa treatment. HEK293 cells or SH-SY5Y cells were treated with LPS (50 ng/ml) or TNFa (10 ng/ml) for 24 h. Similar to the effect of NFκB overexpression on endogenous *USP16* mRNA levels (Fig. 5a), stimulation of LPS resulted in a marked
increase of endogenous USP16 mRNA levels in SH-SY5Y cells (1.55-fold, \( p = 0.0364 \)) (Fig. 5b), but no significant effect in HEK293 cells (data not shown). However, TNFα enhanced the levels of endogenous USP16 mRNA both in HEK293 cells (1.90-fold, \( p = 0.0011 \)) (Fig. 5c) and SH-SY5Y cells (1.85-fold, \( p = 0.0327 \)) (Fig. 5d). In order to confirm p65 directly mediating the effect of TNFα on up-regulating USP16 transcription, TNFα was further applied to a p65 knockout fibroblast cell line (MEF) and USP16 mRNA levels were examined. As shown in Fig. 5e and f, endogenous USP16 mRNA levels was significantly decreased to 0.65 folds by knocking out p65 in MEF cells (\( p = 0.0397 \)), suggesting p65 is a strong activator for endogenous USP16 gene expression. Furthermore, TNFα treatment significantly increased USP16 mRNA levels by 1.12 folds compared with control (\( p < 0.0001 \)), whereas such increase was abolished in MEF p65 KO cells. These data suggest that activation of NFκB signaling pathway by p65, LPS and TNFα upregulates USP16 gene transcription. Taken together, endogenous USP16 gene transcription was enhanced by activators of the NFκB pathway, including LPS and TNFα, and p65 knockout

Fig. 3 USP16 promoter activity is up-regulated by NFκB p65. (a) Schematic diagram of deletion plasmids containing different human USP16 promoter fragments in front of the firefly luciferase reporter gene of the pGL4.10 vector. (b) The deletion plasmids corresponding to pUSP16-N1, –N2, –N3, –N4, –N5 and –N6 were checked by sequencing and restriction enzyme digestion and the digested samples were analyzed on 1% or 3.5% agarose gels. Vector size is 4.2 kb and the USP16 promoter fragment insert size ranges from 69 bp to 2.2 kbp. USP16 promoter deletion plasmids and pMTF-p65 or pMTF were co-transfected into (c, e) HEK293 cells and (d, f) SH-SY5Y cells with pGL4.10 as a control. After transfection for 24 h, the cells were harvested and luciferase activity was presented in relative luciferase units (RLU). The pCMV-Luc luciferase activity was used to normalize for transfection efficiency. The values represent means ± SEM. \( n = 3 \), * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) by two-way ANOVA followed by post-hoc Turkey’s multiple comparisons test.
Fig. 4 Gel mobility shift assay for the USP16 gene promoter. Gel shift and Super gel shift assays were performed as described in the Materials and methods.

**a-d** Double-stranded consensus NFκB oligonucleotides were end-labeled with IR700 dye as probes. Incubation of labeled probe with nuclear extracts formed a shifted DNA-protein complex band (lane 2). For competition assays, different concentrations of unlabeled competition oligonucleotides, consensus NFκB (lanes 3), mutant NFκB (lanes 4), USP16 NFκB (lanes 5 and lanes 6), USP16 NFκB mutant (lanes 7) were added. Anti-NFκB p65 antibody was used for the super gel shift assay. Addition of the anti-NFκB p65 antibody into the reaction mixture produced a supershifted band, indicating the formation of the nuclear protein-USP16-p65 complex (lane 8).

**e-g** USP16 NFκB2, USP16 NFκB3, and USP16 NFκB4 double-stranded oligonucleotides were end-labeled with IR700 dye as probes, respectively. Incubation of labeled probe with nuclear extracts formed a shifted DNA-protein complex band (lane 2). For competition assays, unlabeled competition oligonucleotides, USP16 NFκB (lanes 3), USP16 NFκB mutant (lanes 4), consensus NFκB (lanes 5), mutant NFκB (lanes 6) were added. Anti-NFκB p65 antibody was used for the super gel shift assay. The anti-NFκB p65 antibody supershifted the nuclear protein-USP16-p65 complex (lane 7).

**h** A double-stranded oligonucleotide contains USP16 NFκB2, USP16 NFκB3, and USP16 NFκB4 cis-elements were end-labeled with IR700 dye as probe for EMSA.
abolished the effects of TNFα on upregulating USP16 gene transcription.

**Discussion**

USP16 protein is a histone H2A-specific deubiquitinase with the coding gene located on human chromosome 21. Several previous studies suggest that it plays many roles in gene expression, cell cycle progression, cell self-renewal, and senescence pathways [17, 18]. USP16 affects hematopoiesis and hematopoietic stem cell function [20]. In mouse hematopoietic stem cells (HSCs), knock out of USP16 was associated with a reduction of mature and progenitor cell populations, while HSC number did not change. USP16 was reported to be involved in hepatocellular carcinoma and decreased expression of USP16 by carboxyl-terminal truncated HBx (Ct-HBx) in live tumor cells promoted stem-like properties [22]. By using DS model mice (Ts65Dn), triplication of USP16 contributed to neuronal progenitor defects and abnormal development of mammary epithelium. Overexpression of USP16 in human fibroblast cultures manifested growth impairment and senescence, reminiscent of DS’s cells [17]. Additionally, USP16 was shown to interact with HERC2 to regulate ubiquitin signaling during DNA repair during DNA damage response. This could be essentially important to DS, since cellular response to DNA damage was altered in DS patient cells [19].

In this study, we demonstrated that NFκB enhanced human USP16 gene transcription. We first identified that the 5’ flanking region of USP16 gene, from -1856 bp to +468 bp, showed promotor activity. Then we found several functional regulatory elements by a series of promotor deletion analysis. By using a computer-
based analysis, we determined four putative NFkB binding sites in the promotor region. After conducting EMSA, we provided solid evidence to support that three binding sites (USP16 NFkB2, USP16 NFkB3, and USP16 NFkB4) physically interacts with p65. In this article, we first proved that p65 overexpression could enhance endogenous USP16 mRNA levels through three cis-acting elements. It was unexpected that the promoter activity of the pUSP16-N5 plasmid without any putative p65 binding site was still increased by p65 overexpression. It has been suggested that DNA-binding specificities are different among various NFkB dimers, which is linked to dimer-specific roles in gene regulation [41]. It was reported by a previous study that one c-Rel subunit was able to bind to a nonconsensus half-site of the DNA-binding domain with the other subunit anchoring at the consensus half-site [40]. Therefore, it is possible that a non-canonical NFkB binding site is located in pUSP16-N5, which was regulated by other NFkB family members except p65. As LPS and TNFα are strong activators of NFkB pathway, we tried stimulate USP16 gene by adding LPS and TNFα to cell culture medium and confirmed that both LPS and TNFα stimuli enhanced USP16 transcription. By knocking out p65 in MEF cells, the effects of TNFα treatment on upregulating USP16 gene expression was abolished.

DS has been associated with early onset and higher incidence of aging-related diseases such as AD [5, 42, 43]. DS patients develop early-onset AD (EOAD). Full trisomy of chromosome 21 inevitably causes the development of two pathological characteristics in AD brains, amyloid plaques and neurofibrillary tangles (NFTs). And by the age of 60, approximately two-thirds of individuals with DS suffer from dementia [44, 45]. The mechanisms linking DS to AD remain to be defined. It has been shown that the aging process is related with an impaired or exhausted ability of stem cells to renew themselves. USP16, in this case, may play a role in AD-related pathogenesis in DS. A number of genes on the chromosome 21 plays an important role in the AD pathogenesis. Duplication of amyloid β precursor protein (APP) gene was reported to cause autosomal dominant early-onset Alzheimer disease in five families [46], and various mutations in the APP gene has been identified in AD patients [47]. Our lab recently discovered that BACE2, another the chromosome 21-located gene, was a conditional BACE1 to facilitate AD pathogenesis [48]. NFkB signaling has been implicated in the AD pathogenesis. BACE1 cleaves APP to generate amyloid β protein (Aβ), a central component of neuritic plaques in the AD brains. Previously we found that NFkB p65 expression resulted in increased BACE1 promoter activity and BACE1 transcription [23]. We also demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) and inhibition of GSK3 signaling inhibited BACE1 transcriptional activation [23, 24]. Regulator of Calcineurin 1 (RCAN1) gene, a gene on Chromosome 21, has been implicated in pathogenesis of DS and AD [49]. We showed that RCAN1 expression was elevated in DS and AD, and its overexpression in primary neurons induced caspase-3 dependent apoptosis [3, 7, 8]. We demonstrated that the RCAN1 isoform 4 gene transcription was activated by NFkB signaling [50]. As we have demonstrated that NFkB promotes USP16 gene transcription in this study, we attempt to explore the role of USP16 in AD pathogenesis in future studies.

Although we have identified that USP16 gene transcription was positively affected by NFkB, we also found three negatively regulatory elements in the USP16 promoter region (+427bp~+468bp, +130bp~+150bp, −200bp~−99bp). These three negatively regulatory regions contain a common transcription factor binding site for YY1 (data not shown). The ubiquitous transcription factor YY1 is known to be a multifunctional protein that can either activate or repress gene expression depending upon the cellular context. Ying Yang 1 has been reported to play fundamental roles in embryogenesis, differentiation, replication, and cellular proliferation [51–54]. Ying Yang 1 involved in nervous system development, neuronal differentiation and function [55]. Further studies about whether YY1 could regulate USP16 gene transcription and the potential impacts of USP16 on DS pathogenesis may be warranted.

In conclusion, our study demonstrates that that USP16 gene expression is tightly regulated at transcription level. NFkB signaling regulates the human USP16 gene expression through three cis-acting elements. The results provide novel insights into a potential role of dysregulation of USP16 expression in Alzheimer’s dementia in Down Syndrome.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13041-019-0535-3.

Additional file 1. Supplementary Information.

Abbreviations
AD: Alzheimer’s disease; DS: Down syndrome; NFkB: Nuclear factor kappa-light-chain-enhancer of activated B cells; USP16: Ubiquitin specific peptidase 16

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Authors’ contributions
WS conceived and designed the experiments; SY, JW, SG, DH and IBL performed the experiments; SY, SG, DH, JW, XN, DL, YL, ML, YK, WZ and WS analyzed and contributed reagents/materials/analysis tools; SY, SG, JW, WZ and WZ wrote the paper. All authors reviewed the manuscript.
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Availability of data and materials
Described in Results and Methods section and the authors agree the availability upon request.

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N/A.

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Competing interests
The authors declare that they have no competing interests.

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