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NF-κB regulates the expression of Nucling, a novel apoptosis regulator, with involvement of proteasome and caspase for its degradation

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Nam Hoang Tran, Takashi Sakai, Sun Mi Kim and Kiyoshi Fukui*

The Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan

*Kiyoshi Fukui, Division of Enzyme Pathophysiology, The Institute for Enzyme Research (KOSOKEN), The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Tel: +81 88 633 7430, Fax: +81 88 633 7431, email: kiyo@ier.tokushima-u.ac.jp

Nucling is identified as a novel regulator of apoptosis. In this study, we investigated the nuclear factor-κB (NF-κB)-dependent mechanism of Nucling expression, as well as the degradation pathways mediated by proteasome system and caspase activation. Using chromatin immunoprecipitation assay in wild-type mouse embryonic fibroblasts (WT MEFs), we found that NF-κB p65 could bind to the κB motifs in the promoter regions of Nucling gene at four putative-binding sites. By real-time PCR and immunoblot, we confirmed that Nucling expression was up-regulated by tumour necrosis factor-α (TNF-α) stimulation in WT MEFs, but not in NF-κB p50 knockout MEFs. On the other hand, we investigated the degradation of Nucling in connection with proteasome and caspase by using cycloheximide chase. The results showed that Nucling is a short-lived protein, and its degradation was recovered by proteasome inhibitor MG132. Moreover, under TNF-α stimulation, degradation of Nucling was recovered by pan-caspase inhibitor zVAD-fmk. Taken together, we propose a mechanism of Nucling intracellular metabolism. Nucling expression is induced by canonical NF-κB signalling pathway, whereas Nucling is undergoing proteasome degradation, as well as being cleaved by caspase system under stress conditions. This opens a new perspective for studying the NF-κB dependent regulation mechanism of cell death and survival.

Keywords: apoptosis/caspase/NF-κB/nucling/TNF-α/proteasome.

Abbreviations: Apaf-1, apoptosis activating factor-1; CHX, cycloheximide; ChIP, chromatin immunoprecipitation; Gal-3, galectin-3; KO, knockout; MEFs, mouse embryonic fibroblasts; NF-κB, nuclear factor-κB; PBS, phosphate buffered saline; RT–PCR, reverse transcription-polymerase chain reaction; TNF-α, tumour necrosis factor-α; WB, western blotting; WT, wild-type; p50, p50 sub-unit of NF-κB; p65, p65 sub-unit of NF-κB; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

Nucling has been identified first as a novel, F9 embryonal carcinoma cell-specific molecule (*1*), which recruits and transports the Apaf1/pro-caspase-9/apoptosome complex during stress-induced apoptosis (*2*, *3*). Later studies have shown that Nucling mediates apoptosis by inhibiting expression of galectin-3 (gal-3) through interference with nuclear factor-κB (NF-κB) signalling (*4*), as Nucling can bind to NF-κB p65 and p50 by its N-terminal region containing ankyrin repeats, thus preventing the translocation of NF-κB into the nucleus (*5*, *6*). In the Nucling-knockout (KO) mouse liver, several lines of evidence indicating spontaneous NF-κB activation, up-regulation of TNF-α and gal-3 and a significant decrease in the number of Kupffer cells through apoptosis caused by tumour necrosis factor-α (TNF-α) up-regulation were observed (*7*). The data available so far indicate that Nucling is important for the regulation of apoptosis in general and regulation of NF-κB signals in particular. Nucling is among a number of novel regulators, adding another layer to the mechanism of NF-κB regulation. Further insight into the function of Nucling in interaction with other molecules raises the prospect for better understanding and rational design of therapeutics for many kinds of diseases.

NF-κB has become subject for intensive research for over 2 decades. Referred as a central regulator of stress responses (*8*), NF-κB represents a family of transcription factors that regulates the expression of a large number of genes involved in many biological processes such as innate and adaptive immunity, inflammation, cellular stress responses, cell adhesion, apoptosis and proliferation. NF-κB is generally considered to be an anti-apoptotic, proproliferative and proinflamatory regulator (*9*). Appropriate regulation of NF-κB is critical for the proper function and survival of the cell. NF-κB is induced by hundreds of different stimuli. Active NF-κB, in turn, participates in the control of transcription of hundreds of genes, directly and indirectly (*8*, www.nf-kb.org). The NF-κB transcription factor family in mammals consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) that associate with each other to form dimeric complexes. The p50/p65 heterodimer, most abundant among all types of Rel dimers, is being found in almost all cell types. In the absence of stimuli, NF-κB dimers are retained in the cytosol. TNF-α is a key cytokine that regulates immune responses, cell differentiation and apoptosis. The TNF-α-induced canonical NF-κB activation pathway induces activation of p50/p65 dimer, its nuclear

localization and NF-κB-dependent gene expression (*10*, *11*). NF-κB activation by TNF-α stimulation is one of the most commonly used method for studying NF-κB- dependent gene expression.

Protein degradation is a key cellular process involved in many aspects of the cell. Proteasome plays a major role to maintain cell quality control by removing accumulation of proteins. Commonly, proteins marked by poly-ubiquitination will undergo degradation by proteasomes (*12*, *13*). However, some proteins may also undergo ubiquitin-independent proteasomal degradation (*9*). Many of NF-κB regulated proteins (*e.g.* IκB, p53) (*14*, *15*) are reported to undergo proteasomal degradation. Generally, proteasome inhibition results in blocking NF-κB activation and promotion of apoptosis in most cell lines (*16*).

Caspases are a family of cysteine proteases, which play essential roles in apoptosis. Caspase cascade leads to rapid and irreversible changes in physiological function and morphology, eventually cell death. Caspases are regulated at a post-translational level, ensuring that they can be rapidly activated. The initiation of this cascade reaction can be regulated by caspase inhibitors. The caspase cascade can be activated by granzyme B, death receptors and apoptosome. Nucling is capable of activating caspase cascade by recruiting Apaf1 apoptosome complex (*2*, *3*).

Nucling gene (GenBank Accession Number AB030647) has κB motifs in its promoter regions. Based on this fact we assume that Nucling is a direct target gene of NF-κB transcription factor. In the present study, we examine the binding of p65 with κB consensus sequence in the Nucling promoter regions of mouse chromosome 9. We also demonstrate the role of p50 in Nucling mRNA transcription and Nucling protein expression. Moreover, based on the structure of the Nucling molecule and known mechanism of Nucling in apoptosis regulation, we test the hypothesis that Nucling is undergoing at least two post-translational modifications pathways: proteasomal degradation and proteolytic cleavage by caspase system.

Experimental Procedures

Cells and culture conditions

WT and Nucling-KO pregnant mice were of background B6; 129 as described earlier (*2*, *7*). The background of NF-κB p50 KO pregnant mice were B6; 129P-Nfkb1^{tm1Bal}/J (The Jackson Laboratory). All types of mouse embryonic fibroblasts (MEFs) were obtained by dissecting 16-day pregnant mice, digesting minced embryos with 0.05% trypsin/EDTA in phosphate buffered saline (PBS) at 37°C with vigorously shaking for 1 h. Cells were spun down and cultured in 10 ml of DMEM (Sigma) with 10% foetal calf serum at 37°C in 5% CO_2. Cultures were genotype-confirmed by PCR and split every 3 days.

Chromatin immuno-precipitation assay

Chromatin immunoprecipitation (ChIP) was carried out according to Shang *et al.* (*17*). Briefly, WT MEFs were cross-linked with 1% formaldehyde and sonicated. The chromatin solutions were pre-cleared with the addition of Protein A Sepharose CL-4B beads (GE Healthcare) for 2 h at 4°C. The pre-cleared chromatins were incubated with anti-p65 antibody (Santa Cruz Biotechnology) for overnight, non-immune IgG serum was used a negative control. The complexes were precipitated with Protein A Sepharose CL-4B beads. After washing the beads, cross-linking was reversed by adding 4 μl of 5 M NaCl and incubating at 65°C for 6 h. DNAs were purified by QIAEX II® gel extraction kit (Qiagen). In the promoter region of Nucling gene, four putative κB binding sites with consensus sequence 5′-GGGPuNNPyPyCC-3′ (Pu: Purine, Py: Pyrimidine, N: any nucleotide) were identified respectively, located at 112562, 120701, 121735, 153804 loci in mouse chromosome 9, and the specific primers flanking them were designed using Genetyx software. Primers sequences were as follows: κB1.1 forward 5′-GGG TTA TGT GGC AGT GTG AG-3′ and reverse 5′-GGA ACC TGT CAG CTC TTT GG-3′ (201bp), κB1.2 forward 5′-GGG GAC ATG CCT GAG GTT TC-3′ and reverse 5′-AGT TTA TTG GGG AAT TAG GGT TCC A-3′ (175bp), κB1.3 forward 5′-GGG GAG AGG AAG AGT GTT ACG AAG-3′ and reverse 5′-TCC ACA GTC GGG ACT TGA GG-3′ (283bp), κB2 forward 5′-GGG ACC TTG TCT TTG TGC AGC ACT T-3′ and reverse 5′-GAC CAA GGC ACA GAG ACT TCA TCC CA-3′ (177bp). PCR was performed using Ampdirect (Shimadzu). Amplified bands were visualized by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

Quantitative real-time PCR

NF-κB p50 KO MEFs and WT MEFs were treated with 10 ng/ml TNF-α for 0, 15, 30 and 60 min. For relative quantification, total RNA was isolated using TRIzol® (Invitrogen), in accordance with the manufacturer's instructions. First strand synthesis was performed with 2 μg of total RNA using SuperScript® III First-Strand Synthesis System for RT–PCR (Invitrogen). First strand samples from 100 ng RNA were used for real-time PCR. Real-time PCR was performed using SYRB® Premix Ex Taq™II (Takara) and Applied Biosystems 7500 real-time PCR System according to the manufacturer's recommendations. Relative quantification was achieved by the $\Delta\Delta C_t$-method. Briefly, $\Delta Ct_{sample} = Ct_{target} - Ct_{ref}$; $\Delta Ct_{calibrator} = Ct_{target} - Ct_{ref}$; $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$; relative quantity to calibrator $= 2^{-\Delta\Delta Ct}$; whereas β-actin served as reference, untreated WT MEFs served as calibrator. The PCR primers for Nucling were forward 5′-TGA TCA CCC AGG ACC CGG AAG TTA CC-3′, and reverse 5′-GGT GCT CTT TGA GGG CGA GGA AGT G-3′. Control β-actin primers were: forward 5′-ATG GAG GGG AAT ACA GCC C-3′, and reverse 5′-TTC TTT GCA GCT CCT TCG TT-3′. TNF-α served as positive control for TNF-α -induced up-regulation. TNF-α primers were forward 5′-AGG GTC TGG GCC ATA GAA CT-3′, and reverse 5′-CCA CCA CGC TCT TCT GTC TAC-3′. The reactions were repeated three times. Statistical significance was considered if $P < 0.05$ by Student's *t*-test.

Western blotting

Whole cell lysates were prepared by adding RIPA lysis buffer with freshly added Protease inhibitor cocktail tablets (Roche), followed by scraping and homogenation. The cells were incubated for 15 min, followed by centrifugation at 15,000*g* for 15 min. Supernatant was stored at −80°C as whole cell lysate. Cell fractionation was performed according to Baetz *et al.* (*18*). Western blotting (WB) was carried out according to standard procedures. Loading equivalency was ensured by performing BCA protein assay (Pierce). Antibodies specific to Apaf1 (BD Biosciences), gal-3 (R & D systems), p65, p50 (Santa Cruz Biotechnology), β-actin (Sigma) and the antibody against middle portion of Nucling were used in this study. Detection was done by ECL-plus detection kit (Amersham). Densitometry was calculated by ImageJ 1.42 (National Institutes of Health).

Cycloheximide chase

For detecting degradation of Nucling by proteasome, WT MEFs were incubated in medium containing 35 μg/ml cycloheximide (CHX) in the presence or absence of 40 μM proteasome inhibitor, MG132. For detection of caspase-dependent degradation, CHX chase was conducted with or without 100 nM pan-caspase inhibitor, zVAD-fmk, in both unstimulated condition and in stimulation with 20 ng/ml TNF-α. The cells were lysed at 15, 30, 60 and 120 min after treatment. Expression of Nucling protein in the cell lysate was detected by WB, using β-actin as a loading control.

Results

NF-κB p65 was bound to the κB motifs in the promoter regions of nucling gene

The putative κB binding sites containing consensus sequence (5′-GGGPuNNPyPyCC-3′) were tested for actual *in vivo* binding with anti-p65 antibody in WT MEFs in the presence or absence of TNF-α stimulation. ChIP results demonstrated that NF-κB p65 was actually linked with the four κB binding sites (Fig. 1) located in the Nucling promoter region of mouse chromosome 9, including three sites preceding Nucling Exon 1 and one preceding Exon 2. Input, containing total cellular protein before antibody treatment, was served as positive binding control, while immunoprecipitation with non-immune mouse IgG was served as negative control. PCR bands with appropriate expected sizes were observed following ChIP with anti-p65 antibody. These results suggest that there is actual binding of the p65 to the promoter region of Nucling gene by at least four binding sites, either in the presence or absence of TNF-α stimulation.

Nucling transcription was upregulated by TNF-α in WT MEFs, but suppressed in NF-κB p50 KO MEFs

To confirm that TNF-α stimulation can induce Nucling up-regulation in a time-dependent manner, and NF-κB p50 is indispensable for transcription of Nucling gene, we conducted quantitative real-time PCR with p50 KO MEFs in comparison with WT MEFs. The result showed relative Nucling mRNA quantification with β-actin as internal control and normalized to non-treatment WT MEFs as calibrator. According to the common knowledge, NF-κB activity is transient and cyclic in the presence of continuous inducer for every 30–60 min (*19*). In our investigation throughout a time course of TNF-α treatment for 0, 15, 30 and 60 min, there was no increase of Nucling mRNA in p50 KO MEFs observed (Fig. 2A, grey). In contrast, a significant TNF-α induced increase of Nucling mRNA in WT MEFs was observed in a time-dependent manner (Fig. 2A, black). This result suggests that NF-κB p50 is necessary for transcription of Nucling gene. Meanwhile, TNF-α has been reported to be a target gene of NF-κB, responsive to LPS and TNF stimulation (*20*, *21*). Here we showed relative quantification of TNF-α mRNA following TNF-α treatment in WT MEFs and p50 KO MEFs as positive control of NF-κB-dependent gene up-regulation (Fig. 2B).

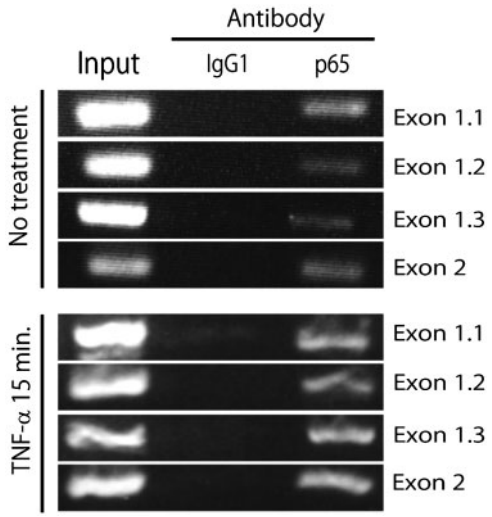

Fig. 1 ChIP detection of NF-κB p65 binding to κB sites in the Nucling's promoter regions located in mouse chromosome 9. In cell lysate from resting and TNF-α stimulated WT MEFs, p65 binding to four putative consensus κB binding sites was confirmed by PCR with the following band size: κB1.1 (201 bp), κB1.2 (175 bp), κB1.3 (283 bp) and κB2 (177 bp). Input and non-immune IgG was used as positive and negative control.

Nucling protein expression was upregulated by TNF-α in WT MEFs, but suppressed in NF-κB p50 KO MEFs

To confirm Nucling protein up-regulation by TNF-α stimulation, and NF-κB p50 is necessary for the translation of Nucling protein, we performed WB with p50 KO MEFs in comparison with WT MEFs. The cells were treated with TNF-α for 0, 15, 30 and 60 min. As shown in Fig. 3A, lane a, Nucling expression in nuclear

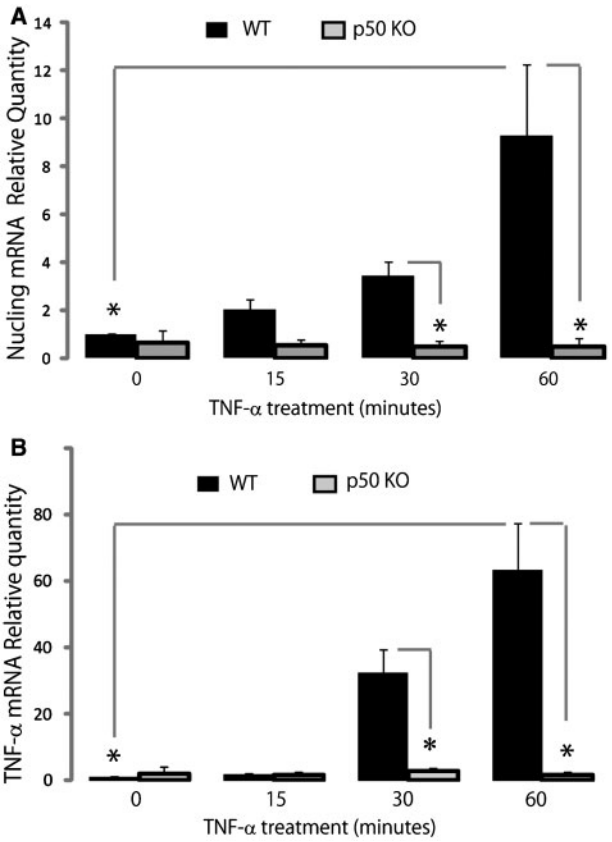

Fig. 2 Quantitative real-time PCR detection of relative transcription level of Nucling mRNA in WT MEFs and p50 KO MEFs. (A) *X*-axis represents TNF-α treatment duration of 0, 15, 30, and 60 min; *Y*-axis represents relative mRNA quantity. All values were calculated by ΔΔCt-method as relative quantity (RQ) value normalized to non-treatment WT MEFs value, using β-actin as internal control. Results were shown as mean ± SE of three independent experiments. $^*P < 0.05$ by Student's *t*-test. (B) Relative quantification of TNF-α mRNA following TNF-α treatment in WT MEFs and p50 KO MEFs shown as positive control of NF-κB-dependent gene up-regulation.

fraction of WT MEFs was up-regulated by TNF-α treatment in a time-dependent manner. This up-regulation of Nucling in WT MEFs is represented quantitatively by densitometry. Nucling protein band was up-regulated after 60 min of treatment (Fig. 3B). On the other hand, almost no Nucling protein was detected in p50 KO MEFs throughout the whole time course of TNF-α treatment (Fig. 3A, lane a). Gal-3, a target gene of NF-κB, was shown as a positive marker for NF-κB induced transcription (Fig. 3A, lane d). Expression of 26 kDa gal-3 was up-regulated in WT MEFs, but strongly suppressed in p50 KO MEFs. Apaf1 recruitment by Nucling in stress conditions was regarded as downstream marker for Nucling expression (*2*). The expression of Apaf1 (130 kDa) was observed in WT MEFs but not in p50 KO MEFs (Fig. 3A, lane e). Although no p50 expression was detected in p50 KO MEFs (Fig. 3A, lane b), p65 expression could be detected in the nuclear fraction of p50 KO MEFs (Fig. 3A, lane c), indicating that without p50, p65 alone could not initiate Nucling gene transcription. β-actin was shown as loading control (Fig. 3A, lane f). Briefly, this WB result consolidated the previous real-time PCR experiment, suggesting that p50 is necessary for Nucling gene translation.

Nucling is a substrate of the proteasomal degradation

Considering that Nucling was found to be located in the cytoplasm around the nucleus (*2*), we tested whether Nucling constitutes a proteolytic substrate of the proteasome. In order to verify the role of proteasome in the degradation of Nucling protein, we performed CHX chase of Nucling in the presence or absence of proteasome inhibitor MG132 (40 μM) for indicated time points, using the WT MEFs. Degradation of Nucling was observed after 15, 30, 60 and 120 min of CHX treatment (Fig. 4A), compared to non-treatment control. Moreover, accumulation of Nucling after proteasome inhibition was also observed (Fig. 4A, compare the bands at each time point). Degradation of Nucling and its accumulation by MG132 treatment could be represented quantitatively by densitometry (Fig. 4B). Since CHX suppresses the synthesis of proteins (*22*, *23*), these results indicate that Nucling accumulation after proteasome inhibition is probably due to reduced degradation of Nucling by the proteasome. In the same way, we also confirmed proteasomal degradation of Nucling in the TNF-α stimulated cells (Fig. 4C and D).

Nucling is degraded by the caspase under TNF-α stimulation

Here we examined the degradation of Nucling by caspase in resting and stimulated MEFs, using the pan-caspase inhibitor zVAD-fmk. CHX chase of Nucling was performed in the presence or absence of zVAD-fmk (100 nM). In resting cells, the degradation of Nucling was observed (Fig. 5A) in a similar pattern to Fig. 4. However, no significant effect of zVAD-fmk treatment was observed at every time point, as shown in Fig. 5B. In the TNF-α stimulated cells, the significant effect of zVAD-fmk treatment was observed after 60 min of treatment (Fig. 5C and D), as zVAD-fmk caused Nucling accumulation at the respective time points. These results indicate that degradation of Nucling after TNF-α stimulation is probably due to proteolytic processing following activation of the caspase system.

Discussion

According to the canonical model, NF-κB dimers in the resting cells are retained in an inactive form in the cytosol through their interaction with IκB proteins. Upon stimulation, IκB kinase (IKK) triggers phosphorylation of IκBs, nuclear translocation of NF-κB and induction of transcription of target genes (*10*).

In ChIP experiments, we examined DNA binding property of p65 and confirmed its binding at the putative κB sites of Nucling gene. It is commonly accepted that gene transcription is induced by p50/p65 dimer. While p65 contains a potent transcriptional activation domain (TAD) that is largely responsible for functional effects attributed to the heterodimeric NF-κB complex, p50 dimer served as repressors of κB-directed transcription, presumably because of the absence of a

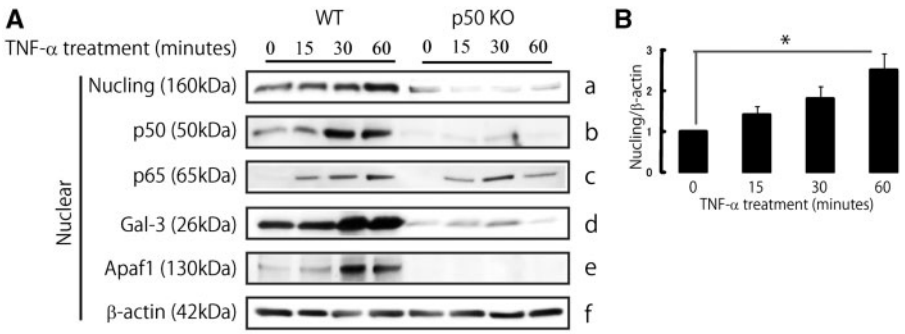

Fig. 3 WB detection of TNF- α induced Nucling expression in WT and p50 KO MEFs. (A) Panel a. Nucling expression at 160 kDa detected by anti-middle portion of Nucling antibody by TNF-α treatment for 0, 15, 30 and 60 min. Nucling up-regulation was observed in nuclear fraction of WT MEFs in contrast with lack of Nucling expression in p50 KO MEFs. Panel b. Confirmation of p50 KO phenotype in p50 KO MEFs. Panel c. p65 availability in nuclear fraction of WT and p50 KO MEFs. Panel d. Expression pattern of Gal-3 as a NF-κB-dependent gene in WT and p50 KO MEFs. Panel e. Apaf1 expression pattern as a molecule being recruited by Nucling in WT and p50 KO MEFs. Panel f. Loading control by β-actin. (B) Densitometric quantification of the band intensity for Nucling expression in WT MEFs, shown as average value of three independent experiments. *$P < 0.05$ by Student's *t*-test.

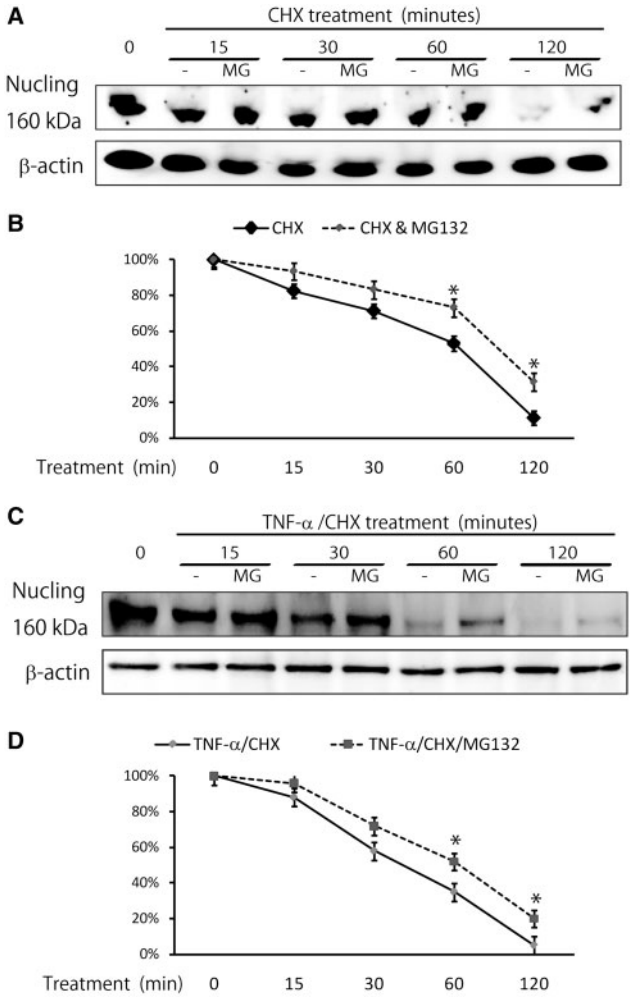

Fig. 4 Degradation of Nucling by the proteasome in WT MEFs. (A) CHX chase of Nucling in resting MEFs. Following addition of 35 μg/μl CHX in the presence or absence of 40 μM MG132 (MG), cells were harvested after 15, 30, 60 and 120 min and the cell lysates were subjected to western blot for detection of Nucling (160 kDa). (B) Degradation curves of Nucling by CHX chase in the presence or absence of MG132 in resting WT MEFs. (C) CHX chase of Nucling in TNF-α stimulated MEFs. Following addition of 35 μg/μl CHX and 20 ng/ml TNF-α in the presence or absence of 40 μM MG132, cells were harvested after 15, 30, 60 and 120 min and the cell lysates were subjected to western blot for detection of Nucling. (D) Degradation curves of Nucling by CHX chase in the presence or absence of MG132 in TNF-α stimulated WT MEFs. Graph represents the means of density of the bands by three different experiments. Expression level of Nucling protein was arbitrarily set to 100% in control culture (untreated or zero time point). $*P<0.05$ by Student's *t*-test.

TAD (*24*). This result suggests that p50/p65 dimer could bind Nucling gene at κB sites, required for gene transcription (*25*). In the real-time PCR (Fig. 2) and WB (Fig. 3) experiments using p50 KO MEFs, we clearly demonstrated that p50 is important for Nucling expression. Given the fact that p65 KO mice suffer a massive degeneration of liver due to apoptosis leading to embryonic lethality at 15–16 days of gestation (*26*), we could not demonstrate similar experiment as done with p50 KO MEFs in order to prove the role of p65 in Nucling mRNA transcription and protein translation. However, the p50 alone possesses no TAD necessary for the target gene expression. This situation could be interpreted that any of three heterodimer combinations of p50: p50/p65, p50/RelB or p50/c-Rel could potentially mediate Nucling expression (*19*).

The results from real-time PCR (Fig. 2) and WB (Fig. 3) experiments also showed a time-dependent manner in NF-κB transcription and synthesis of Nucling in ~60 min duration. This is consistent with the common concept that activation of NF-κB is transient and cyclic in the presence of inducer, nuclear NF-κB binding activity appears and disappears every 30–60 min due to repeated degradation and re-synthesis of IκB and the consequent activation and inactivation of NF-κB, respectively (*11*).

Combined results from our experiments for binding, transcription and translation assays provided an evidence that the classical form of NF-κB consisted of p50/p65 heterodimer could bind to κB sequences in the promoter region of Nucling, initiate mRNA transcription and subsequent translation of Nucling protein and TNF-α could up-regulate this process during a transient cycle ~60 min. However, our data did not exclude probability of Nucling expression to be regulated by an indirect transcription factor of NF-κB, nor limited to canonical pathway of p50/p65 heterodimer, nor exclude existence of non-consensus binding. In other words, NF-κB canonical pathway is one but not necessarily the only pathway for Nucling transcription.

Currently, NF-κB is reported to be a transcription factor of hundreds of genes with different functions: regulators of apoptosis, cytokines/chemokines, immunoreceptors, cell adhesion molecules, acute phase proteins, stress response genes, cell-surface receptors, growth factors, ligands and transcription factors. (www.nf-kb.org). Among the target genes of NF-κB there are pro-survival genes such as Bfl1/A1, Bcl-Xl, Bcl-2, c-FLIP, IAPs, and a fewer number of pro-apoptotic genes such as Fas, Fas-Ligand. As previous studies (*1–7*) had already shown the pro-apoptotic function of Nucling, by this study we added Nucling to the group of regulators of apoptosis, as a pro-apoptotic target of NF-κB.

Since proteasome inhibitor treatment can prevent regulators such as IκB and Nucling from degradation, this will block NF-κB to translocate into nucleus. Apoptosis is increased due to low level of NF-κB dependent anti-apoptotic proteins. At the same time, Nucling in higher concentration may recruit Apaf1 to activate the caspase cascade (*2*). Our result in Fig. 4 is consistent and can explain the common concept that proteasome inhibition will promote apoptosis in most cell lines (*16*).

In the mean time, in unstimulated condition, Nucling was confirmed to become degraded by proteasome (Fig. 4A). This suggests that when the Nucling concentration becomes sufficiently low, it will not be able to prevent NF-κB from nuclear translocation. As a result, the κB binding and transcription will happen, releasing more Nucling into the cytoplasm, as it is being degraded by proteasome again cyclically. Similarly, in TNF-α stimulation, there was an accumulation of Nucling in the presence of proteasome

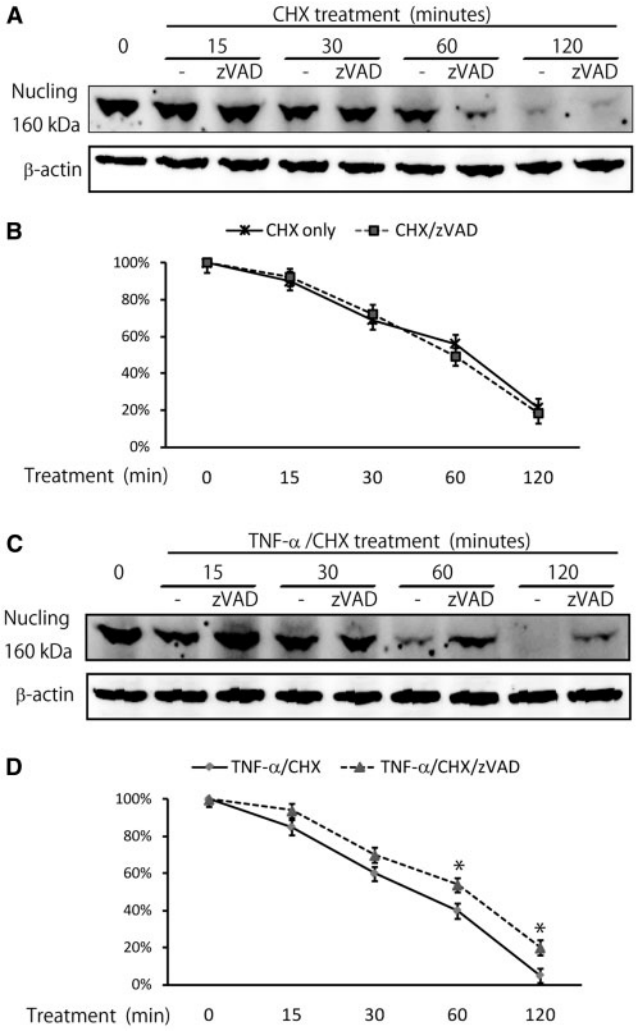

Fig. 5 Degradation of Nucling by the caspase in WT MEFs. (A) CHX chase of Nucling in resting MEFs. Following addition of 35 μg/μl CHX in the presence or absence of 100 nM zVAD-fmk (zVAD), cells were harvested after 15, 30, 60 and 120 min and the cell lysates were subjected to western blot for detection of Nucling (160 kDa). (B) Degradation curves of Nucling by CHX chase in the presence or absence of zVAD in resting WT MEFs. (C) CHX chase of Nucling in TNF-α stimulated MEFs. Following addition of 35 μg/μl CHX and 20 ng/ml TNF-α in the presence or absence of 100 nM zVAD, cells were harvested after 15, 30, 60 and 120 min and the cell lysates were subjected to western blot for detection of Nucling. (D) Degradation curves of Nucling by CHX chase in the presence or absence of zVAD in TNF-α stimulated WT MEFs. Graph represents the means of density of the bands by three different experiments. Expression level of Nucling protein was arbitrarily set to 100% in control culture (untreated or zero time point). $*P<0.05$ by Student's *t*-test.

inhibitor, MG132 (Fig 4B). Shortly, these results suggest that Nucling is undergoing proteasome degradation in resting and stimulated conditions.

In standard way, proteins are undergoing ubiquitination before being degraded by 26S proteasome. In our observations, we are still in search for a specific mechanism of ubiquitination of Nucling in relation with TNF-α stimulation. Besides the ubiquitin-proteasome proteolytic pathway, Nucling may also be degraded in an ubiquitin-independent manner. If it is the case, it could be speculated that in resting cells, free Nucling may undergo the same pathway as IκB-α does, *i.e.* being degraded by 20S proteasome (*9*). However, this possibility remains to be confirmed.

To date, at least 15 members of the caspase family and more than 280 caspase substrates have been identified (*27*). There are two types of apoptotic caspases: initiator caspases and effector caspases. Initiator caspases (*e.g.* CASP8) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (*e.g.* CASP3) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. The initiation of this cascade reaction is regulated by caspase inhibitors. In our experiment, using pan-caspase inhibitor indicated that Nucling is sensitive to caspase activation, however whether Nucling is being cleaved selectively by initiator caspases, or by effector caspases, or being cleaved indirectly by another downstream metabolite, we still need to confirm. On the other hand, by using SVM-based prediction of caspase substrates cleavage sites (*28*), we found that mouse Nucling protein (NP_082559) contains many potential caspase substrate cleavage sites (Supplementary Table S1).

As commonly known, caspase cascade leads to rapid and irreversible changes in physiological function and morphology, eventually cell death. However activation of caspase and cleavage of their substrates cannot be used to determine whether a cell has undergone the point of no return and eventually become dead (*22*). For example, it is reported that caspase activation may lead to activation of MstI and Rho kinases, which promote a broad signalling pathway regulation for cell survival and cell shape (*29*, *30*), including activation of NF-κB. In a similar way, Nucling degradation by caspase activation may open way to NF-κB activation, resulting in cell survival.

CHX chase is a widely used method for analysing protein degradation (*23*). Combined results from our CHX chase experiments provide evidence that Nucling could undergo at least two proteolytic pathways mediated by proteasome and caspase. The results also reveal that Nucling was a short-lived protein, half-life of which is changed in response to different stimuli or inhibitors. Possibilities of other proteolytic pathways or degradation inducers related to Nucling may require further investigation.

Taken together, we propose a mechanism of Nucling expression and degradation. Nucling in the cells is in dynamic state, being continuously degraded and replaced by newly synthesized proteins. Degradation and resysthesis of Nucling is a highly regulated process. In physiological conditions, the cell keeps constant cellular Nucling level by a negative loop mechanism (Fig. 6A): low Nucling level opens the way for NF-κB to translocate to nucleus and to induce Nucling transcription. Then NF-κB is exported immediately by IκB (*10*). Newly synthesized Nucling in turn inhibits further NF-κB nuclear translocation. Excess Nucling undergoes proteasomal degradation until its level becomes low again, inducing resynthesis of Nucling.

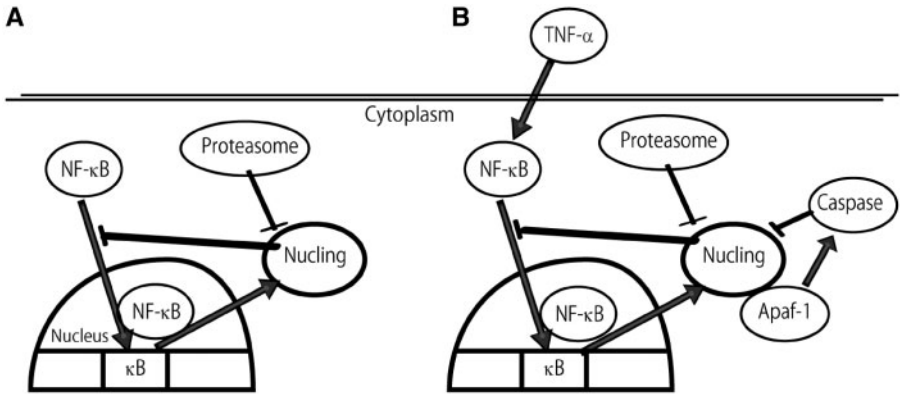

Fig. 6 Proposed mechanism of intracellular Nucling metabolism. (A) The cell keeps optimal balance of cellular Nucling level by a negative loop mechanism: low Nucling level opens the way for NF-κB to translocate to nucleus and to induce Nucling transcription. Then NF-κB is exported immediately by IκB-α. Newly synthesized Nucling in turn inhibits further nuclear translocation of NF-κB. Excess Nucling undergoes proteasomal degradation until its level becomes low again. (B) The cell turns on a pro-survival negative loop mechanism to inactivate Nucling in stress circumstances. TNF-α induced stress initiates recruitment of apoptosome by Nucling, followed by caspase activation. Caspase, in turn, cleaves Nucling to stop apoptosome recruitment.

Under stress conditions, the cell turns on a pro-survival negative loop mechanism to inactivate Nucling (Fig. 6B). Death signal initiates recruitment of Apaf1, cytochrome-*c* to form apoptosome complex (*2*) by Nucling, followed by caspase activation cascade. Caspase, in turn, cleaves Nucling to stop apoptosome recruitment. Low Nucling level may also cause reactivation of NF-κB and pro-survival genes. At this point, how the cell regulates its choice whether to undergo survival or apoptosis may depends on actual interaction among the regulators and pathways.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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