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Research Paper

## P65 controls NF- $\kappa$ B activity by regulating cellular localization of I $\kappa$ B $\beta$

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**Short title:** P65 determines localization of I $\kappa$ B $\beta$

Abbreviations used: CITE, cap-independent translation enhancer; CRM1, exportin-1; DAPI, 4',6-diamidino-2-phenylindole dilactate; HA, haemagglutinin; *ICAM1*, intercellular adhesion molecule 1 gene; I $\kappa$ B, inhibitory  $\kappa$ B; *IP-10*, inducible protein-10 gene; LMB, leptomycin B; LTR, long terminal repeats; NES, nuclear export signal; NF- $\kappa$ B, nuclear factor  $\kappa$ B; *NFKBIA*, inhibitory  $\kappa$ B  $\alpha$  gene; NLS, nuclear localization signal; P/CMV, cytomegalovirus promoter; RHD, rel homology domain; RQ, relative quantification value; SIN, self inactivating; TA, transactivation domain; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

## ABSTRACT

The nuclear factor  $\kappa$ B (NF- $\kappa$ B) controls diverse cellular processes and is frequently misregulated in chronic immune diseases or cancer. The activity of NF- $\kappa$ B is regulated by inhibitory  $\kappa$ B (I $\kappa$ B) proteins which control nuclear-cytoplasmic shuttling and DNA binding of NF- $\kappa$ B. Here, we describe a novel role of p65 as a critical regulator of the cellular localization and functions of NF- $\kappa$ B and its inhibitor I $\kappa$ B $\beta$ . In genetically modified *p65<sup>-/-</sup>* cells, the localization of ectopic p65 is not solely regulated by I $\kappa$ B $\alpha$ , but is largely dependent on the nuclear localization signal (NLS) and the nuclear export signal (NES) of p65. Furthermore, unlike I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  does not contribute to nuclear export of p65. In fact, the cellular localization and degradation of I $\kappa$ B $\beta$  is controlled by the p65 specific NLS and NES. Our data also reveal that, in addition to stimulus-induced redistribution of NF- $\kappa$ B, changes in the constitutive localization of p65 and I $\kappa$ B $\beta$  specifically modulate activation of inflammatory genes. This is a consequence of differences in the DNA binding activity and signal responsiveness between the nuclear and cytoplasmic NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes. Together, our findings indicate that the p65 subunit controls transcriptional competence of NF- $\kappa$ B by regulating the NF- $\kappa$ B/I $\kappa$ B $\beta$  pathway.

**Key words:** Complex, gene expression, cellular localization, inhibitory  $\kappa$ B  $\beta$  (I $\kappa$ B $\beta$ ), nuclear factor  $\kappa$ B (NF- $\kappa$ B), tumour necrosis factor  $\alpha$  (TNF $\alpha$ )

## INTRODUCTION

The nuclear factor  $\kappa$ B is a homo- or heterodimeric inducible transcription factor composed of several mammalian Rel proteins: p65 (RelA), p50 (NF $\kappa$ B1), p52 (NF $\kappa$ B2), c-Rel, and RelB [1]. It controls various genes implicated in immune and stress responses, apoptosis, cell proliferation, and differentiation [2]. To prevent aberrant activation of NF- $\kappa$ B, as it is often seen in inflammatory diseases or cancer [3,4], specific regulation of its transcriptional activity is essential. Controlled cellular localization of NF- $\kappa$ B is a critical mechanism of this regulation [5,6]. In most cells, NF- $\kappa$ B is retained in the cytoplasm by physical association with one of several I $\kappa$ B proteins. The family of I $\kappa$ Bs includes canonical I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  proteins, p50/p105 and p52/p100 precursors, as well as nuclear forms I $\kappa$ B $\zeta$  and Bcl-3 [5]. I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  predominantly interact with ubiquitous forms of NF- $\kappa$ B containing p65, and modulate p65 functions in virtually all tissues [7,8]. The general role of I $\kappa$ Bs in the regulation of NF- $\kappa$ B signalling is well established. However, the molecular mechanisms that determine localization and activity of NF- $\kappa$ B/I $\kappa$ B complexes are not well understood.

It has been established that NF- $\kappa$ B and I $\kappa$ B $\alpha$  continuously shuttle between the nucleus and the cytoplasm. A steady-state cytoplasmic localization of inactive NF- $\kappa$ B/I $\kappa$ B $\alpha$  is achieved due to the dominant nuclear export function of I $\kappa$ B $\alpha$  that shifts dynamic equilibrium of the shuttling [9-11]. Stimuli-induced degradation of I $\kappa$ B $\alpha$  releases NF- $\kappa$ B allowing its rapid accumulation in the nucleus mediated by the NLS activities of NF- $\kappa$ B subunits [12-15]. Nuclear NF- $\kappa$ B binds to  $\kappa$ B DNA consensus sequences and activates a specific subset of genes. An important consequence of the NF- $\kappa$ B activation is an induction of the *NFKBIA* gene by NF- $\kappa$ B and re-synthesis of the I $\kappa$ B $\alpha$  protein. Newly synthesized I $\kappa$ B $\alpha$  dissociates NF- $\kappa$ B from DNA and promotes its nuclear export, thereby providing a negative regulatory feedback mechanism that critically influences the duration of the NF- $\kappa$ B response [16-18].

The regulatory interplay between NF- $\kappa$ B and I $\kappa$ B $\beta$ , a homolog of I $\kappa$ B $\alpha$ , is less clear and a potential feedback mechanism has not been described. In contrast to I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  shows stimuli-induced degradation that is characterized by a slow kinetics [19]. I $\kappa$ B $\beta$  is also not rapidly re-synthesized in a NF- $\kappa$ B dependent manner as described for I $\kappa$ B $\alpha$ . Depending on the cell type or stimulus, I $\kappa$ B $\beta$  may instead undergo persistent degradation, leading to prolonged NF- $\kappa$ B activation [20]. Persistent NF- $\kappa$ B activity is also mediated by a hypophosphorylated I $\kappa$ B $\beta$  that forms a stable complex with NF- $\kappa$ B in the nucleus [21]. Basal phosphorylation of carboxyl-terminal PEST domain in I $\kappa$ B $\beta$  inhibits NF- $\kappa$ B DNA binding and is thought to be primarily responsible for the formation of inactive NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes. The main question here is how these latent NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes are transported back to the cytoplasm when the NF- $\kappa$ B response is completed. Unlike I $\kappa$ B $\alpha$ , which contains a non-conventional nuclear import sequence [22,23] and a classical NES, I $\kappa$ B $\beta$  lacks both of these elements. This strongly argues that I $\kappa$ B $\beta$  cannot, on its own, provide nuclear import or export functions. It has been demonstrated that cytoplasmic  $\kappa$ B-Ras protein interacts with the latent NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes and prevents their nuclear import in resting cells [24]. However, this finding cannot explain how cytoplasmic localization of the NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes is achieved in a post-induction period. Furthermore, it has been demonstrated that  $\kappa$ B-Ras binds to only a fraction of the NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes [25]. Thus, it is necessary to clarify the mechanisms that regulate cellular distribution of the NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes which do not bind to  $\kappa$ B-Ras. The contribution of other proteins to the control of the I $\kappa$ B $\beta$  localization may provide an explanation.

Here, we report that cellular localization of NF- $\kappa$ B and I $\kappa$ B $\beta$  is controlled by the NLS and NES of p65. Furthermore, we show that the constitutive nuclear localization of NF- $\kappa$ B and

I $\kappa$ B $\beta$  mediated by p65 modulates the basal and the cytokine-induced activation of inflammation associated *IP-10* and *ICAM1* genes. The changes in gene expression correlated with differences in the DNA binding activity of NF- $\kappa$ B and the stimulus-induced degradation of I $\kappa$ B $\beta$  observed for cytoplasmic and nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$ . Together, these findings reveal a novel role of p65 in the regulation of the NF- $\kappa$ B/I $\kappa$ B $\beta$  pathway.

## EXPERIMENTAL

### Antibodies

Anti-p65(A) (sc-109), anti-p65(C20) (sc-372), anti-I $\kappa$ B $\alpha$ (H-4) (sc-1643), and anti-I $\kappa$ B $\beta$ (C20) (sc-945) (Santa Cruz), anti-HA(HA.11) (Covance), anti- $\alpha$ -tubulin (Sigma), HRP-conjugated anti-rabbit and anti-mouse (Sigma), Cy3-conjugated anti-rabbit (Jackson Immunology), and FITC-conjugated anti-mouse (Covance) antibodies were used for immunological applications.

### Plasmids

A myc-tagged *p65WT* was cloned into retroviral pRRL vector [26] by substituting the *GFP* gene. A cap-independent translation enhancer (*CITE*) sequence fused to an antibiotic resistance *bsd* gene was introduced into *SalI* site immediately downstream of *p65* coding sequence. To obtain a final pRRL-myc-p65WT plasmid, a woodchuck hepatitis virus posttranscriptional regulatory element (*WPRE*) sequence [27] was introduced into *EcoRI* downstream of *bsd*. pRRL-myc-p65NLS<sub>mut</sub>, pRRL-myc-p65NES<sub>mut</sub> and pRRL-myc-p65NLS/NES<sub>mut</sub> were generated by site-directed mutagenesis according to the QuickChange protocol (Stratagene). Deletion mutants RHDL<sub>+NES</sub> and RHDL<sub>-NES</sub> were generated by PCR using primers 5'-GCGGATCCACCATGGAGCAGAAGCTGATCAGCGAGGAGGACCTG-ATGGACGAACTGTTCCCCCTC-3' / 5'-TCGCTCGAGTTACAGGTCTTCATCATCAA-CTGC-3' and 5'-TCGCTCGAGTTACAGGGCCTCTGACAGCG-3', respectively. Control plasmid contains the *CITE/bsd* insert instead of the *GFP* gene. The pRRL-HA-I $\kappa$ B $\beta$  plasmid was generated by cloning a PCR fragment coding for the human HA-tagged I $\kappa$ B $\beta$ 1 isoform [28] into *BamHI* and *SalI* sites of pRRL containing *CITE/pac* and *WPRE* sequences downstream of the insert. To generate the pLPCX-HA-I $\kappa$ B $\alpha$  plasmid, human I $\kappa$ B $\alpha$  cDNA was amplified by PCR as HA-tagged version and cloned into *BglII* and *SalI* of pLPCX vector (Clontech). The envelope plasmid pMD.G and the packaging plasmid pCMV- $\Delta$ R8.91 have previously been described [29].

### Cells and Retroviral Transduction

*P65*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) have been described elsewhere [30]. Cells were cultured in D-MEM medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin/streptomycin and non-essential amino acids at 37°C and 5% CO<sub>2</sub>. Virus production and transduction of cells were performed as described previously [29]. Infected cells were selected using blasticidin S (2.5  $\mu$ g/ml) or puromycin (3  $\mu$ g/ml).

### Immunoblot Assay

For immunoblotting, cells were washed with PBS and lysed in ice-cold NP-40 lysis buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin). The lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Whatman), and immunoprobed with the indicated antibodies according to manufacturers' recommendations. Enhanced chemiluminescence detection (Pierce) was used in immunoblot assays.

### Immunofluorescence

MEFs were plated at a density of  $4.5 \times 10^4$  cells per chamber on poly-l-lysine-coated chamber slides (Lab-Tek) and incubated overnight at 37°C and 5% CO<sub>2</sub>. The next day, cells were starved in serum free medium for 14 h and stimulated with TNF $\alpha$  (20 ng/ml) for the indicated times. To inhibit nuclear export, leptomycin B (40 ng/ml) was added to the cell medium for the last 4 h of starvation. For immunofluorescent detection of proteins, cells were fixed in 4% paraformaldehyde for 10 min. After permeabilization in PBS containing 0.2% Triton-X-100 for 5 min and blocking in 0.5% BSA for 20 min, slides were incubated with primary (1:300) antibodies followed by Cy3- or FITC-conjugated secondary antibodies (1:250). DNA was stained with a solution of PBS containing 300 nM DAPI for 5 min. The chamber slides were rinsed with PBS, air-dried, and mounted with Vectashield medium (Vector Lab). Immunofluorescence was analyzed with fluorescence microscope Olympus BX51 and images were captured using Olympus DP71 CCD camera. The representative images were used to determine the ratio of nucleus-to-cytoplasm fluorescence using Olympus Soft Imaging System (Cell B).

### Quantitative Real-Time PCR

Total RNA was isolated with a RiboPure kit (Ambion) and reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturers' recommendations. Quantitative real-time PCR was performed using mouse *NFKBIA*-, *IP-10*-, *ICAM1*-, and  $\beta$ -*actin*-specific primers and SYBR Green PCR Core Reagents (Applied Biosystems) with subsequent detection of the melting curves by StepOne Real-Time PCR System (Applied Biosystems). Results were normalized to  $\beta$ -*actin* expression and quantified using StepOne Software (v2.0) (Applied Biosystems).

### Electrophoretic Mobility Shift Assay (EMSA)

To prepare nuclear extracts for EMSA, cells were starved for 14 h and treated with TNF $\alpha$  (10 ng/ml) for 20 min or left untreated. Cell fractionation was performed as previously described [31]. Nuclear extracts (5  $\mu$ g) were incubated with a <sup>32</sup>P-labelled DNA probe [31] in 20  $\mu$ l of buffer (10 mM Tris-HCl pH 7.5, 65 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, 0.1  $\mu$ g/ $\mu$ l BSA, 10% glycerol, 0.05  $\mu$ g/ $\mu$ l poly dI-dC) for 45 min at room temperature. Supershift was produced by adding 1  $\mu$ g of anti-p65(C-20) antibody to the mixture 5 min prior to loading. Samples were subjected to 4% PAGE and analyzed by autoradiography. Densitometric analysis was performed on digitized images using ImageQuant 5.2 software.

## RESULTS

### Constitutive and post-induction localization of p65 is regulated by its intrinsic NLS and NES activities

To study the role of p65 specific NLS and NES, *p65*<sup>-/-</sup> MEFs were genetically complemented with the wild-type or mutated p65 proteins by retroviral transduction. The mutants include full-length p65 mutated in NLS and/or NES (*p65NLS*<sub>mut</sub>, *p65NES*<sub>mut</sub>, and *p65NLS/NES*<sub>mut</sub>) as well as C-terminal deletion variants (*RHDL*<sub>+NES</sub> and *RHDL*<sub>-NES</sub>) lacking the transactivation domain (Fig. 1A). The use of genetically modified *p65*<sup>-/-</sup> cells excludes dimerization of the mutants with endogenous p65 that can mask or reduce any possible effects of the mutations. The expression level of the recombinant full-length p65 proteins in complemented *p65*<sup>-/-</sup> MEFs was comparable to that of the endogenous p65 in normal mouse embryonic fibroblasts (Fig.1B and S1A). The ectopic expression of p65WT or mutants restored the level of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in the cells (Fig. 1B). This suggested that the ectopic p65 proteins were able to

activate transcription of the *NFKBIA* gene [17,18] and/or to enhance stability of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  [32,33], the functions previously assigned to endogenous p65. The relevance of the complementation has further been confirmed by revealing comparable TNF $\alpha$ -induced degradation and re-synthesis of I $\kappa$ B $\alpha$ , nuclear-cytoplasmic re-distribution of the endogenous and ectopic p65, and activation of the *NFKBIA* and *IP-10* genes in the *p65*<sup>+/+</sup> and *p65*<sup>-/-</sup> MEFs expressing ectopic p65WT (Fig. S1 and S2).

Immunofluorescence microscopy of the quiescent and TNF $\alpha$ -stimulated *p65*<sup>-/-</sup> cells transduced with virally encoded p65 proteins revealed that mutation of the NLS and/or NES of p65 substantially changed steady-state and/or induced cellular distribution of the ectopic proteins (Fig. 2A and C). We observed elevated nuclear staining of p65NES<sub>mut</sub> in unstimulated cells whereas the wild-type, NLS and NLS/NES mutants of p65 were predominantly localized in the cytoplasm under similar conditions. The estimated nucleus-to-cytoplasm fluorescence ratio for p65NES<sub>mut</sub> was approximately 2.3 times higher than that observed for p65WT despite comparable expression of the I $\kappa$ B proteins (Fig. 2D and C). When cells were treated with TNF $\alpha$ , p65WT and p65NES<sub>mut</sub> efficiently accumulated in the nucleus displaying similar nucleus-to-cytoplasm ratios after 20 min of TNF $\alpha$  stimulation. This suggested that only a fraction of p65NES<sub>mut</sub> or its specific complexes was constitutively nuclear, and that the cytoplasmic portion of the protein could be induced to accumulate in the nucleus. Further analysis of the TNF $\alpha$ -stimulated cells revealed that, in contrast to p65WT, a post-induction nuclear export of p65NES<sub>mut</sub> was less efficient and delayed in time. After 60 min of TNF $\alpha$  stimulation, the ratios of nuclear-to-cytoplasm fluorescence specific for p65WT and p65NES<sub>mut</sub> were 0.35 and 0.86, respectively. Since degradation and re-synthesis of I $\kappa$ B $\alpha$  were similar in both cell lines, we concluded that nuclear export of NF- $\kappa$ B in the post-induction phase was dependent on the NES of p65 and could not be attributed to the NES function of I $\kappa$ B $\alpha$  alone. In agreement with others [12,14], we found that the induced nuclear accumulation of p65 required the presence of an intact NLS in the protein. Mutation of the NLS in p65 substantially reduced its nuclear import despite the normal kinetics of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  degradation. Moreover, inhibition of the nuclear import of p65 was accompanied by a reduction of the post-induction re-synthesis of I $\kappa$ B $\alpha$ . Simultaneous mutation of the NLS and NES elements in p65 partially restored the re-synthesis of I $\kappa$ B $\alpha$ , suggesting that the NF- $\kappa$ B response may depend on a ratio of its nuclear-cytoplasmic shuttling determined by the intrinsic NLS and NES activities of the p65 subunit (Fig. 2A and D).

Expression of RHDL<sub>+NES</sub> and RHDL<sub>-NES</sub> in the *p65*<sup>-/-</sup> cells restored a steady-state level of I $\kappa$ B $\alpha$  but prevented the p65-dependent re-synthesis of the inhibitor during a course of TNF $\alpha$  stimulation (Fig. 2E). This allowed us to investigate TNF $\alpha$ -induced cellular distribution of the ectopic proteins without I $\kappa$ B $\alpha$ -mediated nuclear import/export. Similar to p65NES<sub>mut</sub>, RHDL<sub>-NES</sub> displayed increased nuclear staining in unstimulated cells (Fig. 2B and C). Its NES proficient counterpart RHDL<sub>+NES</sub> exhibited a cellular distribution comparable to that of p65WT, e.g. was found to localize predominantly in the cytoplasm. In response to TNF $\alpha$ , RHDL<sub>-NES</sub> translocated into the nucleus reaching a nuclear-to-cytoplasm ratio of 1.3. By contrast, RHDL<sub>+NES</sub> could not efficiently accumulate in the nucleus, with a large portion of the protein localizing in the cytoplasm at all time points of TNF $\alpha$  stimulation. The maximal ratio of the nuclear-to-cytoplasm fluorescence detected for this mutant was only 0.6. These data suggest that the NES of p65 operates to maintain nuclear export of the protein independently of I $\kappa$ B $\alpha$ . Despite the presence of NES in RHDL<sub>+NES</sub>, the protein was detected in both cytoplasmic and nuclear compartments upon TNF $\alpha$  treatment, indicating that NES can not fully compensate for the NLS function in this mutant. We therefore conclude that predominantly cytoplasmic localization of the p65 protein requires the intact NES of p65 and

presence of I $\kappa$ B $\alpha$ . This is in agreement with previous findings suggesting the role of I $\kappa$ B $\alpha$ -specific NES in the regulation of nuclear-cytoplasmic shuttling of NF- $\kappa$ B/I $\kappa$ B $\alpha$  [9-11]. When the NES function of p65 and re-synthesis of I $\kappa$ B $\alpha$  are compromised, as in the case of RHDL<sub>NES</sub>, the protein remains predominantly nuclear over the entire period of TNF $\alpha$  stimulation (Fig. 2B and C). Remarkably, the expression of I $\kappa$ B $\beta$  was not abrogated in the cells complemented with the deletion mutants of p65. However, the presence of I $\kappa$ B $\beta$  could not compensate for a loss of I $\kappa$ B $\alpha$  and prevent nuclear accumulation of RHDL<sub>NES</sub> in the TNF $\alpha$ -treated cells. Thus, we hypothesized that the expression of I $\kappa$ B $\beta$  is not sufficient for the nuclear export of NES-deficient p65 mutants.

### **P65 controls cellular localization of I $\kappa$ B $\beta$**

Next we investigated the effects of the p65 mutations on the localization of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  and their complexes. The cells were infected with viruses encoding HA-tagged I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$ . This enhanced expression of the individual I $\kappa$ B isoforms and their specific NF- $\kappa$ B/I $\kappa$ B complexes in the cells (Fig. 3A and S3). Immunofluorescent staining for p65 and the two I $\kappa$ Bs revealed that both p65 and I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  proteins were mainly cytoplasmic when p65WT was used (Fig. 3B). TNF $\alpha$ -induced degradation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  allowed p65WT to accumulate in the nucleus. At the later time points of the treatment, ectopic I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  were re-synthesized from a constitutive viral promoter and co-stained with the p65WT predominantly in the cytoplasm. Similar cytoplasmic localization of I $\kappa$ B $\alpha$  was observed in unstimulated or TNF $\alpha$ -treated cells complemented with either p65NES<sub>mut</sub>, p65NLS<sub>mut</sub> or p65NLS/NES<sub>mut</sub> suggesting that the deficiency in NLS and/or NES of p65 did not change cellular distribution of I $\kappa$ B $\alpha$ . Interestingly, inactivation of the NES in p65 resulted in a constitutive nuclear staining of both p65 and I $\kappa$ B $\beta$  (Fig. 3B and S3). An enriched nuclear presence of p65NES<sub>mut</sub> and I $\kappa$ B $\beta$  was also observed in TNF $\alpha$ -treated cells suggesting that at least a fraction of NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes is regulated by the NES of p65. The use of the truncated RHDL<sub>+NES</sub> and RHDL<sub>-NES</sub> mutants (Fig. S3) revealed that the effect of the p65 NES dysfunction on I $\kappa$ B $\beta$  was not dependent on I $\kappa$ B $\alpha$ . Thus, our results indicate that the NES of p65 is required to maintain predominantly cytoplasmic localization of I $\kappa$ B $\beta$ . By contrast, p65NLS<sub>mut</sub> or p65NLS/NES<sub>mut</sub> were found to co-localize with I $\kappa$ B $\beta$  mainly in the cytoplasm under all conditions tested. This indicates that inactivation of the NLS in p65 prevents constitutive nuclear accumulation of p65 and I $\kappa$ B $\beta$ , the phenotype observed for the p65NES<sub>mut</sub> expressing cells. Based on this, we assume that the NLS of p65 is also involved in the regulation of the I $\kappa$ B $\beta$  compartmentalization. The mutual effect of the p65 mutants and I $\kappa$ B $\beta$  on their cellular localization suggests that formation of a complex between p65 and I $\kappa$ B $\beta$  is required for this phenomenon. This was confirmed by co-immunoprecipitation experiments demonstrating that p65WT and all mutants were able to form I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  specific complexes (Fig. S4).

### **Nuclear-cytoplasmic shuttling of p65 and I $\kappa$ B $\beta$**

Since the mutation of the NLS and/or NES of p65 influenced localization of I $\kappa$ B $\beta$ , we assumed that the NF- $\kappa$ B/I $\kappa$ B $\beta$  complex may shuttle between the nucleus and the cytoplasm. To explore this possibility, the unstimulated cells were treated with LMB, an inhibitor of CRM1-mediated nuclear export. Within 4 h of LMB treatment, both p65WT and I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  accumulated in the nucleus (Fig. 4). This indicates that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , and their complexes shuttle between the nucleus and the cytoplasm. Mutation of the NLS or NES of p65 did not substantially change the ability of p65 and I $\kappa$ B $\alpha$  to be trapped by LMB in the

nucleus. However, inactivation of the NLS in p65 reduced LMB-mediated nuclear accumulation of p65 and I $\kappa$ B $\beta$  suggesting that NF- $\kappa$ B/I $\kappa$ B $\alpha$  and NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes may possess a different rate of nuclear import. It is also evident from our results that NLS from other proteins may contribute to nuclear import of NF- $\kappa$ B/I $\kappa$ B $\alpha$  and NF- $\kappa$ B/I $\kappa$ B $\beta$  when p65 specific NLS is mutated. Those NLS may originate from other Rel proteins present in NF- $\kappa$ B/I $\kappa$ B $\alpha$  and NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes (Fig. S4).

### Constitutive nuclear localization of p65 and I $\kappa$ B $\beta$ modulates activity of NF- $\kappa$ B

To explore the role of NF- $\kappa$ B and I $\kappa$ B $\beta$  shuttling between the nucleus and the cytoplasm, we analyzed possible effects of changes in a steady-state localization of p65 and I $\kappa$ B $\beta$  on gene expression. Since mutation of the NES in p65 shifted both p65 and I $\kappa$ B $\beta$  to the nucleus, we compared basal and TNF $\alpha$ -induced expression of p65-dependent genes in cells ectopically expressing I $\kappa$ B $\beta$  and p65WT or p65NES<sub>mut</sub> (Fig. 5). Quantitative RT-PCR of the *IP-10* and *ICAM1* gene products revealed that their basal expression was about 1.6 times higher in the cells expressing constitutive nuclear p65NES<sub>mut</sub>/I $\kappa$ B $\beta$  complex when compared to control cells. Although nuclear retention of p65NES<sub>mut</sub> and I $\kappa$ B $\beta$  elevated a basal NF- $\kappa$ B activity, it was not sufficient to trigger the full transcriptional activation by NF- $\kappa$ B. As shown in Fig. 5, the expression of *IP-10* and *ICAM1* was remarkably stimulated by TNF $\alpha$ . Interestingly, the induced expression of these genes was reduced by up to 50% in cells expressing nuclear p65NES<sub>mut</sub>/I $\kappa$ B $\beta$  when compared to control cells. This suggests that nuclear localization of p65 and I $\kappa$ B $\beta$  renders their complex less responsive to TNF $\alpha$  stimulation. At the same time, the expression of the *NFKB1A* gene did not differ significantly in those cells (Fig. 5), indicating that only a subset of genes is dependent on a steady-state localization of p65 and I $\kappa$ B $\beta$ . Involvement of other complexes, e.g. NF- $\kappa$ B/I $\kappa$ B $\alpha$ , in the regulation of *NFKB1A* may be an explanation. Similar results have been observed in the p65WT or p65NES<sub>mut</sub> complemented cells without ectopic expression of I $\kappa$ B $\beta$  (Fig. S5).

Analysis of the NF- $\kappa$ B DNA binding activity in the nuclear extracts of both cell lines revealed that the basal level of DNA binding of p65NES<sub>mut</sub> was elevated when compared to p65WT (Fig. 6A). By contrast, the TNF $\alpha$ -induced DNA binding was higher in cells expressing p65WT. This correlated well with differences observed for the basal and induced expression of *IP-10* and *ICAM1* in those cell lines. The DNA binding of NF- $\kappa$ B is regulated by the I $\kappa$ B component of the NF- $\kappa$ B/I $\kappa$ B complexes [34]. Since degradation of I $\kappa$ B is required for the NF- $\kappa$ B activation in response to various stimuli [13,19], we studied TNF $\alpha$ -induced degradation of I $\kappa$ B $\beta$  in cells expressing predominantly cytoplasmic or nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes (Fig. 6B). Degradation of ectopic (Fig.6B) as well as endogenous I $\kappa$ B $\beta$  proteins (Fig.2D) was reduced in cells expressing p65NES<sub>mut</sub> compared to control cells. Hence, nuclear retention of I $\kappa$ B $\beta$  protected it from TNF $\alpha$ -induced degradation that correlated with attenuation of the *IP-10* and *ICAM1* expression. A similar resistance to signal-induced degradation has previously been reported for the nuclear fraction of I $\kappa$ B $\alpha$  [35].

## DISCUSSION

Changes in localization and processing of key NF- $\kappa$ B/I $\kappa$ B complexes are important regulatory steps in NF- $\kappa$ B signalling. Previous studies have implicated a critical role of I $\kappa$ B $\alpha$  in the control of nuclear-cytoplasmic distribution of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complexes. The importance of the CRM1-dependent nuclear export function of I $\kappa$ B $\alpha$  in maintaining predominantly cytoplasmic localization of inactive NF- $\kappa$ B has been demonstrated [9-11]. Although the proposed mechanism clarifies the function of I $\kappa$ B $\alpha$ , it does not explain all aspects of the NF-

$\kappa$ B regulation. For instance, it cannot be applied to the regulation of NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes, as none of the known NES sequences are found in I $\kappa$ B $\beta$ . Recent studies have demonstrated that the regulation of NF- $\kappa$ B/I $\kappa$ Bs is much more complex and may require a functional interplay between NF- $\kappa$ B and I $\kappa$ B proteins. The crystal structure of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  and NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes reveals that the NLS of one NF- $\kappa$ B subunit does not contact I $\kappa$ B and is mostly solvent-exposed suggesting its possible contribution to the nuclear import of the complexes [36-38]. Furthermore, a putative NES has been identified in p65 but any functional relevance of this element in NF- $\kappa$ B/I $\kappa$ B complexes remained to be investigated [39]. Hence, it is possible that NF- $\kappa$ B itself is critically involved in the cellular localization of NF- $\kappa$ B/I $\kappa$ B complexes.

In this study we have addressed an essential role of the p65 subunit of NF- $\kappa$ B in the regulation of localization and functions of NF- $\kappa$ B and its inhibitor I $\kappa$ B $\beta$ . We genetically reconstituted expression of p65 or its mutants in p65<sup>-/-</sup> MEFs and demonstrated that both NLS and NES of p65 are essential for the regulation of its nuclear-cytoplasmic distribution before and after TNF $\alpha$  stimulation. Mutation of the p65 NES enriched constitutively the nuclear fraction of NF- $\kappa$ B which likely represented nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes, as shown in our later experiments. However, the main fraction of p65NES<sub>mut</sub> remained cytoplasmic and co-localized with I $\kappa$ B $\alpha$ . Although I $\kappa$ B $\alpha$  retained a large portion of NES deficient p65 in the cytoplasm of unstimulated cells, it failed to rapidly export this mutant from the nucleus after TNF $\alpha$  treatment suggesting a contribution of the p65 NES to the termination of the NF- $\kappa$ B response. Interestingly, the activity of the p65 NES appears to be essential but not sufficient for the termination of the nuclear localization of NF- $\kappa$ B in a post-induction period. Despite the presence of NES in RHD<sub>L+NES</sub>, it was detected in both nuclear and cytoplasmic compartments when I $\kappa$ B $\alpha$  re-synthesis was compromised in TNF $\alpha$ -treated cells. Thus, we propose that p65 and I $\kappa$ B $\alpha$  operate together to rapidly terminate NF- $\kappa$ B activation and to restore the cytoplasmic pool of inactive NF- $\kappa$ B/I $\kappa$ B $\alpha$ . This statement is in a good agreement with studies indicating that the NES of p65 is located outside of the region involved in the binding to I $\kappa$ B $\alpha$  [40]. It is likely that the NES of p65 is exposed and accessible for the nuclear export protein CRM1 when NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex is formed. Inactivation of the NLS in p65 not only reduced nuclear import of NF- $\kappa$ B in response to TNF $\alpha$  but also prevented the phenotype observed for the p65NES<sub>mut</sub>. This suggests that the NF- $\kappa$ B response is determined by the ratio of its intrinsic NLS and NES activities.

I $\kappa$ B $\beta$  has been shown to rescue the lethal phenotype observed for I $\kappa$ B $\alpha$ -deficient mice when it was expressed under the control of the promoter and regulatory sequence of the *NFKBIA* gene [41]. These data indicate that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  share significant similarities in their biochemical activities and may possess overlapping functions. Surprisingly, we found that I $\kappa$ B $\beta$  could not substitute for I $\kappa$ B $\alpha$  when the NES of p65 was mutated. In the absence of I $\kappa$ B $\alpha$ , newly re-synthesized I $\kappa$ B $\beta$  did not promote nuclear export of RHD<sub>L-NES</sub> at the late time points of TNF $\alpha$  stimulation. Furthermore, in contrast to I $\kappa$ B $\alpha$  which co-localized with p65NES<sub>mut</sub> in the cytoplasm, I $\kappa$ B $\beta$  was predominantly nuclear when co-expressed with p65NES<sub>mut</sub>. Our results therefore suggest that, unlike I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  is not directly involved in nuclear export of p65. In fact, we have shown that the cytoplasmic localization of I $\kappa$ B $\beta$  is mediated by the NES of p65. Another important observation is that the NLS of p65 participates in the nuclear import of I $\kappa$ B $\beta$  supporting our hypothesis that p65 controls NF- $\kappa$ B/I $\kappa$ B $\beta$  compartmentalization.

A previous report indicates that the NES of p65 is sensitive to LMB which is a specific inhibitor of the nuclear export receptor CRM1 [39]. Given the role of the NLS and NES of p65 in the cellular localization of I $\kappa$ B $\beta$  it is logical to assume that complexes containing p65

and I $\kappa$ B $\beta$  may shuttle between the nucleus and the cytoplasm in a CRM1-dependent manner. Indeed, incubation of cells in the presence of LMB led to nuclear accumulation of a substantial portion of the p65 and I $\kappa$ B $\beta$  proteins indicating their nuclear-cytoplasmic shuttling. This observation contrasts with previous reports suggesting that I $\kappa$ B $\beta$ , but not I $\kappa$ B $\alpha$ , functions as a static cytoplasmic inhibitor of NF- $\kappa$ B which does not undergo dynamic shuttling [42,43]. The discrepancies could be explained by the differences in experimental settings, e.g. by the distinct time of LMB treatment. Similar discrepancies caused by different conditions of LMB treatment have been reported for NIK and I $\kappa$ B $\epsilon$ . Both proteins were initially identified as non-shuttling proteins [10,43], but later they were shown to shuttle between the nucleus and the cytoplasm using a prolonged LMB treatment [44,45]. It is likely that NF- $\kappa$ B/I $\kappa$ B $\beta$  is characterized by a slower rate of nuclear import when compared to the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex. Hence, a short treatment with LMB used in the previous studies would be sufficient to trap NF- $\kappa$ B/I $\kappa$ B $\alpha$  in the nucleus whereas NF- $\kappa$ B/I $\kappa$ B $\beta$  would still remain predominantly cytoplasmic. We have demonstrated that inactivation of the NLS of p65 reduced LMB-mediated nuclear staining of p65 and I $\kappa$ B $\beta$ , but not I $\kappa$ B $\alpha$ , suggesting that NF- $\kappa$ B/I $\kappa$ B $\alpha$  and NF- $\kappa$ B/I $\kappa$ B $\beta$  possess a different rate of nuclear import. It has previously been shown that signalling molecules of the NF- $\kappa$ B pathway constitutively shuttle between the nucleus and the cytoplasm exhibiting distinct kinetics of nuclear import [44]. For example, I $\kappa$ B $\alpha$  showed a rapid nuclear accumulation and it was detected in the nucleus after 30 min of LMB treatment. In contrast, nuclear accumulation of IKK1 required exposure to LMB for 2 to 4 h. The kinetics of accumulation in the nucleus will not only depend on the biochemical properties of NLS and NES but also on complex formation with other proteins that influence nuclear import or export and dissociation parameters of these complexes. Interestingly,  $\kappa$ B-Ras has been reported to bind to the ankyrin repeat domain of I $\kappa$ B $\beta$  and to retain NF- $\kappa$ B/I $\kappa$ B $\beta$  in the cytoplasm. Similarly, a cytoplasmic retention of NF- $\kappa$ B/I $\kappa$ B $\alpha$  mediated by interaction with G3BP2 protein has previously been described [46]. It is possible that  $\kappa$ B-Ras and G3BP2 affect the dynamics of nuclear-cytoplasmic shuttling of NF- $\kappa$ B/I $\kappa$ Bs by modulating the specific ratio of their NLS and NES activities.

The cellular distribution of individual NF- $\kappa$ B/I $\kappa$ Bs appears to be very dynamic and specifically regulated suggesting that their compartmentalization may play a more complex regulatory role than simply shutting down NF- $\kappa$ B-induced gene expression. In this study, we attempted to address how cellular localization of NF- $\kappa$ B/I $\kappa$ B $\beta$  affects basal and stimuli-induced expression of the NF- $\kappa$ B responsive genes. For this purpose, we specifically modulated a steady-state cellular distribution of NF- $\kappa$ B/I $\kappa$ B $\beta$  by mutating the p65 specific NES. As discussed above, this led to constitutive accumulation of NF- $\kappa$ B/I $\kappa$ B $\beta$  in the nucleus whereas NF- $\kappa$ B/I $\kappa$ B $\alpha$  remained predominantly cytoplasmic. The constitutive presence of NF- $\kappa$ B/I $\kappa$ B $\beta$  in the nucleus increased basal NF- $\kappa$ B activity that resulted in an elevated level of *ICAM1* and *IP-10* expression. The dynamic dissociation/association of the nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  could explain such a basal activity of NF- $\kappa$ B. A similar increase in basal expression of those genes was observed in various autoimmune and chronic inflammatory disorders or in tumours which are often characterized by constitutive nuclear NF- $\kappa$ B activity [4,47,48]. Further studies are needed to investigate the possible role of nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes in up-regulation of inflammatory genes linked to the pathogenesis of these diseases. Interestingly, we found that, similar to I $\kappa$ B $\alpha$  [35], nuclear retention of I $\kappa$ B $\beta$  reduced its TNF $\alpha$ -induced degradation. Consistent with this, p65 has previously been shown to regulate the stability of I $\kappa$ B $\beta$  but the mechanism was not discussed [32]. In our studies, we observed that the nuclear retention of I $\kappa$ B $\beta$  correlated with a decrease in activation of the *ICAM1* and *IP-10* but not *NFKB1A* expression in response to TNF $\alpha$ . Thus, we propose that changes in

steady-state localization of NF- $\kappa$ B/I $\kappa$ B $\beta$  may specifically regulate a subset of NF- $\kappa$ B-dependent genes. The differences in NF- $\kappa$ B response observed for the cytoplasmic and the nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes may be dictated by a distinct repertoire and mode of function of various NF- $\kappa$ B regulators in those cellular compartments. For example, NF- $\kappa$ B/I $\kappa$ B $\beta$  can undergo different posttranslational modifications or resides in distinct complexes depending on their actual compartmentalization.

The exact cellular role of nuclear NF- $\kappa$ B/I $\kappa$ B $\beta$  complexes remains currently unclear. Based on our results, it is tempting to speculate that localization of NF- $\kappa$ B/I $\kappa$ B $\beta$  may be affected by changes of its nuclear import/export rates under some physiological conditions to modulate constitutive and/or stimuli-induced NF- $\kappa$ B activity. This is supported by studies demonstrating that nuclear expression of I $\kappa$ B $\beta$  is associated with specific stages of B-cell differentiation [49]. In contrast to pre-B cells, mature WEHI-231 B-lymphoma cells were characterized by expression of a hypophosphorylated form of I $\kappa$ B $\beta$  highly abundant in the nucleus. It was hypothesized by the authors that nuclear I $\kappa$ B $\beta$  is responsible for constitutive NF- $\kappa$ B activity generally observed for this cell line. There is also evidence that nuclear localization of I $\kappa$ B $\beta$  is associated with the pathology of HIV-1 virus infection. I $\kappa$ B $\beta$  was present in the nucleus of HIV-1-infected myeloid cells and participated in the ternary complex formation with NF- $\kappa$ B and DNA maintaining persistent NF- $\kappa$ B activity [50].

In summary, we propose that the nature of NF- $\kappa$ B responses is determined by the specific compartmentalization of the NF- $\kappa$ B and I $\kappa$ B $\beta$  proteins which largely depends on the NLS and NES functions of p65. Further definition of the mechanisms regulating cellular localization of I $\kappa$ B $\beta$  by p65 may help to develop strategies for specific targeting of genes downstream of the NF- $\kappa$ B/I $\kappa$ B $\beta$  pathway.

#### AUTHOR CONTRIBUTION

Taras Valovka performed research and contributed to experimental design, data analysis and manuscript writing. Michael O. Hottiger contributed to data analysis and manuscript writing.

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## FIGURE LEGENDS

### Figure 1 Genetic complementation of $p65^{-/-}$ MEFs

(A) Schematic representation of retroviral constructs coding for wild-type or mutated p65 proteins. The critical residues and inactivating mutations in NLS and NES of p65 are underlined. (B) Mock transduced and complemented  $p65^{-/-}$  MEFs were analyzed for the expression of ectopic p65 and endogenous I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  proteins by immunoblot analysis.

### Figure 2 Cellular distribution of p65 is regulated by its NLS and NES elements

(A) and (B) Nuclear-cytoplasmic re-distribution of the wild-type and mutated forms of p65 in response to TNF $\alpha$ . The complemented  $p65^{-/-}$  MEFs were starved for 14 h and then stimulated with TNF $\alpha$  (20 ng/ml) for the indicated times. The ectopic proteins were visualized by indirect immunofluorescence. Scale bar, 20  $\mu$ m. (C) The graphs summarize three experiments and present the nucleus-to-cytoplasm (Nuc/Cyt) ratio of p65 fluorescence in the cells (mean $\pm$ s.d.). (D) and (E) Degradation and re-synthesis of the endogenous I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in response to TNF $\alpha$ . The cells were treated as in (A) and (B). Whole cell lysates were immunoblotted with the indicated antibodies.

### Figure 3 The p65 specific NLS and NES control cellular localization of I $\kappa$ B $\beta$

(A) Ectopic expression of the p65, HA-I $\kappa$ B $\alpha$  and HA-I $\kappa$ B $\beta$  proteins in the complemented  $p65^{-/-}$  MEFs. The cells were infected with viruses coding HA-I $\kappa$ B $\alpha$  or HA-I $\kappa$ B $\beta$  and analyzed for the expression of the ectopic proteins by immunoblot analysis. (B) Co-localization of the wild-type and the mutated forms of p65 (red) with I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  (green) was analyzed by immunofluorescence in unstimulated and TNF $\alpha$ -treated cells. Scale bar, 20  $\mu$ m.

**Figure 4 Nuclear-cytoplasmic shuttling of p65 and IκBβ**

The cells were starved and treated with LMB (40 ng/ml) for the last 4 h of starvation. The p65 and HA-IκB proteins were visualized as in Fig. 3B. DAPI (blue) was used to localize nuclei. Scale bar, 20 μm.

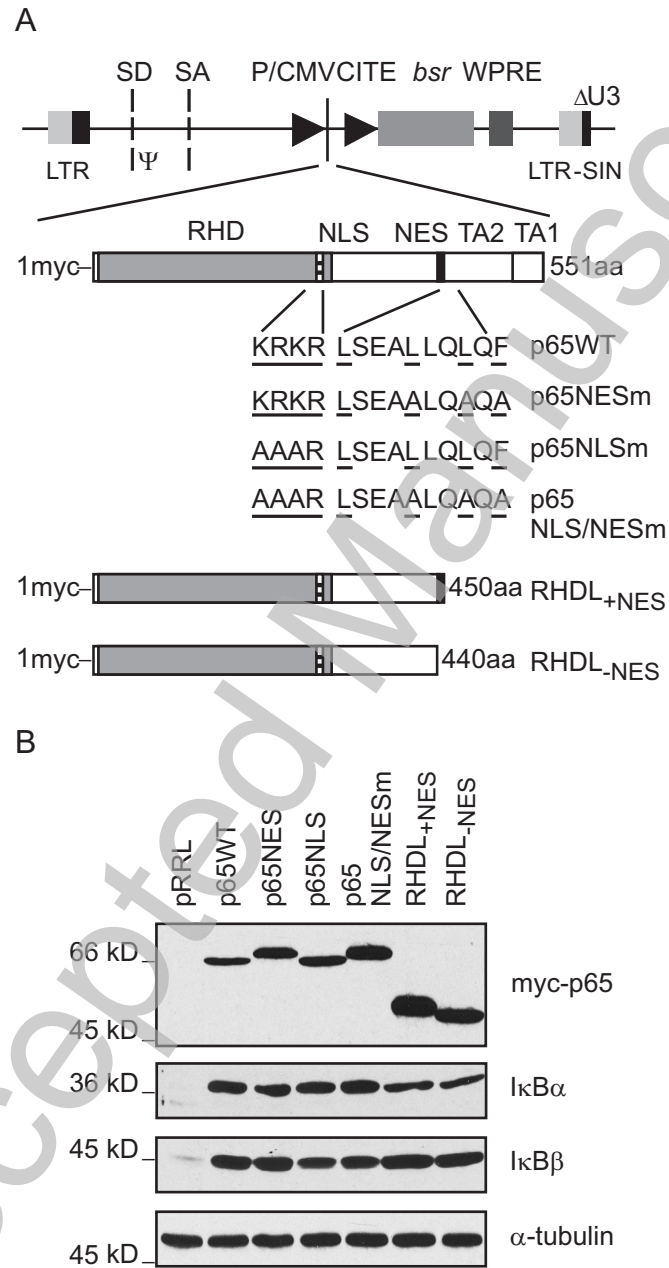
**Figure 5 Nuclear localization of p65 and IκBβ elevates basal but reduces TNFα-induced expression of inflammatory genes**

Expression of the *IP-10*, *ICAM1* and *NFKB1A* genes in response to TNFα. The gene specific mRNAs were quantified by RT-qPCR at the indicated time points of TNFα treatment (10 ng/ml) using a relative standard curve method. Data are mean±s.d. of three experiments. RQ, relative quantification value.

**Figure 6 Nuclear retention of p65 and IκBβ affects DNA binding activity of NF-κB and stability of IκBβ**

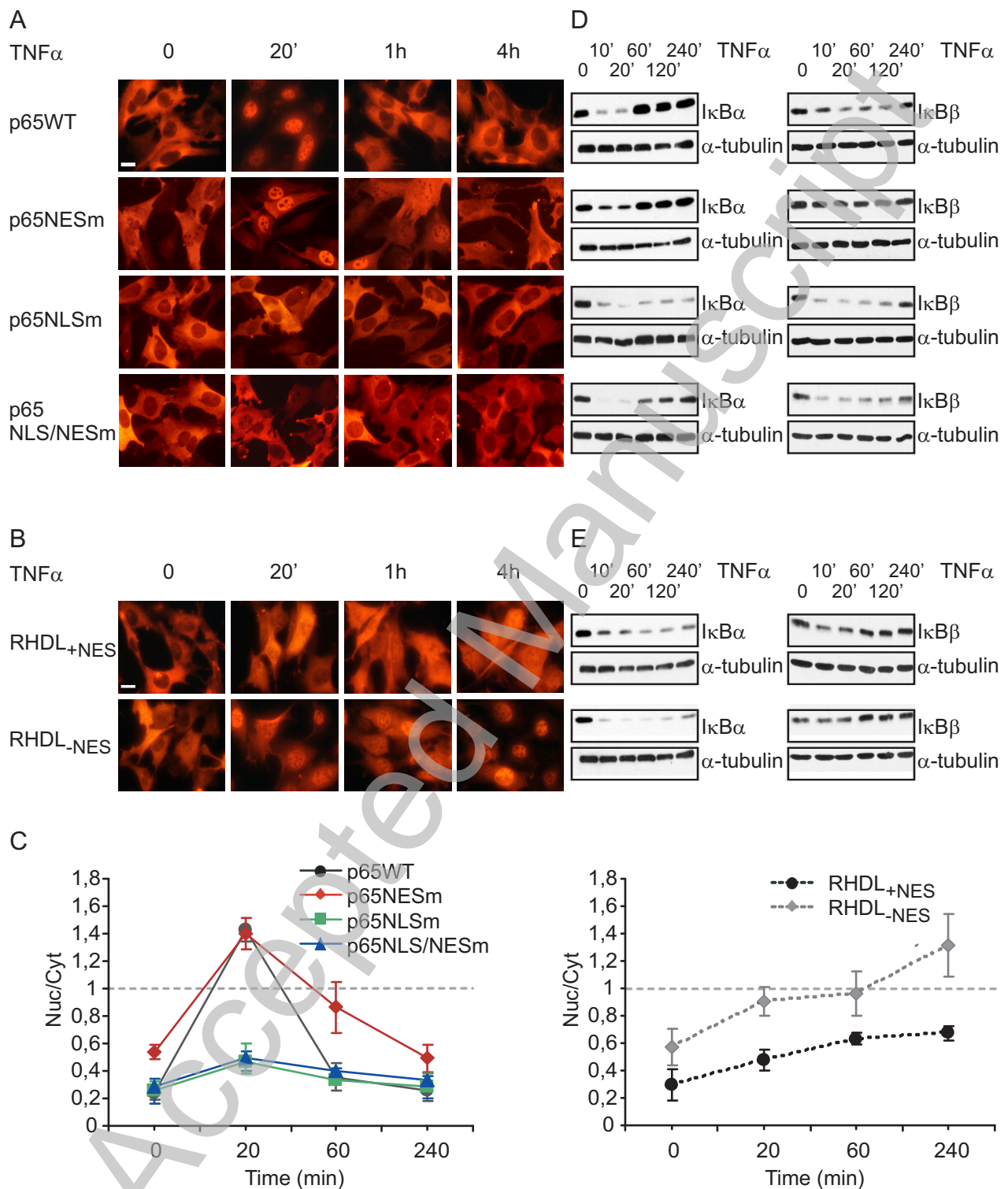
(A) EMSA of nuclear extracts from the cells expressing cytoplasmic p65<sup>WT</sup>/IκBβ and nuclear p65<sup>NES<sub>mut</sub></sup>/IκBβ using a radioactively labelled NF-κB consensus oligonucleotide. Distinct lines of the same EMSA gel are separated by boxes. Quantification of the bound probe was performed by densitometric analysis of the representative gel. (B) Degradation of HA-IκBβ in cells expressing p65<sup>WT</sup> or p65<sup>NES<sub>mut</sub></sup> in response to TNFα. Cells were starved and treated with TNFα (10 ng/ml) for 20 min. Whole cell lysates were subjected to immunoblot analysis.

**Figure 1**



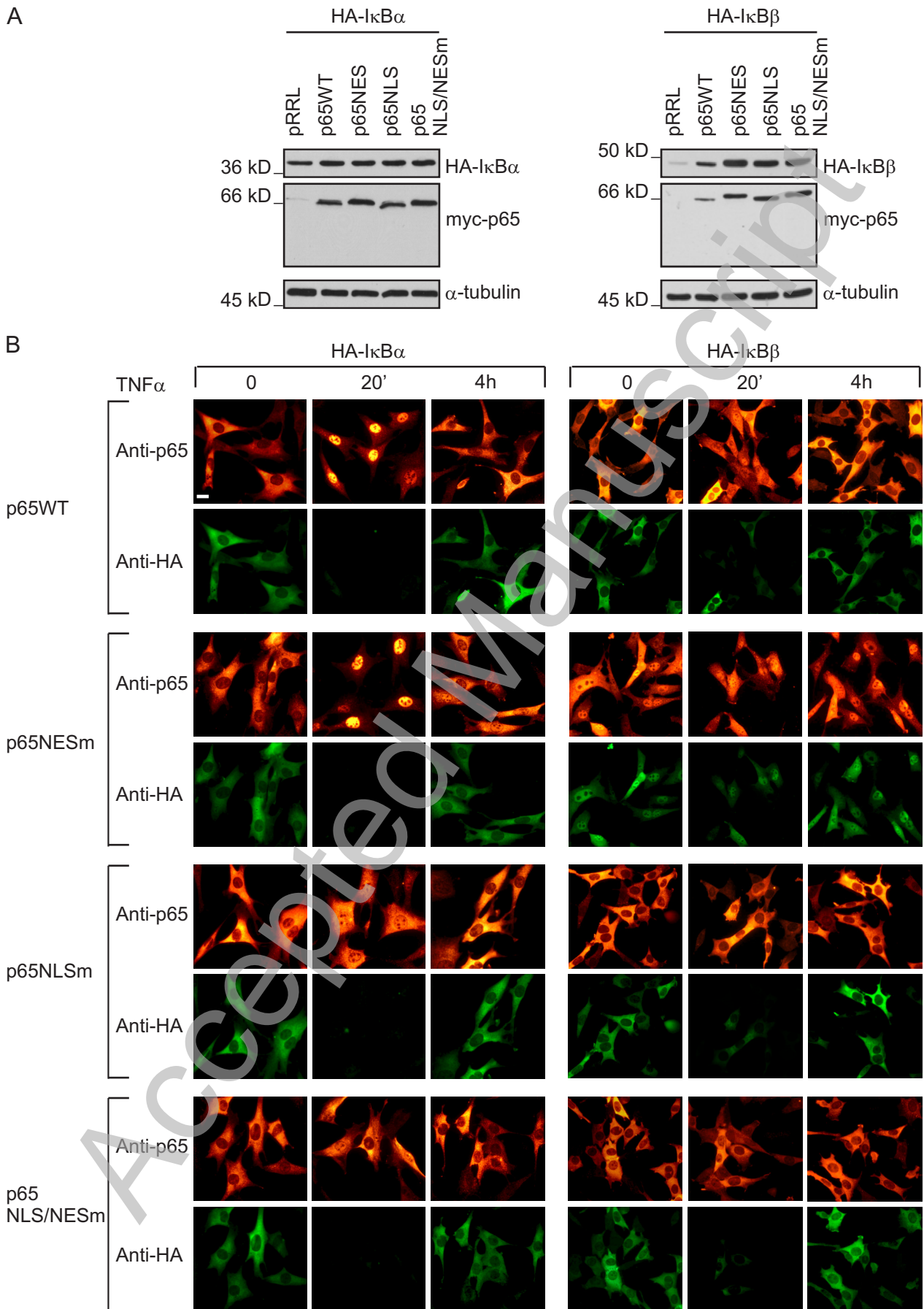
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**Figure 2**



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**Figure 3**



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Figure 4

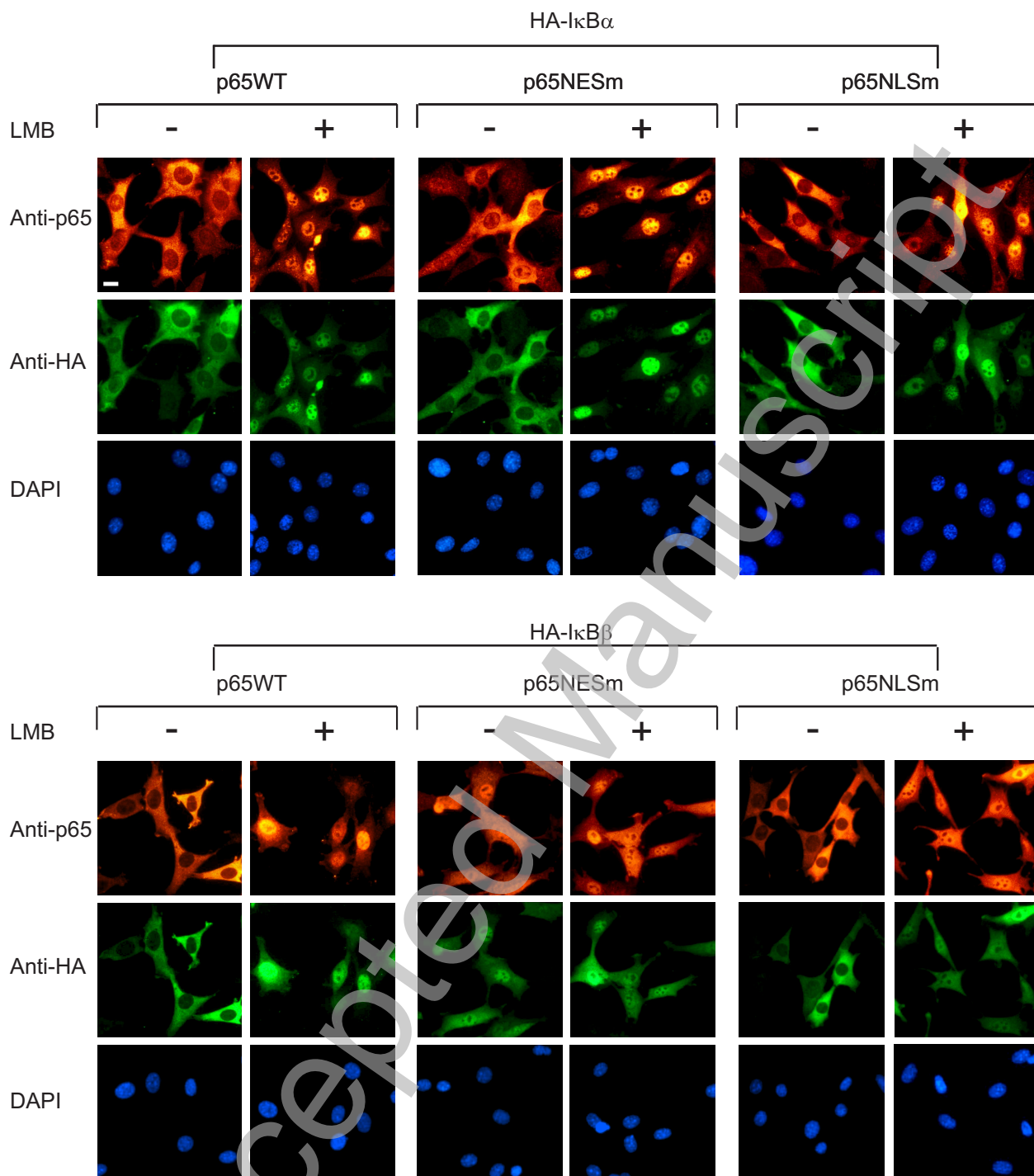
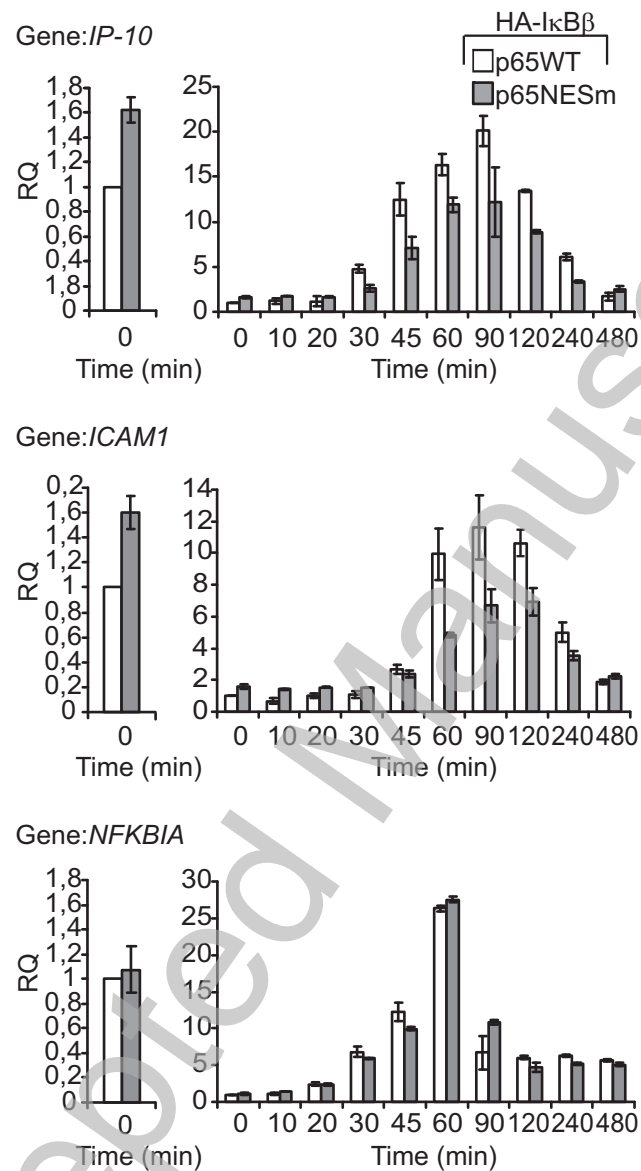
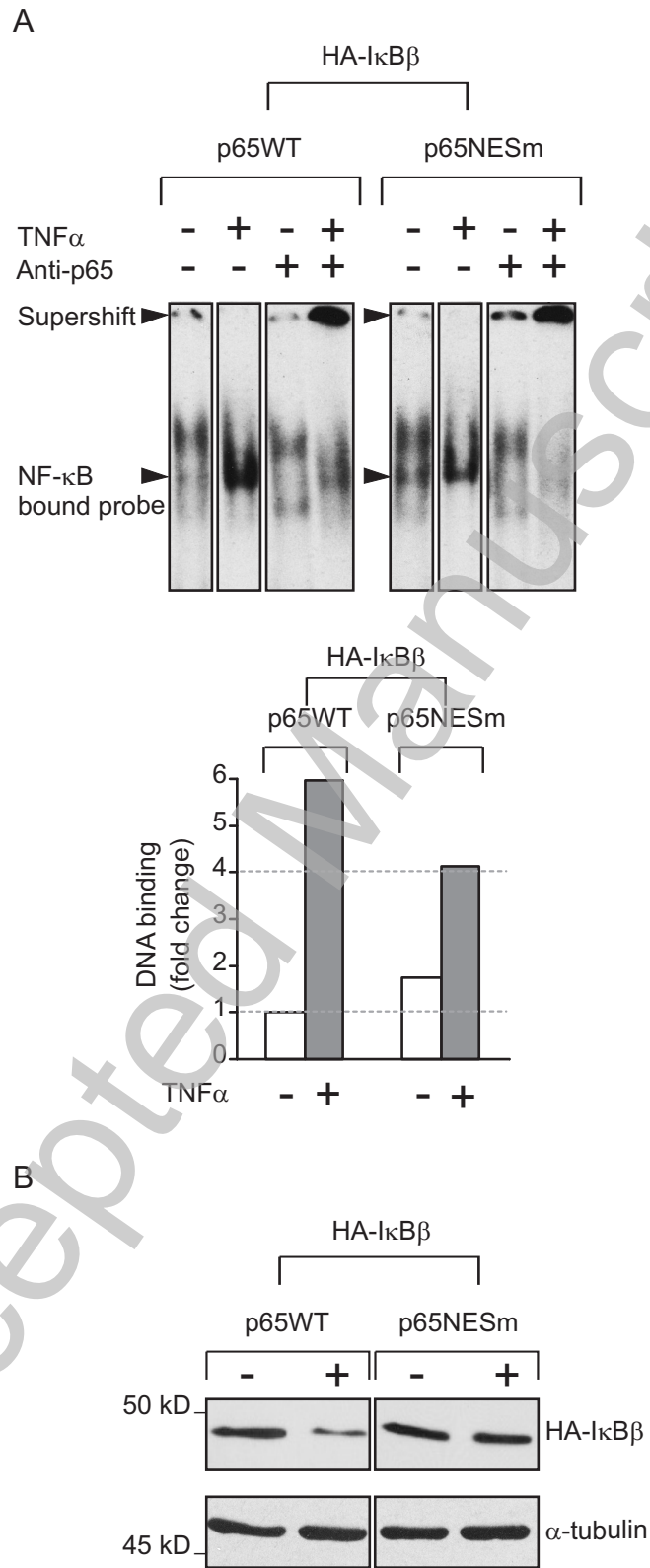


Figure 5



**Figure 6**



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