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DDX6 is involved in the pathogenesis of inflammatory diseases via NF-κB activation

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ABSTRACT

The IL-6 amplifier was originally discovered as a mechanism for the enhanced activation of NF-κB in non-immune cells. In the IL-6 amplifier, IL-6-STAT3 and NF-κB stimulation is followed by an excessive production of IL-6, chemokines, and growth factors to develop chronic inflammation preceding the development of inflammatory diseases. Previously, using a shRNA-mediated genome-wide screening, we found that *DEAD-Box Helicase 6* (*DDX6*) is a candidate positive regulator of the amplifier. Here, we investigate whether DDX6 is involved in the pathogenesis of inflammatory diseases via the IL-6 amplifier. We found that *DDX6*-silencing in non-immune cells suppressed the NF-κB pathway and inhibited activation of the IL-6 amplifier, while the forced expression of DDX6 enhanced NF-κB promoter activity independent of the RNA helicase activity of DDX6. The imiquimod-mediated dermatitis model was suppressed by the siRNA-mediated gene downregulation of *DDX6*. Furthermore, silencing *DDX6* significantly reduced the TNF-α-induced phosphorylation of p65/RelA and IκBα, nuclear localization of p65, and the protein levels of IκBα. Mechanistically, DDX6 is strongly associated with p65 and IκBα, but not TRADD, RIP, or TRAF2, suggesting a novel function of DDX6 as an adaptor protein in the NF-κB pathway. Thus, our findings demonstrate a possible role of DDX6 beyond RNA metabolism and suggest DDX6 is a therapeutic target for inflammatory diseases.

Keywords

DDX6; NF-κB; IL-6; Inflammation

1. Introduction

Whereas healthy inflammation is indispensable for physiological defense, chronic inflammation causes not only autoimmune diseases but also metabolic diseases, neurodegenerative diseases, and some mental illness [1-3]. We have previously identified the IL-6 amplifier as an essential molecular mechanism in chronic inflammation [4,5]. The IL-6 amplifier describes the hyper-activation of NF-κB in non-immune cells, including endothelial cells and fibroblasts, induced by the synergistic activation of NF-κB and IL-6-STAT3. It enhances a local production of NF-κB target genes, including *IL-6*, chemokines, and growth factors, which are crucial for the pathogenesis of disease models including rheumatoid arthritis (RA) and inflammatory diseases in patients [6,7]. Indeed, some IL-6 amplifier targets, such as epiregulin, are highly expressed in the serum of patients with RA or multiple sclerosis compared to healthy controls [5,8]. These findings indicate a novel therapeutic strategy based on the IL-6 amplifier. Moreover, we performed a genome-wide screen, using short hairpin (sh)RNA and subsequently identified more than 1,000 genes as candidates of positive regulators of the amplifier including *DEAD-Box Helicase 6* (*DDX6*) [3].

The DEAD-box helicase family is a large group of proteins that share a conserved motif containing the amino acid sequence Asp-Glu-Ala-Asp (DEAD). It is essential for the regulation of gene expression, RNA processing, and other cellular processes [9]. DDX6 was identified as a translational repressor of mRNA decapping [10]. DDX6 is involved in several other RNA processes, including miRNA-mediated RNA degradation and translation inhibition [11,12] and viral RNA replication and expression [13,14], as well as acting as a RNA co-sensor and signaling enhancer for RIG-I [15] and regulator of

stress granules formation [16] and phase separation [17]. Finally, DDX6 has been associated with several diseases including RA, systemic lupus erythematosus (SLE), and systemic sclerosis (SSc) [18-22]. However, the direct link between DDX6 and inflammatory diseases has not been well-established.

Here, we demonstrate the importance of DDX6 for IL-6 amplifier activation based on the following: We show that the downregulation of *DDX6* suppresses the expression of NF-κB targets *in vitro*; the silencing of *DDX6* suppresses the development of an inflammatory disease *in vivo*; the forced expression of DDX6 enhances IL-6 and NF-κB promoter activity; the inhibition of *DDX6* expression significantly reduces the TNF-α-mediated phosphorylation of p65/RelA and inhibitor of κBα (IκBα), nuclear localization of p65, and the protein levels of IκBα; and the binding of DDX6 to several kinds of TNFR-NF-κB signaling molecules, including IκBα, SKP1, and p65, but not TRADD, RIP, or TRAF2. Therefore, DDX6 acts as an adaptor protein for IL-6 amplifier activation, suggesting that blockade of the DDX6-NF-κB axis in non-immune cells is a possible therapeutic target for inflammatory diseases.

2. Materials and methods

2.1. Cell lines and stimulation H4, primary human dermal fibroblasts (HDF), primary human synovial fibroblasts (HSF), HEK293T, and 293FT cells were cultured according to the manufacturer's instructions, as described previously [23]. Cytokine stimulations were performed, as previously outlined [23]. Briefly, the cytokine stimulation was performed by seeding cells in a 96-well plate (1×10^4 cells/well). After a 2-hour serum starvation, the cells were treated with human IL-6 (100 ng/mL; R&D Systems) and human soluble IL-6 receptor (100 ng/mL; R&D Systems), and/or human or mouse TNF-α (50 ng/mL; PeproTech).

2.2. Antibodies For immunoblotting and confocal microscopy, the following antibodies were employed: anti-DDX6 (14632-1-AP, Proteintech), anti-TRAF2 (4724, Cell Signaling Technology), anti-FLAG M2 (Sigma-Aldrich), Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) (715-035-150, Jackson Immuno Research Laboratories), Peroxidase-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) (711-035-152, Jackson Immuno Research Laboratories), Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Invitrogen), and Hoechst 33342 trihydrochloride trihydrate (H3570, Life Technologies).

2.3. Plasmids *DDX6*-encoding complementary DNA (cDNA) was subcloned into pEF-BOS vector, as previously described [23].

2.4. Real-time PCR Cellular total RNA was prepared from the cells using a SuperPrep II Cell Lysis Kit for qPCR (Toyobo). The 7300 fast real-time PCR system (Applied Biosystems) along with SYBR Green PCR master mix (Kapa Biosystems) was employed for assessing the relative mRNA expression levels, which were normalized to the *glycerol-3-phosphatase dehydrogenase* (*GAPDH*) mRNA expression level, as

previously described [23]. The PCR primer pairs utilized in the real-time PCR are listed in Table 1.

2.5. RNA interference and transfection. For transient silencing (si)RNA-mediated gene silencing, the cells were transfected with 10 nM of the corresponding siRNA oligonucleotide duplexes (for human *DDX6*, 1: s4011, 2: s4012, Ambion; for human p65, SASI_Hs01_00171090, Sigma-Aldrich; for human si-nontarget, Sigma Mission SIC-001s; Sigma-Aldrich) using Lipofectamine RNAi Max (Invitrogen) according to the manufacturer's instructions, as previously described [23]. For stable shRNA-mediated gene silencing, pLKO.1-puro-based lentiviral vectors obtained from the Sigma Mission shRNA library (for sh-*DDX6*-1, TRCN0000074694; sh-*DDX6*-2, TRCN0000074696, Sigma-Aldrich; control non-targeting shRNA in pLKO.1-puro 1864, Addgene) were used. The lentiviral particles were prepared following the previously outlined procedure [24]. The H4 cells were exposed to the lentiviral preparations in the presence of polybrene (8 μg/mL; Sigma-Aldrich). Following a 24-hour incubation, 1 μg/mL puromycin was introduced, and the cells were maintained for one week to facilitate the selection for infected cells.

2.6. Western blotting. Cell lysate preparation and Western blotting were conducted following established procedures [23]. For the simultaneity of experimental results, monitoring the quantitative changes in protein expression for each objective on a single membrane. Therefore, during the Western blotting process, membrane strips cut in the vicinity of the expected molecular weights post-transfer for blotting were utilized.

2.7. Mouse strains. C57BL/6 mice were acquired from Japan SLC. All mice were housed in specific pathogen-free conditions in accordance with the protocol of Hokkaido University. The mouse experiments and protocols were approved by the

Institutional Animal Care and Use Committee of Hokkaido University. The experiments were carried out under the committee's guidelines. The mice utilized in the experiments were 6–8 weeks old.

2.8. Skin inflammation model. Topical application of imiquimod (Mochida Pharmaceuticals, Japan), a Toll-like receptor 7 agonist, induced skin inflammation in one ear of C57BL/6 mice [23]. On days 1, 2, and 3, control, *DDX6* or *NF-κB p65* siRNA (Dharmacon, SMARTpool) was applied to both ears. On day 0, a mixture of 2.8 μL of cream-based ointment (Johnson & Johnson) and 4.7 μL of 20 μM siRNA was administered to the skin of the ears [23]. The ear skin thickness was assessed using a micro-caliper.

2.9. Immunoprecipitation. Cell lysates were prepared, following the previously outlined procedure [25]. Briefly, 2×10^6 HEK 293T cells were transfected with pEFBOS-Flag-DDX6 or pEFBOS-Flag empty vector using polyethyleneimine (PEI). After a 48-hour incubation, the cells were lysed in 500 μL of Buffer-B [20 mM Tris (pH 7.8), 100 mM KCl, 10% glycerol, 0.2% Triton X-100], with the addition of a protease inhibitor and phosphatase inhibitor cocktail (Thermo). Precleared supernatants were incubated with anti-FLAG antibody-conjugated agarose beads (Sigma-Aldrich). After washing with Buffer-B, the immunoprecipitants were heated in SDS sample buffer for 3 min at 95℃ and then utilized for immunoblotting.

2.10. Luciferase reporter assay. pEFBOS-Flag-DDX6 or an empty vector and pGL4.32 [luc2P/NF-κB RE/Hygro] (Promega) or pGL4.21-human *IL-6* promoter (−1178/+13), and pGL4.74 [hRluc/TK] (Promega), as an internal control, were transfected into HEK293T cells using PEI, as previously described [23]. Luciferase activities were assessed using the dual-luciferase reporter assay system (Promega) as per the

manufacturer's guideline. The results were normalized with Renilla luciferase.

2.11. Confocal laser scanning microscopy. To assess p65 translocation, sh-*DDX6*-transfected and control (Mock)-transfected cells were subjected to TNF-α stimulation for 0, 15, and 30 minutes on μ-Slides (Ibidi). The cells were treated with 4% paraformaldehyde for 20 minutes, permeabilized using Perm/Wash solution (Cytofix/Cytoperm Kit, BD Biosciences), and then exposed to an anti-p65 antibody for 1 hour. Following rinsing with Perm/Wash solution, the cells were treated with Hoechst 33342 nuclear stain and anti-rabbit Alexa Fluor 488-conjugated secondary antibody for one hour. The translocation of p65 to the nucleus was evaluated by observing the cells using the LSM5 Pascal confocal microscopy system (Carl Zeiss).

2.12. Statistical analysis. Differences between two groups were analyzed using Student’s two-tailed t-tests. For multiple comparisons, one-way analysis of variance with Dunnett's post hoc test was employed. *P*-values below 0.05 were considered statistically significant.

3. Results

3.1. DDX6 is involved in NF-κB activation *in vitro.*

To investigate whether DDX6 is involved in activation of the IL-6 amplifier in non-immune cells, we first used a human neuroglioma cell line, H4, in which the IL-6 amplifier was previously reported to be augmented by the simultaneous stimulation of IL-6 and TNF-α [26]. H4 cells with *DDX6* siRNA, similar to *p65* siRNA, showed significantly less expressions of *DDX6* and *IL-6* than did mock-transfected cells after stimulation with IL-6 plus TNF-α (Fig. 1A). Next, we performed the same experiments using primary human fibroblast cell lines derived from dermal (HDF) and joint synovial (HSF) tissues. These cells showed higher *IL-6* production following simultaneous IL-6 and TNF-α stimulation. Consistently, a significant reduction in the *IL-6* expression level was observed with *DDX6*-siRNA treatment (Fig. 1B,C). Next, we evaluated the specificity of the gene expression caused by DDX6-mediated IL-6 amplifier activation. Silencing *DDX6* specifically suppressed the expression of the NF-κB-target gene *CCL2* [27], but not the STAT3 target *STAT3* itself [28], in H4 cells (Fig. 2A). Consistently, we found that the forced expression of DDX6 enhanced the NF-κB response element and IL-6 promoter activities following TNF-α stimulation in HEK293T cells (Fig. 2B). The forced expression of helicase-dead DDX6 mutant (DDX6 E247Q) [29] enhanced those promoter activities like wild-type DDX6 (Fig. 2C). Thus, these results showed that DDX6 is involved in NF-κB activation *in vitro, while* the helicase activity of DDX6 is dispensable for DDX6-mediated NF-κB activation.

3.2. DDX6 is involved in NF-κB-mediated skin inflammation.

Next, we employed an NF-κB-mediated skin inflammation to evaluate the biological

relevance of DDX6 in IL-6 amplifier-mediated inflammation, siRNA of *DDX6*, *p65* or mock was applied to the imiquimod-induced ear swelling mouse model [30,31]. *DDX6*-siRNA treatment, similar to *p65*-siRNA application, significantly reduced imiquimod-induced ear inflammation (Fig. 3), indicating that DDX6 in non-immune cells contributes to the inflammation response by activating NF-κB *in vivo*.

3.3. DDX6 is associated with NF-κB signaling molecules.

We next investigated the molecular mechanisms that explain how DDX6 specifically activates the NF-κB pathway. shRNA-mediated silencing of *DDX6* in H4 cells (Fig. 4A) dramatically suppressed the TNF-α-induced phosphorylation of p65 (Ser536) and IκBα (Ser32/36) and IκBα degradation (Fig. 4B). Consistently, the nuclear localization of p65 was reduced in *DDX6*-silenced H4 stimulated with TNF-α for 15 and 30 min (Fig. 4C). Then, to assess whether DDX6 can associate with molecules related to NF-κB pathway activation, we performed a coimmunoprecipitation of DDX6 with molecules in the NF-κB pathway using Flag-tagged DDX6 in HEK293T cells with or without TNF-α stimulation. We found that DDX6 binds several kinds of TNF-TNFR-NF-κB signaling molecules, including IκBα, SKP1, and p65, but not TRADD, RIP, or TRAF2, independent of TNF-α stimulation (Fig. 4D). Thus, these results suggest that DDX6 mediates the activation of the NF-κB signaling pathway by accelerating the phosphorylation and nuclear localization of p65 most likely as an adaptor protein in SKP1/Cullin/F-box (SCF) complex.

4. Discussion

A shRNA-lentivirus-mediated genome-wide screening previously identified DDX6 as a candidate positive regulator of the IL-6 amplifier, which is a mechanism for the enhanced activation of NF-κB in non-immune cells [3]. Here, we investigated this possibility further. We show that silencing DDX6 in non-immune cells suppressed IL-6 amplifier activation; the forced expression of DDX6 enhanced IL-6 amplifier activation; the downregulation of DDX6 suppressed a NF-κB-mediated inflammation model in C57BL/6 mice; and DDX6 binds several TNFR-NF-κB signaling molecules, including IκBα, SKP1, and p65, but not TRADD, RIP, or TRAF2. It was reported that DDX6 is possibly an RNA helicase based on its structure [29]. Therefore, we constructed RNA helicase dead DDX6 and analyzed its reporter activity on NF-κB responsive elements. DDX6 enhanced the reporter activity on NF-κB responsive elements regardless of the RNA helicase activity, suggesting that DDX6 functions as an adaptor molecule that binds with IκBα, SKP1, and p65.

It has been reported that the DDX6 SNP rs10892286 is associated with several autoimmune diseases including SSc [22]. Furthermore, DDX6-CXCR5 on chromosome 11p23.3 has been associated with Sjögren's disease (SjD) and SLE by genome-wide association studies (GWAS) [21,32-35]. Because we previously demonstrated that the IL-6 amplifier is involved in the development of SSc, SjD and SLE [3,4,36], combined with the results here, we hypothesize that DDX6 acts on the NF-κB pathway via the IL-6 amplifier during the development of those inflammatory diseases. Although we have not investigated how the risk allele of rs10892286 is related to the development of inflammatory diseases, it may increase *DDX6* mRNA to enhance the NF-κB pathway in the development of autoimmune diseases.

It is well documented that SKP1 functions as a fundamental constituent within the SCF complex and serves as the adaptor protein, playing a crucial role in binding with CUL1 and facilitating the recruitment of various F-box proteins to assemble the SCF complex [37]. β-TrCP is also a component of the SCF complex: it functions as an E3 ubiquitin ligase, recognizing phosphorylated IκBα, and catalyzes the ubiquitination of IκBα, leading to its degradation through the SCF pathway [38,39]. Mutant forms of β-TrCP lacking the essential F-box motif, which required for the interaction with SKP1, are unable to facilitate the ubiquitination of IκBα [40,41]. Thus, mechanistically, DDX6 may facilitate the ubiquitination of IκBα to promote IκBα protein degradation through its bridging interaction with SKP1 and IκBα. Based on our findings, this mechanism may also accelerate the nuclear translocation of p65. Additionally, while the binding affinity is weak, the interaction of DDX6 with p65 implies a positive effect on the NF-κB pathway. Because we showed that Bmi1 is an adapter protein in the SCF complex, which activates the NF-κB pathway [42], it will be interesting to investigate which of DDX6 and Bmi1 in the SCF complex is critical for activation of the NF-κB pathway. In conclusion, our findings revealed DDX6 in non-immune cells may play a role in the IL-6 amplifier to enhance NF-κB signaling by acting as an adaptor protein in the SCF complex regardless of its RNA helicase activity. Therefore, the DDX6-NF-κB axis is a novel diagnostic and therapeutic target for inflammatory diseases.

CRediT authorship contribution statement

Seiichiro Naito: Investigation, Formal analysis. **Hiroki Tanaka**: Investigation. **Jiang Jing-Jing**: Investigation. **Masato Tarumi**: Investigation. **Ari Hashimoto**: Resources. **Yuki Tanaka**: Validation. **Kaoru Murakami**: Validation. **Simpei I. Kubota**: Validation. **Shintaro Hojo**: Validation. **Shigeru Hashimoto**: Project administration, Methodology, Writing- Original draft preparation. **Masaaki Murakami**: Conceptualization, Writing- Review & Editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1. *DDX6*-silencing suppresses *IL-6* expression in non-immune cells.

(A) Knockdown efficiency of a *DDX6*-specific small interfering RNA (si-*DDX6*) was evaluated by assessing *DDX6* mRNA levels in transfected H4 cells (left). *IL-6* mRNA levels were measured in mock, si-*p65* (positive control) or si-DDX6 H4 cells that were stimulated with IL-6 and TNF-α (right). (B) and (C) show *DDX6* and *IL-6* mRNA levels of HDF and HSF under the same conditions as Fig. 1A, respectively. Data represent the mean ± S.D. * $p < 0.05$, ** $p < 0.01$; ns, not significant. *In vitro* experiments were performed at least three times.

Fig. 2. DDX6 induces the IL-6 amplifier via NF-κB activation.

(A) mRNA levels for *CCL2* and *STAT3* were measured in H4 cells stimulated with IL-6 and TNF-α and transfected with mock, si-*p65* or si-*DDX6*. (B) Luciferase assay using reporter plasmids containing *IL-6* promoter (left) and NF-κB response element (right) in HEK293T cells with or without overexpressed DDX6 in the presence or absence of TNF-α stimulation. (C) Luciferase assay using NF-κB RE reporter in HEK293T cells expressing mock, wild-type or helicase-dead DDX6 expression with TNF-α stimulation. Data represent the mean ± S.D. * $p < 0.05$, ** $p < 0.01$; ns, not significant. *In vitro* experiments were performed at least three times.

Fig. 3. DDX6 is involved in NF-κB activation *in vivo*.

C57BL/6 mice were treated with imiquimod on the ears for 3 days to induce skin inflammation. Gene knockdown was performed by applying siRNA (for *DDX6*, *p65*, or control) on the ears daily between day 0 to 3. Ear thickness was measured daily, and its

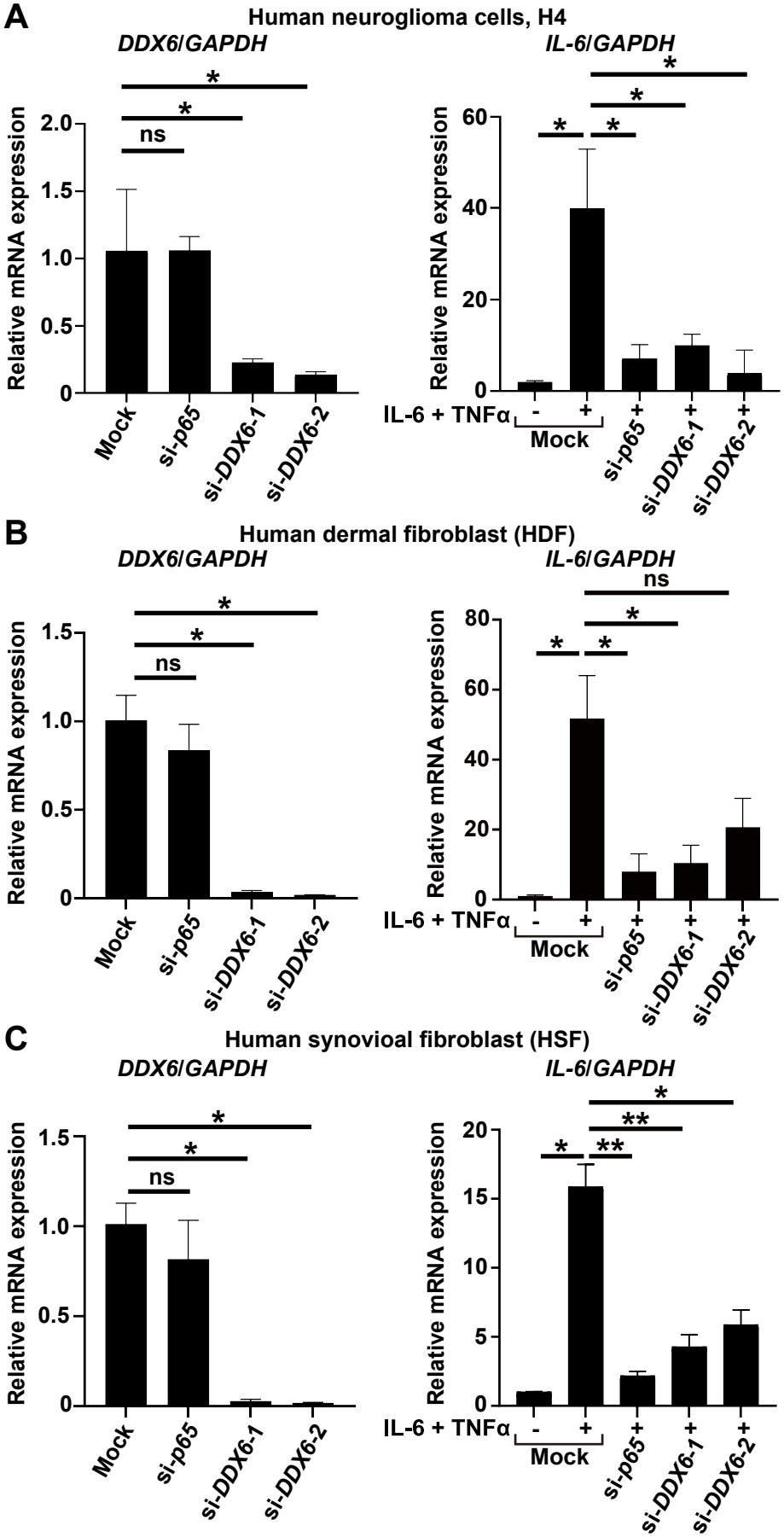
variation relative to day 0 is shown (n=6, each group). Means ± SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. DDX6 binds to NF-κB signaling molecules.

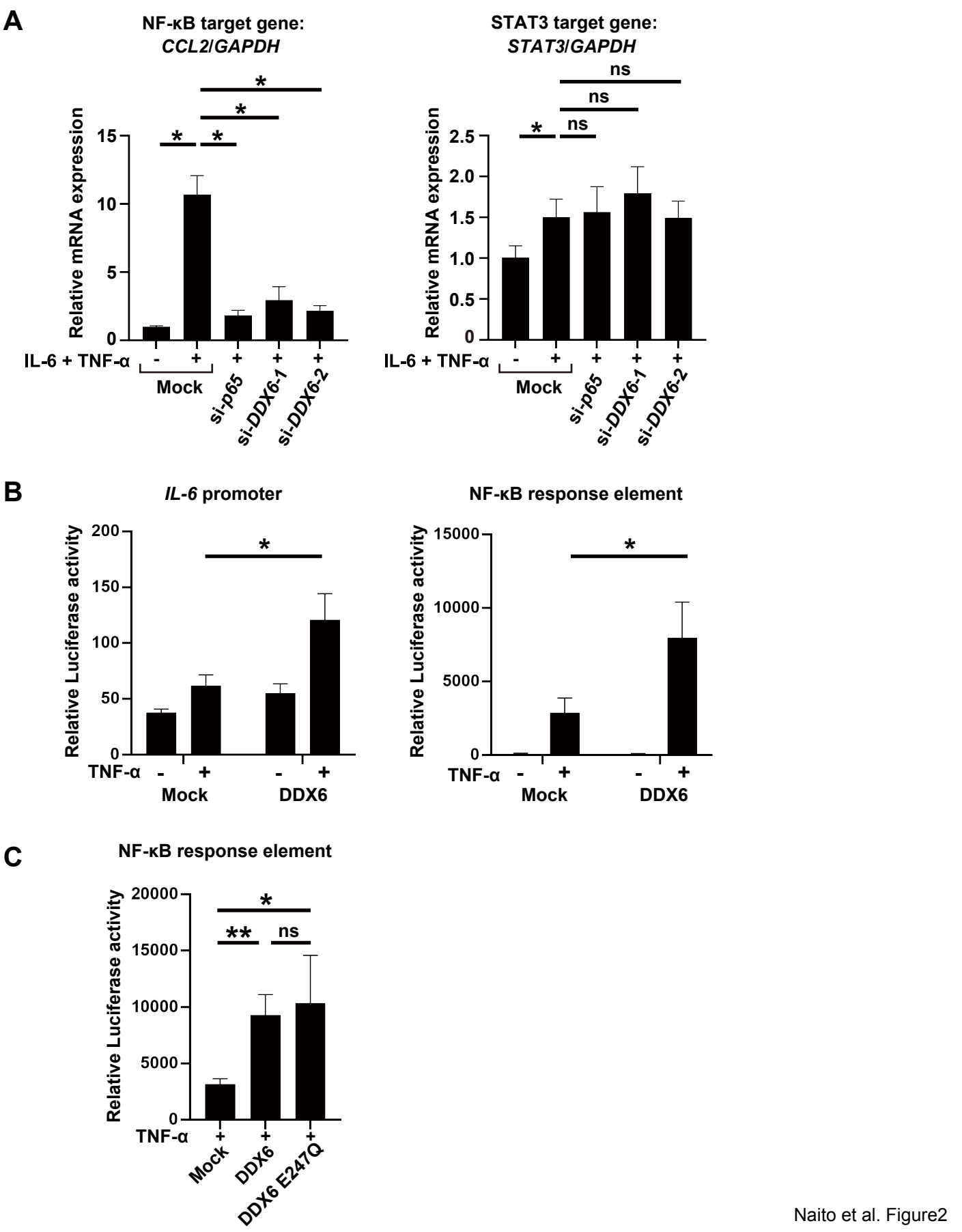
(A) *IL-6* levels were measured in H4 cells stimulated with IL-6 and TNF-α for 3 h and transfected with control shRNA (Mock) or *DDX6* shRNA (sh-*DDX6*). (B) H4 cells were treated with TNF-α for 0, 5, and 15 min. Protein levels of phosphorylated p65 (S536), p65, phosphorylated IκBα (S32/36), IκBα, DDX6, and tubulin were measured. (C) The nuclear translocation of p65 was assessed by immunofluorescence staining in H4 cells with TNF-α stimulation for 0, 15, 30, and 60 min. At least 300 cells were counted in each group. Right panels show quantification of the cellular localization. (D) Immunoprecipitation was performed on HEK293T cells expressing Flag-tagged DDX6 or empty vector with TNF-α stimulation for 5 min. IP, Immunoprecipitation; IB, Immunoblotting.

Table 1. Primer sequences for qPCR.

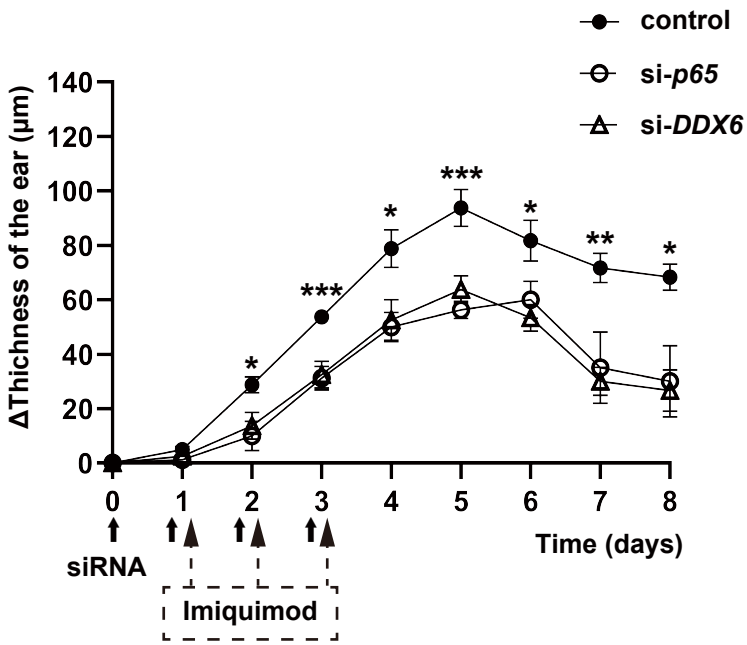
Gene	Sequence	
Human *GAPDH*	Forward	5’-GAGCAACGGAGGCG-3’
	Reverse	5’-CGCCCGGAAGAGGG-3’
Human *IL-6*	Forward	5’-GGAGACTTGCCTGGTGAAAA-3’
	Reverse	5’-GTCAGGGGTGGTTATTGCAT-3’
Human *CCL2*	Forward	5’-CAGCCAGATGCAATCAATGCC-3’
	Reverse	5’-TGGAATCCTGAACCCACTTCT-3’
Human *STAT3*	Forward	5’- ACCAGCAGTATAGCCGCTTC-3’
	Reverse	5’- GCCACAATCCGGGCAATCT-3’
Human *DDX6*	Forward	5’-CCGAAATGGCTTATGCCGCAATC-3’
	Reverse	5’-GGAGATAGGTCTCTGCCAGCTT-3’



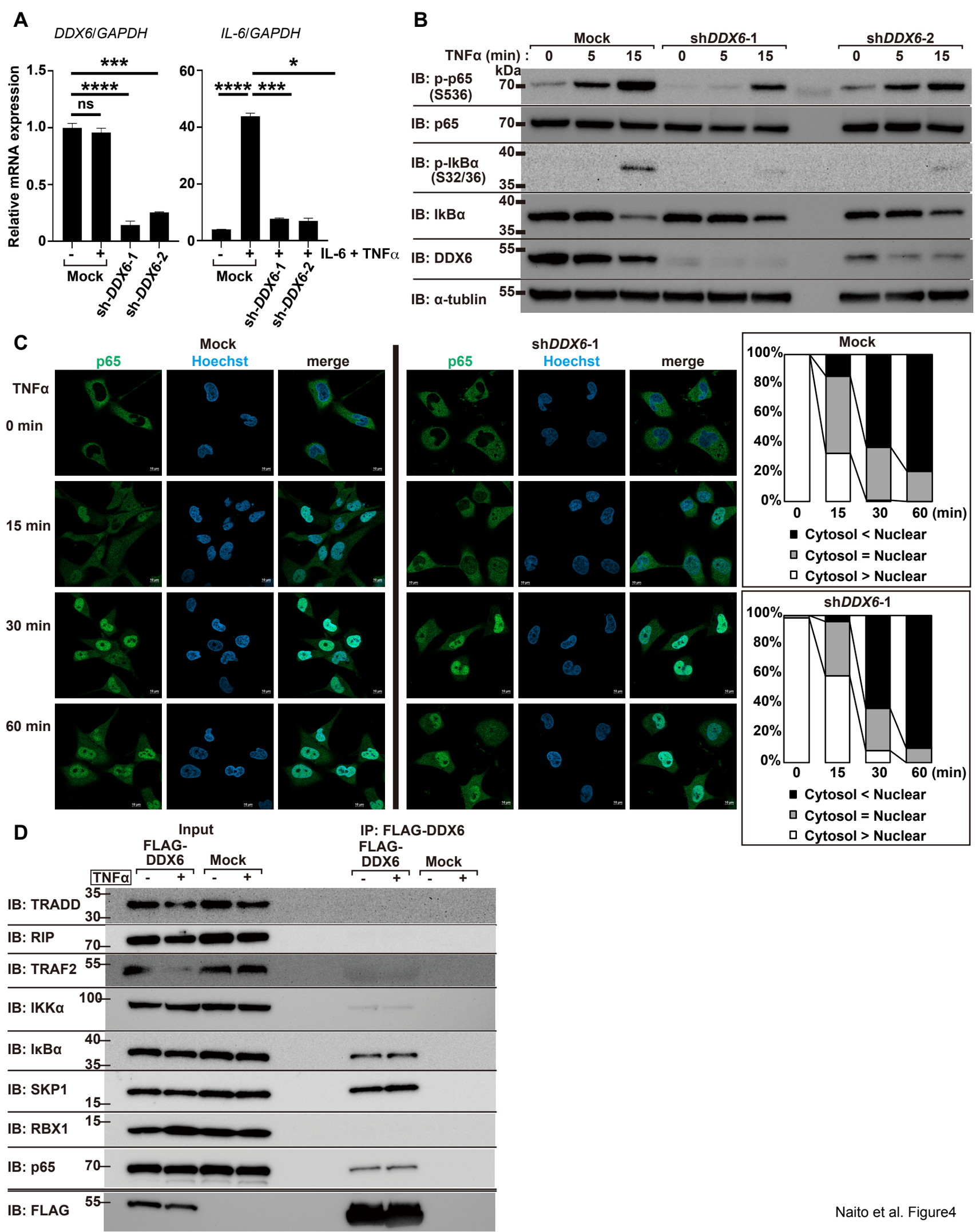
Naito et al. Figure1



Naito et al. Figure2



Naito et al. Figure3



Naito et al. Figure4