MOLECULAR AND CELLULAR BIOLOGY, July 2011, p. 2802–2816 Vol. 31, No. 14
0270-7306/11/$12.00 doi:10.1128/MCB.00253-10

Exogenous Signal-Independent Nuclear IκB Kinase Activation Triggered by Nkx3.2 Enables Constitutive Nuclear Degradation of IκB-α in Chondrocytes[▽]

Yeryoung Yong, Seung-Won Choi, Hye-Jeong Choi, Hyung Wook Nam, Jeong-Ah Kim, Da-Un Jeong, Don Young Kim, Yu Sam Kim, and Dae-Won Kim*

Department of Biochemistry, Yonsei University, Seoul 120-749, Republic of Korea

Received 7 March 2011/Returned for modification 6 April 2011/Accepted 11 May 2011

NF-κB is a multifunctional transcription factor involved in diverse biological processes. It has been well documented that NF-κB can be activated in response to various stimuli. While signal-inducible NF-κB activation mechanisms have been extensively characterized, exogenous signal-independent intrinsic NF-κB activation processes remain poorly understood. Here we show that IκB kinase β (IKKβ) can be intrinsically activated in the nucleus by a homeobox protein termed Nkx3.2 in the absence of exogenous IKK-activating signals. We found that ubiquitin chain-dependent, but persistent, interactions between Nkx3.2 and NEMO (also known as IKKγ) can give rise to constitutive IKKβ activation in the nucleus. Once the Nkx3.2-NEMO-IKKβ complex is formed in the nucleus, IKKβ-induced Nkx3.2 phosphorylation at Ser148 and Ser168 allows βTrCP to be engaged to cause IκB-α ubiquitination independent of IκB-α phosphorylation at Ser32 and Ser36. Taken together, our results provide a novel molecular explanation as to how an intracellular factor such as Nkx3.2 can accomplish persistent nuclear IKK activation to enable intrinsic and constitutive degradation of IκB in the nucleus in the absence of exogenous NF-κB-activating signals, which, in turn, plays a role in chondrocyte viability maintenance.

NF-κB regulates expression of a variety of genes associated with immune responses, proliferation, differentiation, and apoptosis (21, 23, 39). Numerous studies have described in detail the molecular mechanisms of signal-dependent NF-κB activation. In particular, it is well established that diverse NF-κB-activating signals trigger IκB kinase β (IKKβ) activation in the cytoplasm to permit signal-dependent IκB-α phosphorylation, which, in turn, causes its ubiquitination and proteasomal degradation, leading to NF-κB activation (3, 11, 13, 18, 21, 23, 39). While these extensive studies pertain to the signal-inducible NF-κB activation process, exogenous signal-independent intrinsic NF-κB activation mechanisms are largely unknown.

Nkx3.2 (also termed Bapx1) is initially expressed in chondrocyte precursor cells during development, and later, its expression is maintained in chondrocytes of various skeletal elements (19, 20, 25, 29, 37, 47). We have previously demonstrated that Nkx3.2 supports chondrocyte survival by constitutively activating p65-RelA, leaving p105 and p100 NF-κB unaffected (28). However, it remains to be understood how Nkx3.2, a nuclear homeobox protein, can indeed trigger constitutive IκB degradation and give rise to intrinsic NF-κB activation in chondrocytes in the absence of exogenous NF-κB-activating signals.

It has been suggested that polyubiquitin chains play a role in creating multiprotein complexes in signal-inducible NF-κB activation pathways (4, 9, 27, 34, 35, 41, 48). For instance, the ability of NEMO (also known as IKKγ) to recognize and interact with polyubiquitin chains has been shown to be critical for NEMO to establish functionally active IKK complexes in the cytoplasm in response to NF-κB-activating signals (4, 9, 34, 41, 48). While these findings pertain to signal-inducible events, a role for polyubiquitin chain-dependent complex formation during intrinsic NF-κB activation in the nucleus has not been investigated.

In this work, we demonstrate that ubiquitination-dependent complex formation between Nkx3.2 and NEMO allows persistent IKKβ activation in the nucleus in the absence of exogenous NF-κB-activating signals. Besides, according to the current paradigm of signal-dependent NF-κB activation, IκB proteins must be phosphorylated in its N terminus prior to its ubiquitination; however, we found that IKKβ-induced phosphorylation of Nkx3.2 allows βTrCP-mediated IκB-α ubiquitination independently of IκB-α phosphorylation at Ser32 and Ser36. Our results reveal a novel mechanism of intrinsic IKKβ activation leading to constitutive nuclear IκB degradation and NF-κB activation, which, in turn, supports cell viability maintenance in chondrocytes.

MATERIALS AND METHODS

Cell lines, chemical reagents, and antibodies. ATDC5 cells were maintained in Dulbecco modified Eagle medium (DMEM)–Ham's F-12 medium (1:1 dilution) supplemented with 5% fetal bovine serum (FBS). HEK293, 293T, C3H10T1/2, MEF-3T3, human polydactyl chondrocyte, and human fetal chondrocyte cells were grown in DMEM supplemented with 10% FBS. NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum. To generate cell lines stably expressing vector, wild-type Nkx3.2 ($Nkx3.2^{WT}$), Nkx3.2(K260/262R) ($Nkx3.2^{KR}$), or Nkx3.2(S148/168A) ($Nkx3.2^{SA}$) ATDC5 cells were transfected with the indicated pcDNA constructs and selected for neomycin resistance. Recombinant mouse tumor necrosis factor alpha (TNF-α) was obtained from R&D Systems, and BAY 11-7085 (BAY), IKK2 inhibitor-IV, and MG132 were

* Corresponding author. Mailing address: Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, 262 Seongsanno Seodaemoon-Gu, Seoul 120-749, Republic of Korea. Phone: 82-2-2123-5696. Fax: 82-2-362-9897. E-mail: kimdw@yonsei.ac.kr.

▽ Published ahead of print on 23 May 2011.

purchased from Calbiochem. Radioactive chemicals were purchased from Amersham. Anti-Myc rabbit polyclonal and anti-Myc monoclonal (9E10) antibodies were purchased from Upstate Biotechnology. Anti-hemagglutinin (HA) monoclonal antibody was purchased from Roche, and anti-V5 was obtained from Invitrogen. We obtained anti-Flag monoclonal M2 antibody, normal rabbit immunoglobulin G, and normal goat serum from Sigma, and anti-NEMO antibody was obtained from Santa Cruz Biotechnology. Anti-Nkx3.2 antibodies were purchased from Abcam and used where indicated, except in the work described below (see Fig. 3A). Anti-Nkx3.2 antibodies used in the work described below (see Fig. 3A) were custom-made by SeouLin Biosciences Co., and detailed information on the antibody production is available upon request. Anti-IKKβ, anti-cleaved PARP1, and anti-cleaved Caspase-3 antibodies were purchased from Cell Signaling Technology, and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was obtained from Abfrontier. Secondary antibodies for immunocytochemistry were obtained from Jackson Laboratory.

Expression plasmids and molecular cloning. Nkx3.2 expression plasmids were generated by subcloning expression constructs into pCS or pcDNA empty vectors. Point mutations of Nkx3.2, IκB, IKKβ, or NEMO were generated by two-step PCR mutagenesis of wild-type constructs and verified by DNA sequencing.

Transient-transfection and reporter assay. Transfection was performed using FuGene 6 (Roche) or VivaMagic (Vivagen). For reporter assays, 200 ng of the 4× κB-Luc reporter construct, 20 ng of pRL-TK normalization plasmid, and 200 ng of the indicated expression constructs per well of a 12-well plate were used. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega), and all transfections were performed in duplicate.

Coimmunoprecipitation and immunoblotting. Cells were washed in phosphate-buffered saline (PBS) and lysed in buffer containing 50 mM Tris at pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM imidazole, 1 mM NaF, 1.15 mM Na_2MoO_4, 1 mM Na_3VO_4, 4 mM $C_4H_4Na_2O_6$, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.5% or 1% NP-40, and protease inhibitors (complete, EDTA free; Roche). The cell extracts were centrifuged for 10 min at 13,000 × *g* at 4°C, and the supernatants were subjected to coimmunoprecipitation (co-IP) and Western blot analysis. Visualization of the immunoblots was performed using the ECL detection kit (GE Healthcare).

siRNA, shRNA and reverse transcription-PCR (RT-PCR). Double-stranded small interfering RNAs (siRNAs) for mouse IKKα, IKKβ, NEMO, and βTrCP and control siRNA were purchased from Santa Cruz and were transfected using Oligofectamine (Invitrogen). The Nkx3.2 short hairpin RNA (shRNA) lentiviral construct was purchased from Sigma, and lentiviral particles for Nkx3.2 shRNA were custom-made by Macrogen Co. as previously described (8). Total RNA was isolated using the RNAspin minikit (GE Healthcare). The SuperScript III cDNA synthesis kit (Invitrogen) was employed for cDNA synthesis. PCRs were performed with the following oligonucleotides, consisting of targeted mouse cDNA sequences: GAPDH, 5′-TTTGTGATGGGTGTGAACCACG-3′ (sense) and 5′-TTGTGAGGGAGATGCTCAGTGTTG-3′ (antisense); βTrCP, 5′-TGTTTGCAGTACAGAGACAGGCTG-3′ (sense) and 5′-ATGTAGGTGTATGTCCGAGAAGGG-3′ (antisense); Nkx3.2, 5′-AACCGTCGCTACAAGACCAAACG-3′ (sense) and 5′-GGGACGCAGGAATCCTTCTTTG-3′ (antisense); and Bcl-x_L, 5′-AACATCCCAGCTTCACATAACCCC-3′ (sense) and 5′-CCCGTAGAGATCCACAAAAGTGTCC-3′ (antisense).

Apoptosis assay. ATDC5 and stable cell lines were treated with 10 μM BAY 11-7085, an NF-κB inhibitor, and assayed for apoptosis. For RNA knockdown of Nkx3.2, ATDC5 cells were infected with Nkx3.2-shRNA lentiviruses for 60 h. For overexpression of kinase-dead IKKβ ($IKK\beta^{KM}$) or the D304N mutation of NEMO ($NEMO^{D304N}$), ATDC5 cells were infected with lentivirus for 48 h. For fluorescence-activated cell sorter (FACS) analyses, cells were trypsinized, washed with PBS containing 5% calf serum and 0.09% sodium azide, and then washed with 1× annexin V binding buffer (10 mM HEPES at pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cultures were then incubated with annexin V-fluorescein isothiocyanate (FITC) (BD Bioscience), and fluorescent cells were counted using FACSCalibur (BD Bioscience).

Mass spectrometric analysis. For in-gel digestion, Coomassie blue-stained protein spots were excised from the gel and destained with 50 mM NH_4HCO_3-50% acetonitrile. Samples were digested with 1 μg trypsin in 50 mM NH_4HCO_3 at 37°C for 18 h. The digested peptides were extracted in 0.1% formic acid-50% acetonitrile and dried in a Speed Vac. Peptides were analyzed by nanoscale liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS) to determine the amino acid sequences and phosphorylation sites. Ultimate nanoLC systems, combined with the FAMOS autosampler and Switchos column switching valve (LC Packings), were used. Samples were loaded onto a trap column (1-cm by 200-μm inside diameter [i.d.] 5-μm Zorbax 300SB-C_{18}; Agilent, CA) and washed with loading solvent (0.1% formic acid in H_2O) at a flow rate of 4 μl/min for 10 min to remove salts. Subsequently, a Switchos II column switching device transferred flow paths to the analytical column (15-cm by 75-μm i.d. 5-μm Zorbax 300SB-C_{18}; Agilent, CA). The nanoflow eluted at a rate of 200 nl/min using a 90-min gradient elution from 0% solvent A to 32% solvent B, where solvent A was 0.1% formic acid-5% acetonitrile and solvent B was 0.1% formic acid-90% acetonitrile. The column outlet was coupled directly to the nanoESI source, which was interfaced to the QSTAR mass spectrometer (Applied Biosystems). The data acquisition time was set to 3 s per spectrum over an *m/z* range of 400 to 1,500 Da for nanoLC-MS/MS analyses. Database searches were performed with a National Center for Biotechnology Information (NCBI) nonredundant database using the MASCOT software package (Matrix Sciences).

***In vitro* kinase assay.** To measure the total IKK activity, pcDNA or Nkx3.2 stable ATDC5 cells were treated with 5 ng/ml TNF-α for 1 h, where indicated, and lysed with buffer containing 50 mM Tris at pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM imidazole, 1 mM NaF, 1.15 mM Na_2MoO_4, 1 mM Na_3VO_4, 4 mM $C_4H_4Na_2O_6$, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.5% NP-40, and protease inhibitors (complete, EDTA free; Roche). For detection of nuclear IKK activity, pcDNA or Nkx3.2 stable ATDC5 cells, control ATDC5 cells, human polydactyl chondrocytes, C3H10T1/2 cells, and NIH 3T3 cells were scraped in PBS containing phosphatase inhibitors and resuspended with NEBA buffer (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA) containing phosphatase inhibitors, protease inhibitors, and 1 mM DTT. After 30 min of incubation on ice and vigorous vortexing, cells were centrifuged for 30 min at 10,000 × *g* at 4°C, and the supernatant was add to NEBC buffer (100 mM HEPES at pH 7.9, 750 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1% NP-40) containing phosphatase inhibitors, protease inhibitors, and 1 mM DTT for the cytosolic fraction. Cell pellets were resuspended with NEBD (20 mM HEPES at pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 25% glycerol, and 0.2% NP-40) containing phosphatase inhibitors, protease inhibitors, and 1 mM DTT. After 30 min of incubation on ice with vortexing every 5 min, cells were centrifuged for 10 min at 13,000 × *g* at 4°C. The supernatant was added to NEBE buffer (20 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 0.2% NP-40) containing phosphatase inhibitors, protease inhibitors, and 1 mM DTT for the nuclear fraction. Anti-NEMO immunoprecipitates prepared from whole-cell, cytosolic, or nuclear extracts were incubated with 1 μg glutathione *S*-transferase (GST)-IκB and [γ-^{32}P]ATP in kinase buffer (20 mM HEPES at pH 7.9, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, phosphatase inhibitors, and protease inhibitors) for 30 min. To measure Nkx3.2-associated IKK activity, transfected ATDC5 cells were lysed with buffer containing 50 mM Tris at pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM imidazole, 1 mM NaF, 1.15 mM Na_2MoO_4, 1 mM Na_3VO_4, 4 mM $C_4H_4Na_2O_6$, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.5% NP-40, and protease inhibitors. Anti-HA immunoprecipitates were treated with 5 μM IKK2 inhibitor-IV from Calbiochem for 5 min, where indicated, and then incubated with 1 μg GST-IκB and [γ-^{32}P]ATP in kinase buffer (20 mM HEPES at pH 7.9, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, phosphatase inhibitors, and protease inhibitors) for 30 min. Reactions were terminated by the addition of 10 mM EDTA and resolved by SDS-PAGE, followed by autoradiography.

Immunocytochemistry. Cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100–PBS containing 5% goat serum. Cells were then incubated with the indicated primary antibodies in PBS containing 1% goat serum and 0.02% Tween 20, followed by washing and incubation with the mixture of secondary antibody and 10 μM DAPI (4′,6-diamidino-2-phenylindole) in PBS containing 1% goat serum and 0.02% Tween 20. Samples were washed three times with PBS, treated with antifading solution, and processed for fluorescence microscopy.

RESULTS

Nuclear localization of IKKβ in a chondrogenic cellular background. It has been well established that IKKβ activation plays an instrumental role in signal-inducible IκB degradation and NF-κB activation (3, 11, 13, 18, 36). Since Nkx3.2-mediated RelA activation also involves IκB-α degradation, IKKβ may also be associated with Nkx3.2-mediated NF-κB activation in chondrocytes. However, previous studies have indicated that IKKβ functions in the cytoplasm to induce IκB phosphorylation in response to NF-κB-activating signals (6, 24, 26, 30, 46),

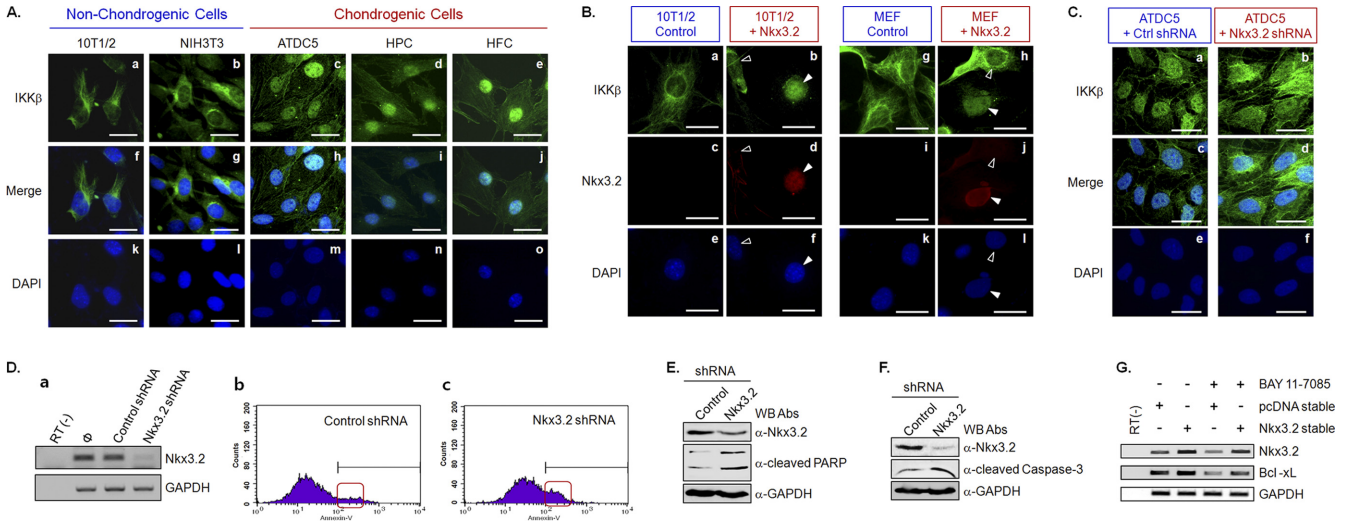

FIG. 1. Nuclear accumulation of IKKβ is required for cell survival in chondrocytes. (A) Subcellular localization of IKKβ in chondrogenic and nonchondrogenic cells. C3H10T1/2 cells (a, f, and k), NIH 3T3 cells (b, g, and l), ATDC5 cells (c, h, and m), HPC (d, i, and n), or HFC (e, j, and o) were immunostained with anti-IKKβ antibody (a to e) and stained with DAPI (k to o). The scale bars represent 25 μm. (B) Nkx3.2 induces nuclear translocation of endogenous IKKβ in nonchondrogenic cells. C3H10T1/2 cells (a to f) or MEFs (g to l) were transfected with an empty vector (a, c, e, g, i, and k) or the Nkx3.2-3Flag expression vector (b, d, f, h, j, and l) for 48 h and processed for immunocytochemistry using anti-IKKβ antibody (a, b, g, and h) or anti-Flag antibody (c, d, i, and j) (for Nkx3.2). Untransfected (open triangle) and transfected (closed triangle) cells are indicated. The scale bars represent 25 μm. (C) Nkx3.2 shRNA decreases nuclear localization of IKKβ. ATDC5 cells were infected with control or Nkx3.2 shRNA lentiviruses. After 48 h, immunocytochemistry was performed with anti-IKKβ antibody (a and b). The scale bars represent 25 μm. (D) RNA knockdown of endogenous Nkx3.2 causes spontaneous apoptosis in ATDC5. Control or Nkx3.2 shRNA lentiviral infection was performed with ATDC5 cells. After 60 h, GAPDH and Nkx3.2 mRNA levels were assessed by RT-PCR (a), and the percentage of apoptotic cells in control shRNA virus-infected ATDC5 cells (b) (6.7% of total cells) and Nkx3.2 shRNA virus-infected ATDC5 cells (c) (18.9% of total cells) was estimated by FACS. (E and F) Nkx3.2 shRNA can induce programmed cell death in HFC and HPC. Control or Nkx3.2 shRNA lentiviral infection was performed in human fetal chondrocytes (E) or human polydactyl chondrocytes (F) for 72 h, and total cell lysates were analyzed for apoptosis by Western blotting (WB) as indicated. Abs, antibodies. (G) IKK inhibition can modulate Nkx3.2-mediated induction of Bcl-x_L in ATDC5 cells. Vector or Nkx3.2 stable ATDC5 cells were treated with 10 μM BAY, and the levels of Bcl-x_L were estimated by RT-PCR analyses.

whereas exogenous signal-independent RelA activation mediated by Nkx3.2 in the chondrocyte takes place in the nucleus (28). Hence, as an initial step to reveal the involvement of IKKβ in Nkx3.2-mediated NF-κB activation, we examined subcellular localization of IKKβ in chondrogenic and nonchondrogenic cells. As would be expected, IKKβ resides mainly in the cytoplasm in nonchondrogenic cells such as C3H10T1/2 and NIH 3T3 cells (Fig. 1A, panels a and b). However, interestingly, IKKβ was detected primarily in the nucleus in chondrogenic cells such as mouse ATDC5 cells, human polydactyl chondrocytes (HPC), and human fetal chondrocytes (HFC) (Fig. 1A, panels c to e). In addition, Nkx3.2 transfection into nonchondrogenic cells such as C3H10T1/2 cells and mouse embryonic fibroblasts (MEFs) induced nuclear localization of endogenous IKKβ (Fig. 1B, panels b and h). Conversely, 48 h of lentiviral infection of Nkx3.2 shRNA into chondrogenic ATDC5 cells effectively increased cytoplasmic localization of endogenous IKKβ (Fig. 1C, compare panels a and b or panels c and d). Since we have observed from our multiple experiments that the total amounts of endogenous IKKβ (both mRNA and protein) are not significantly altered by either Nkx3.2 overexpression or knockdown (data not shown), these results suggest that the majority of IKKβ proteins can be retained in the nucleus with high levels of nuclear Nkx3.2 under control shRNA-infected conditions, whereas significant amounts of IKKβ proteins can be translocated to the cytoplasm as a consequence of Nkx3.2 knockdown. However, this interpretation of Fig. 1C may have to be taken carefully, as these phenomena may have been caused by nuclear membrane breakdown because the cells could be at the early stages of apoptosis due to Nkx3.2 knockdown. Nevertheless, the results shown in Fig. 1A to C together may indicate a reasonable correlation between Nkx3.2 expression levels and the nuclear localization levels of IKKβ. Furthermore, prolonged Nkx3.2 shRNA treatment (i.e., 60 to 72 h) was sufficient to trigger spontaneous programmed cell death in multiple chondrogenic cells, including mouse ATDC5 cells (Fig. 1D), human fetal chondrocytes (Fig. 1E), and human polydactyl chondrocytes (Fig. 1F), as judged by annexin V binding (see Fig. 4D), Procyclic acidic repetitive protein (PARP) cleavage (Fig. 1E), and Caspase-3 activation (Fig. 1F). Besides, we have found that stable overexpression of Nkx3.2 can elevate the levels of Bcl-x_L, a well-known antiapoptotic NF-κB target (38), while treatment with BAY 11-7085, an IKK inhibitor, can reduce Bcl-x_L expression (Fig. 1G, middle). Besides, importantly, the remaining levels of Bcl-x_L in BAY-treated pcDNA and BAY-treated Nkx3.2 stable cells are significantly different; i.e., BAY-treated Nkx3.2 stable cells maintain reduced but appreciable levels of Bcl-x_L (Fig. 1G, middle, compare lanes 3 and 4). Therefore, these results together indicate that Nkx3.2, which can be associated with chondrocyte-specific nuclear localization of IKKβ, plays a role in cell type-specific viability maintenance in chon-

drocytes by modulating the levels of Bcl-x_L. On the other hand, NEMO was basically detected both in the cytoplasm and in the nucleus regardless of cellular background (data not shown), and we were unable to observe coherent cell type-dependent differences in subcellular localization of NEMO between chondrogenic and nonchondrogenic cells that we examined.

Nkx3.2-mediated NF-κB activation requires IKKβ and NEMO. Since the results shown in Fig. 1 suggest that IKKβ may function in the nucleus, we next examined whether IKKβ plays a role in Nkx3.2-mediated NF-κB activation. Interestingly, unlike IKKα, IKKβ siRNA completely eliminated the ability of Nkx3.2 to functionally activate NF-κB, as judged by NF-κB-dependent reporter assays (Fig. 2A). The efficiencies of IKKα and IKKβ siRNAs were tested as shown in Fig. 2B. In addition, Nkx3.2-mediated NF-κB activation was potentiated by wild-type IKKβ ($IKK\beta^{WT}$) and diminished by kinase-dead IKKβ ($IKK\beta^{KM}$) (Fig. 2C). While these results suggest that IKKβ plays a role in Nkx3.2-mediated NF-κB activation, it has been shown that $IKK\beta^{KM}$ can inhibit IKKα function (1). Thus, to more carefully assess the involvement of IKKα and IKKβ in this process, we employed $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ MEF-3T3 cells (15, 22). Consistent with the findings shown in Fig. 2A and C, Nkx3.2 coexpression was able to induce functional activation of NF-κB in control and $IKK\alpha^{-/-}$ MEFs but not in $IKK\beta^{-/-}$ MEFs; moreover, reintroduction of IKKβ into $IKK\beta^{-/-}$ cells successfully restored the ability of Nkx3.2 to activate NF-κB (Fig. 2D). Thus, these results indicate that IKKβ is a significant component of Nkx3.2-mediated NF-κB activation.

Next, to further characterize the involvement of IKKβ, we examined the effects of $IKK\beta^{KM}$ on Nkx3.2-mediated IκB ubiquitination. Consistent with the results shown in Fig. 2C, $IKK\beta^{KM}$ efficiently inhibited IκB ubiquitination induced by Nkx3.2 (Fig. 2E, top), while $IKK\alpha^{KM}$ moderately enhanced IκB ubiquitination induced by Nkx3.2 (data not shown). However, importantly, the interaction between Nkx3.2 and IκB (Fig. 2E, middle) was not affected by $IKK\beta^{KM}$. Therefore, these findings suggest that Nkx3.2 requires IKKβ not to complex with IκB-α but to trigger IκB ubiquitination.

IKKγ (also known as NEMO), a regulatory subunit of IKK, has been shown to play an essential role in functionally activating catalytic subunits of IKK (5, 11, 18, 26, 31, 44). Since we found that IKKβ was functionally associated with Nkx3.2-mediated NF-κB activation, we next investigated whether NEMO is also involved in this process. While Nkx3.2 effectively induced protein degradation of IκB-α, as expected, NEMO siRNA substantially decreased such activity of Nkx3.2 (Fig. 2F, top). In addition, Nkx3.2-mediated NF-κB activation was also significantly diminished by NEMO siRNA (Fig. 2G). These results indicate that NEMO, along with IKKβ, is also a necessary component of Nkx3.2-mediated induction of IκB degradation and NF-κB activation.

One technical note can be stated here. As can be seen in Fig. 2A and G, loss of IKKβ or NEMO did not reduce basal activity of the NF-κB-dependent reporter gene in ATDC5 cells, which is somewhat confusing. However, this is likely a technical issue, and the following has been our interpretation. Chondrogenic lineage cells including ATDC5 characteristically have significant levels of steady-state NF-κB activation in the absence of exogenous stimuli, as we have previously shown (28). These already activated endogenous NF-κB proteins, which preexist prior to transfection, would be capable of activating NF-κB-dependent reporter gene expression immediately after the transfected reporter DNA plasmids are taken up by the cells. On the other hand, we have repeatedly observed that IKKβ (or NEMO) knockdown transfection requires at least 24 h or more to give rise to a meaningful reduction of endogenous IKKβ (or NEMO) mRNAs and proteins. Therefore, within the time frame of our conventional reporter assays (i.e., 36 to 48 h), IKKβ (or NEMO) knockdown treatment using siRNA transfection may not be an effective means to cause an evident and rapid decrease in basal NF-κB activity because the activation of transfected NF-κB-dependent reporter DNA by already activated endogenous NF-κB proteins would not be significantly inhibited by IKKβ (or NEMO) siRNA transfection at least for the first 24 h. In contrast, additional NF-κB-dependent reporter gene activation induced by transfected Nkx3.2 would be more efficiently inhibited by parallel transfection of IKKβ (or NEMO) siRNAs.

Nkx3.2 ubiquitination is necessary for Nkx3.2-NEMO interaction. As our results suggest that both IKKβ and NEMO are functionally associated with Nkx3.2, we next explored the possibility that Nkx3.2 may form a complex with IKKβ and/or NEMO. Interestingly, we were able to detect endogenous protein complexes of Nkx3.2-NEMO-IKKβ in ATDC5 cells by co-IP assays using anti-Nkx3.2 (Fig. 3A) or anti-NEMO (Fig. 3B) antibodies. In addition, interestingly, endogenous Nkx3.2 proteins in ATDC5 cells showed laddering electrophoretic migration patterns, and some of them displayed evidently higher molecular weights (MWs) than the theoretically calculated MW of Nkx3.2, which is 35,146. Besides, as can be seen in Fig. 3B, Nkx3.2 proteins showing the highest MWs were more enriched in the anti-NEMO IPs than in total cell lysates (Fig. 3B, compare Nkx3.2 signals in the second and fifth panels). Therefore, these results suggest that a posttranslational modification of Nkx3.2 may promote Nkx3.2-NEMO complex formation.

It is a possible that NEMO-associated Nkx3.2 showing higher MWs may represent ubiquitinated forms of Nkx3.2. Besides, it has been demonstrated that NEMO recognizes a necessary cofactor by sensing its ubiquitination during IKK complex activation processes (4, 9, 34, 41, 48). Therefore, we next investigated whether Nkx3.2 ubiquitination plays a role in Nkx3.2-NEMO complex formation. To do this, we first sought to find a mutant form of Nkx3.2 defective in basal ubiquitination, since Nkx3.2-mediated NF-κB activation is supposed to occur in the absence of exogenous stimuli. Thus, we generated a large number of Lys-to-Arg mutants of Nkx3.2, evaluated their basal (i.e., exogenous signal-independent) ubiquitination levels, and eventually found that the K260/262R mutation is most effective in eliminating basal ubiquitination of Nkx3.2 (Fig. 3C, top, lane 4 [the laddering electrophoretic migration patterns represent ubiquitinated forms of Nkx3.2]). The K244/251R mutation of Nkx3.2 in Fig. 3C, lane 3, was included as an internal negative control for K-to-R mutations. Mass spectrometric analyses further confirmed that the Nkx3.2 residues K260 and K262 are indeed ubiquitinated *in vivo* (data not shown). Thus, we employed Nkx3.2(K260/262R) ($Nkx3.2^{KR}$) as a mutant form of Nkx3.2 defective in basal ubiquitination. In addition, it can be noted here that, although most of the above-

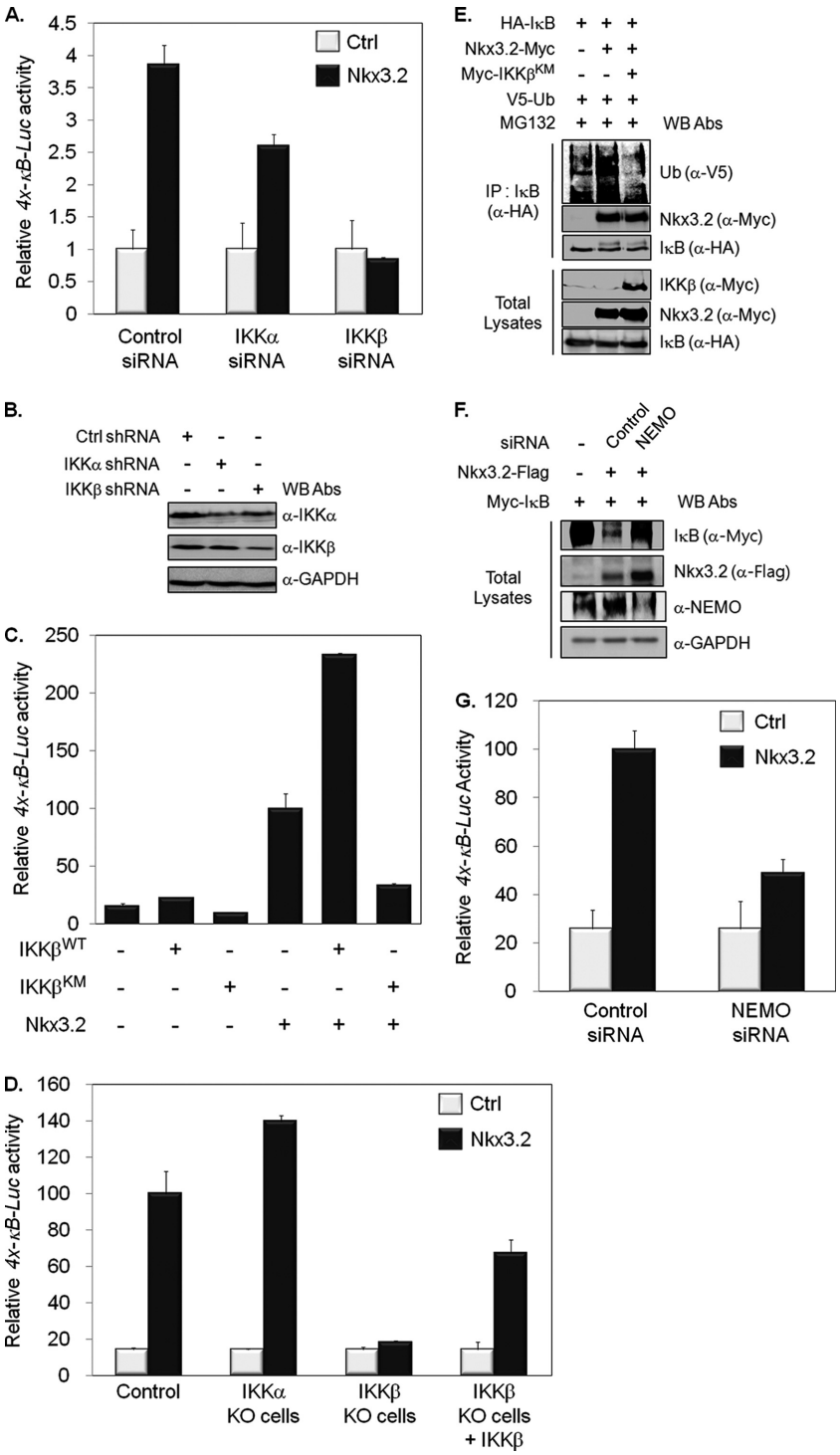

FIG. 2. IKKβ and NEMO are required for Nkx3.2-mediated NF-κB activation. (A) IKKβ siRNA but not IKKα siRNA eliminate Nkx3.2-mediated NF-κB activation. ATDC5 cells were transfected with control (Ctrl), IKKα, or IKKβ siRNA. Thirty hours later, cells were cotransfected with the 4× κB-Luc reporter gene and an empty vector or the Nkx3.2-3HA expression vector. After an additional 30 h, luciferase assays were performed. (B) IKKα and IKKβ knockdown by siRNAs. ATDC5 cells were transfected with control, IKKα, or IKKβ siRNA. After 40 h, total lysates were analyzed by Western blotting. (C) IKKβ plays a critical role in Nkx3.2-mediated NF-κB activation. The 4× κB-Luc reporter was transfected into ATDC5 cells with the $IKK\beta^{WT}$ or $IKK\beta^{KM}$ expression vector in the absence or presence of Nkx3.2. Cells were harvested after 48 h, and luciferase assays were performed. (D) Nkx3.2 is not able to activate NF-κB in $IKK\beta^{-/-}$ cells. Control MEF-3T3, $IKK\alpha^{-/-}$ MEF-3T3, or $IKK\beta^{-/-}$ MEF-3T3 cells were transfected with the 4× κB-Luc reporter in the absence or presence of expression constructs for Nkx3.2 or IKKβ. Luciferase activity was measured after 48 h of transfection. KO, knockout. (E) IKKβ is required for Nkx3.2-mediated IκB ubiquitination. Expression constructs for 3HA-IκB and 2V5-ubiquitin (Ub) were transfected into 293T cells in the absence or presence of the Nkx3.2-3Myc or 6Myc-$IKK\beta^{KM}$ expression plasmid for 24 h. Transfected cells were exposed to 20 μM MG132 for the final 6 h of incubation. Anti-HA

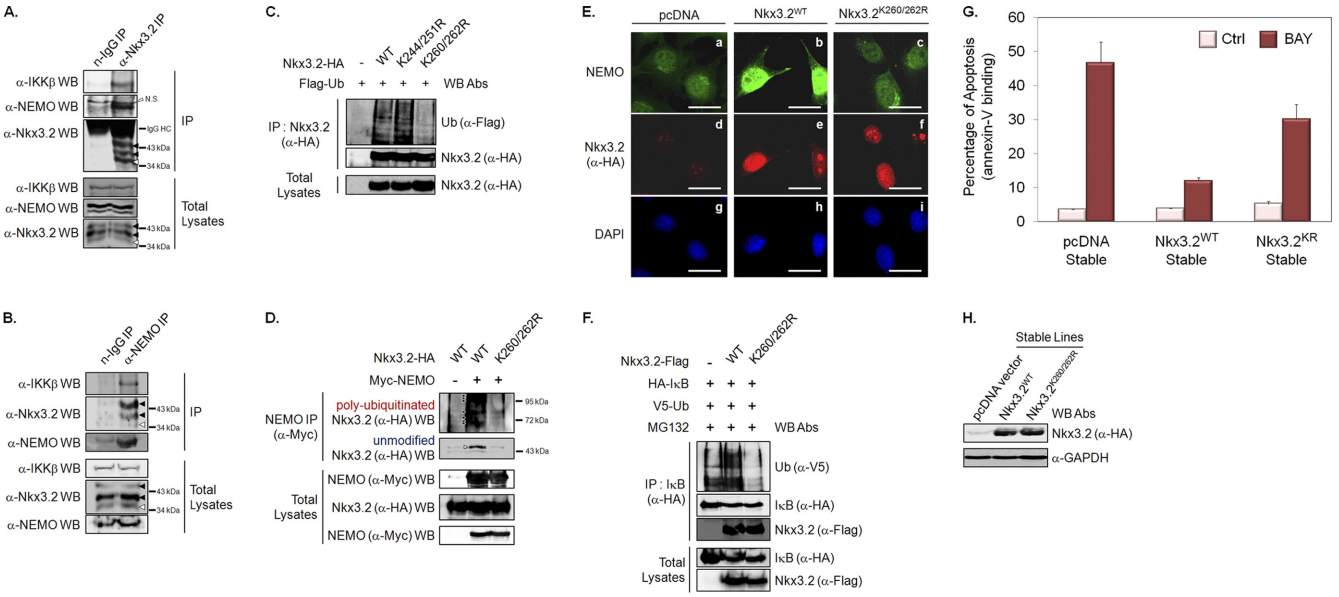

FIG. 3. Nkx3.2 ubiquitination is necessary for Nkx3.2 to interact with NEMO and to inhibit chondrocyte apoptosis. (A and B) Detection of the endogenous protein complex of Nkx3.2-NEMO-IKKβ. ATDC5 cell extracts were incubated with 5 μg of normal rabbit IgG (n-IgG) (A and B), anti-NEMO (A), or anti-Nkx3.2 (B) antibodies. Immunoprecipitates and total lysates were analyzed by Western blotting as indicated. Modified and unmodified Nkx3.2 proteins are marked with closed and open triangles, respectively. N.S., nonspecific; HC, heavy chain. (C) The K260/262R mutation decreases basal ubiquitination of Nkx3.2 in chondrocytes. The 3Flag-ubiquitin expression plasmid was transfected into ATDC5 cells in the absence or presence of 3HA-tagged Nkx3.2^{WT}, Nkx3.2(K244/251R), or Nkx3.2^{KR} expression plasmids. After 48 h, anti-HA immunoprecipitates and total lysates were analyzed by Western blotting. (D) The ubiquitination-defective Nkx3.2 mutant fails to interact with NEMO. 293T cells were transfected with 3HA-tagged Nkx3.2^{WT} or Nkx3.2^{KR} expression constructs with an empty vector or the 6Myc-NEMO expression vector for 24 h. Anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. Polyubiquitinated and nonubiquitinated forms of Nkx3.2 are indicated with closed (top panel) and open (second panel) triangles, respectively. (E) Nkx3.2 ubiquitination at Lys260 and Lys262 is required for Nkx3.2 to enhance nuclear translocation of NEMO. pcDNA vector (a, d, and g), Nkx3.2^{WT} (b, e, and h), or Nkx3.2^{KR} (c, f, and i) stable ATDC5 cells were immunostained with anti-NEMO antibody (a to c) or anti-HA antibody (d to f) (for Nkx3.2). The scale bars represent 25 μm. (F) Nkx3.2^{KR} is unable to cause IκB ubiquitination. Expression vehicles encoding 6Myc-IκB and 2V5-ubiquitin were transfected into 293T cells in the absence or presence of 3HA-tagged Nkx3.2^{WT} or Nkx3.2^{KR} expression plasmids for 24 h, including a final 6 h of exposure to MG132. Anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. (G and H) Nkx3.2^{KR} is not able to delay chondrocyte apoptosis. (G) Vector, Nkx3.2^{WT}, or Nkx3.2^{KR} stable ATDC5 cells were treated with 10 μM BAY, the percentage of apoptotic cells was estimated by three independently repeated FACS analyses, and the average values are presented. (H) Comparable levels of Nkx3.2 in Nkx3.2^{WT} or Nkx3.2^{KR} stable ATDC5 cells were confirmed by Western blotting.

described experiments related to Nkx3.2 ubiquitination were performed in chondrogenic ATDC5 cells, the Nkx3.2 ubiquitination process does not require particular chondrocyte-specific components since our multiple experiments verified that that exogenously expressed Nkx3.2 proteins can be effectively ubiquitinated in various nonchondrogenic cellular backgrounds as well.

Next, the effects of the K260/262R mutation of Nkx3.2 on its ability to interact with NEMO were examined. Interestingly, NEMO IP effectively coprecipitated polyubiquitinated forms of Nkx3.2^{WT}, whereas Nkx3.2^{KR} fails to interact with NEMO (Fig. 3D, top). In addition, nonubiquitinated Nkx3.2^{WT} was also detected in the Nkx3.2-NEMO complex (Fig. 3D, second panel, lane 2). However, it may not be the case that nonubiquitinated Nkx3.2 possesses affinities sufficient to form a stable complex with NEMO because nonubiquitinated Nkx3.2^{KR} was not capable of interacting with NEMO (Fig. 3D, second panel, lane 3). As a matter of fact, one important detail to be considered for more careful interpretation is that the NK family homeoproteins are capable of forming homodimers through the interactions between their homeodomains (45). Apart from this report, we have confirmed for ourselves that Nkx3.2 proteins tagged with different epitopes (e.g., HA and FLAG tagged) can be effectively dimerized (data not shown). Therefore, considering these results, it seems that nonubiquitinated Nkx3.2^{WT} can be included in Nkx3.2-NEMO complexes due to its ability to dimerize with ubiquitinated Nkx3.2^{WT}. On the other hand, such a possibility is not applicable for nonubiq-

immunoprecipitates and total lysates were analyzed by Western blotting. (F) Nkx3.2-mediated IκB degradation requires NEMO. ATDC5 cells were transfected with control or NEMO siRNA. At 12 h posttransfection, the cells were transfected with the 6Myc-IκB expression plasmid in the absence or presence of the Nkx3.2-3Flag expression constructs. After an additional 60 h, total lysates were analyzed by Western blotting. (G) NEMO siRNA reduces Nkx3.2-mediated NF-κB activation. ATDC5 cells were transfected with control or NEMO siRNA. Thirty hours later, cells were transfected with the 4× κB-Luc reporter with an empty vector or the Nkx3.2-3HA expression vector. After an additional 30 h, cells were harvested, and luciferase assays were performed.

uitinated Nkx3.2^{KR}, which is defective in being ubiquitinated. Thus, taken together, Nkx3.2 ubiquitination at Lys260 and Lys262 may play a critical role in stable complex formation between Nkx3.2 and NEMO.

Then we investigated whether Nkx3.2 can enhance nuclear localization of NEMO in a ubiquitination-dependent manner since the Nkx3.2-mediated NF-κB activation pathway is presumed to function in the nucleus (28). Immunostaining of endogenous NEMO in pcDNA vector, Nkx3.2^{WT}, and Nkx3.2^{KR} stable cells revealed that stable overexpression of Nkx3.2^{WT} in ATDC5 increases nuclear staining of endogenous NEMO, while Nkx3.2^{KR} lacks this activity (Fig. 3E, panels a to c). Since various previous experiments performed in our laboratory have indicated that the total amounts of endogenous NEMO (both mRNA and protein) are not significantly modulated by either Nkx3.2 overexpression or knockdown (data not shown), we interpret these results as follows: a subpopulation of endogenous NEMO proteins that are scattered in the cytoplasm of vector stable ATDC5 cells can be further recruited into the nucleus by the elevated levels of nuclear Nkx3.2 in Nkx3.2 stable ATDC5 cells. Furthermore, considering the nature of immunofluorescence microscopy, concentrated staining (e.g., restricted nuclear staining) is generally more effective in generating intensive signals than scattered staining (e.g., spread cytoplasmic staining). Therefore, the results shown in Fig. 3D and E together may suggest that Nkx3.2 ubiquitination plays a role in stabilizing a nuclear complex of Nkx3.2 and NEMO.

Next, to assess the functional importance of this ubiquitination-dependent Nkx3.2-NEMO interaction, we investigated whether Nkx3.2 ubiquitination is necessary for Nkx3.2-mediated IκB ubiquitination. Consistent with other results, we found that the ability of Nkx3.2 to enhance IκB ubiquitination was eliminated in Nkx3.2^{KR} (Fig. 3F). In addition, while stable overexpression of Nkx3.2^{WT} in ATDC5 cells effectively delayed chondrocyte apoptosis induced by NF-κB inhibition, as previously shown (28), Nkx3.2^{KR} stable cells failed to exhibit such activity (Fig. 3G). Comparable expression levels of Nkx3.2 in Nkx3.2^{WT} and Nkx3.2^{KR} stable cells were verified by Western blotting (Fig. 3H). Thus, these results indicate that Nkx3.2 ubiquitination at Lys260 and Lys262 is required for Nkx3.2 to be capable of inducing NF-κB activation and accumulating functionally active NF-κB proteins in the cell, which allows Nkx3.2 stable cells to remain viable against apoptotic challenges caused by NF-κB inhibition longer than vector stable cells. We believe that this phenomenon is associated with the differences in the rate of Bcl-x_L decrease.

The ubiquitin chain sensing ability of NEMO is required for Nkx3.2-NEMO interaction. Since we found that Nkx3.2 ubiquitination is critical for Nkx3.2-NEMO complex formation, we next investigated whether the ability of NEMO to recognize and interact with polyubiquitin chains plays a role in this process. To this end, we employed a polyubiquitin chain binding-defective NEMO mutant termed NEMO(D304N) (7, 40, 41). Co-IP assays revealed that the interaction between Nkx3.2 and NEMO can be significantly attenuated by the D304N mutation of NEMO (Fig. 4A, second panel), and polyubiquitinated forms of NEMO were detected in the Nkx3.2-NEMO^{WT} complex (Fig. 4A, top panel, lane 2, closed triangles). These results suggest that polyubiquitin chain recognition by NEMO may not be required for the initial contacts between Nkx3.2 and NEMO but plays a crucial role in further stabilizing the Nkx3.2-NEMO complex.

Next, we evaluated the functional importance of NEMO's ability to sense and bind polyubiquitin chains in Nkx3.2-mediated NF-κB activation. Interestingly, NEMO^{D304N} coexpression efficiently inhibited NF-κB activation by Nkx3.2 (Fig. 4B). Besides, Nkx3.2-induced IκB ubiquitination was enhanced by NEMO^{WT} and diminished by NEMO^{D304N} (Fig. 4C, top panel). However, importantly, the interaction between Nkx3.2 and IκB was not affected by either NEMO^{WT} or NEMO^{D304N} (Fig. 4C, second panel). Therefore, these results demonstrate that, similar to the requirement of IKKβ, NEMO's ability to sense and bind polyubiquitin chains is necessary for Nkx3.2 to induce IκB ubiquitination subsequent to Nkx3.2-IκB complex formation.

Since our results suggest that sensing polyubiquitin chains of Nkx3.2 by NEMO is critical for Nkx3.2-induced NF-κB activation, we next asked whether polyubiquitin chain-dependent Nkx3.2-NEMO interaction plays a role in chondrocyte viability. Interestingly, lentiviral infection of NEMO^{D304N} alone was sufficient to cause significant apoptosis in ATDC5 cells in the absence of additional apoptotic stimuli (Fig. 4D). Besides, it was intriguing to observe that NEMO^{D304N} overexpression does not induce programmed cell death in nonchondrogenic cells such as NIH 3T3 and HEK293 cells (Fig. 4D). Therefore, these results indicate that the ability of NEMO to sense polyubiquitin chains is crucial for chondrocyte-specific viability maintenance.

The polyubiquitin chain-dependent Nkx3.2-NEMO interaction establishes a functional IKK complex in the nucleus independent of the exogenous NF-κB-activating signal. The results obtained up to this point lead us hypothesize that stable interactions between Nkx3.2 and NEMO may, in turn, trigger the recruitment of IKKβ for the establishment of a functionally competent IKK complex. To test this hypothesis, the effects of Nkx3.2^{KR} and NEMO^{D304N} on Nkx3.2-NEMO-IKKβ complex formation were examined. Co-IP assays revealed that Nkx3.2^{KR}, compared to Nkx3.2^{WT}, has a significantly reduced ability to form a complex with NEMO and IKKβ (Fig. 4E, top and second panels). Besides, Nkx3.2-IKKβ co-IP can be significantly enhanced by NEMO^{WT} and effectively abolished by NEMO^{D304N} (Fig. 4F, top panel). Furthermore, interestingly, we have found that both NEMO^{WT} and NEMO^{D304N} are competent in forming a complex with IKKβ (Fig. 4G). Therefore, these results clearly indicate that NEMO^{D304N} affects Nkx3.2-NEMO-IKKβ complex formation, since the D304N mutation of NEMO attenuates its ability to stably interact with Nkx3.2 rather than IKKβ. Taken together, we conclude that ubiquitination-dependent interactions between Nkx3.2 and NEMO play a crucial role in establishing and stabilizing the complex of Nkx3.2-NEMO-IKKβ.

Next, we sought to determine whether Nkx3.2 is capable of functionally activating IKK in addition to its ability to form a complex with IKKβ and NEMO. To address this, IKK-specific *in vitro* kinase assays were performed. The IKK activities measured from whole-cell extracts and cytosolic fractions were not dramatically different between Nkx3.2 stable and pcDNA vector stable ATDC5 cells (Fig. 4H, lanes 1, 2, 5, and 6), and TNF-α treatment elicited an additional increase in IKK activity

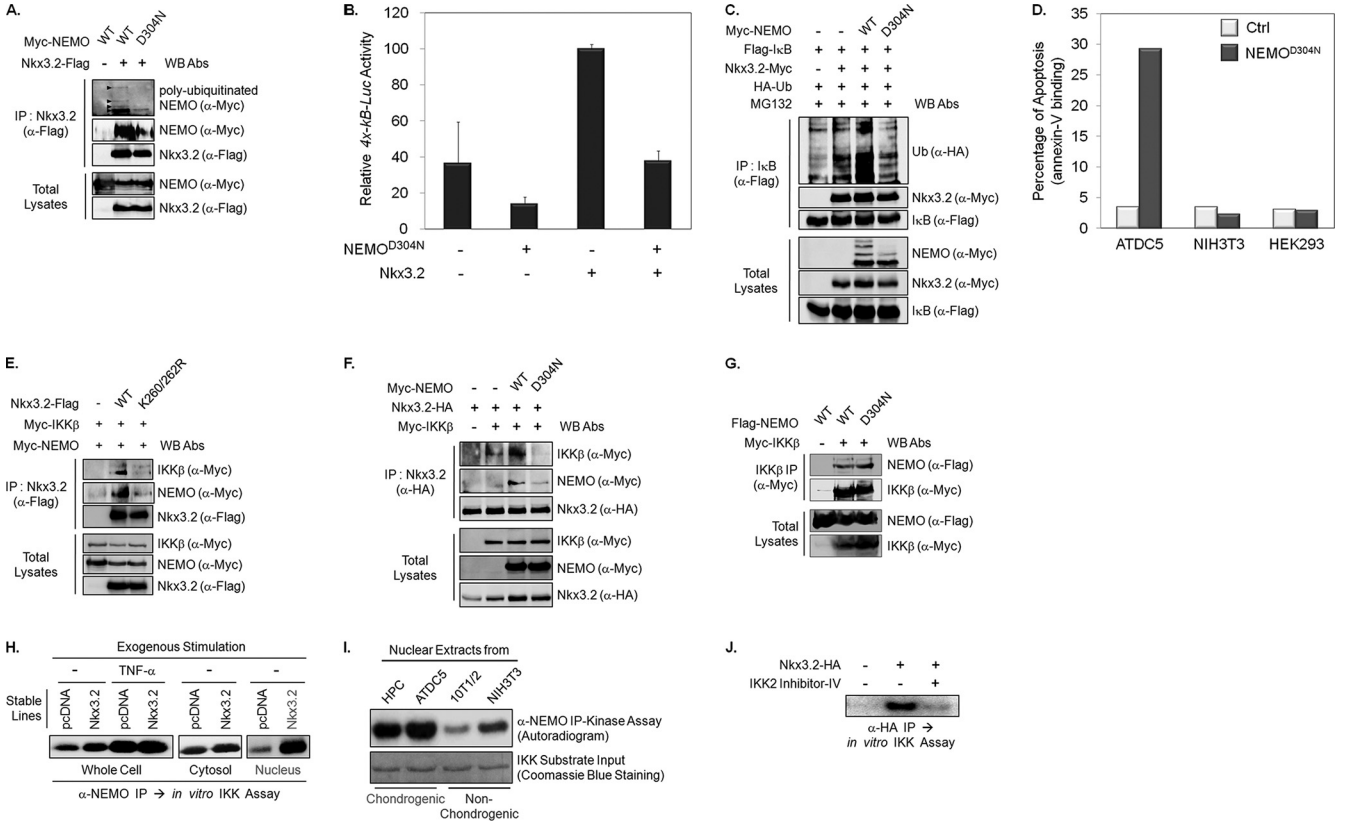

FIG. 4. The polyubiquitin chain sensing ability of NEMO is required for Nkx3.2-NEMO interaction and for IKKβ recruitment to the Nkx3.2-NEMO complex. (A) The polyubiquitin chain binding-defective NEMO mutant is unable to form a complex with Nkx3.2. 293T cells were transfected with 6Myc-tagged $NEMO^{WT}$ or $NEMO^{D304N}$ expression vectors in the absence or presence of the Nkx3.2-3Flag expression plasmid. After 40 h of transfection, the cells were harvested and subjected to anti-Flag immunoprecipitation. The anti-Flag immunoprecipitates and total lysates were analyzed by Western blotting. (Top panel) Polyubiquitinated forms of NEMO are marked with closed triangles. (B) $NEMO^{D304N}$ inhibits Nkx3.2-mediated NF-κB activation. The 4× κB-Luc reporter gene was transfected into ATDC5 cells with an empty vector or the Nkx3.2-3HA expression plasmid in the absence or presence of the 6Myc-$NEMO^{D304N}$ expression construct. Luciferase activity was measured from total cell extracts at 48 h after transfection. (C) Nkx3.2-mediated IκB ubiquitination is enhanced by $NEMO^{WT}$ and diminished by $NEMO^{D304N}$. Expression constructs for 3Flag-IκB and 3HA-ubiquitin (Ub) were cotransfected into 293T cells with the Nkx3.2-3Myc expression vector in the absence or presence of 6Myc-tagged $NEMO^{WT}$ or $NEMO^{D304N}$ expression plasmids. After 24 h of transfection, which included a final 6 h of MG132 incubation, the cells were harvested. Anti-Flag immunoprecipitates and total lysates were analyzed by Western blotting. (D) The polyubiquitin chain binding ability of NEMO is necessary for chondrocyte survival. ATDC5, NIH 3T3, or HEK293 cells were infected with vector- or $NEMO^{D304N}$-encoding lentiviruses. The percentage of apoptotic cells was estimated by FACS. (E) Nkx3.2 ubiquitination is required for Nkx3.2-IKKβ-NEMO complex formation. 293T cells were transfected with 6Myc-NEMO and 6Myc-IKKβ expression constructs in the absence or presence of the 3Flag-tagged $Nkx3.2^{WT}$ or $Nkx3.2^{KR}$ expression plasmid. After 24 h, anti-Flag immunoprecipitates and total lysates were analyzed by Western blotting. (F) $NEMO^{WT}$ stabilizes and $NEMO^{D304N}$ destabilizes the Nkx3.2-NEMO-IKKβ complex. ATDC5 cells were transfected with the Nkx3.2-3HA expression construct with or without the 6Myc-IKKβ expression vector in the absence or presence of 6Myc-tagged $NEMO^{WT}$ or $NEMO^{D304N}$ expression plasmids. The cells were harvested after 24 h of transfection, and anti-HA immunoprecipitates and total lysates were analyzed by Western blotting. (G) Both $NEMO^{WT}$ and $NEMO^{D304N}$ are able to interact with IKKβ. Expression constructs for 6Myc-IKKβ were cotransfected into ATDC5 cells with 3Flag-tagged $NEMO^{WT}$ or $NEMO^{D304N}$ expression plasmids. The cells were harvested after 30 h, and anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. (H) Nuclear IKK activity can be augmented by stable overexpression of Nkx3.2. Vector or Nkx3.2 stable ATDC5 cells were treated with 5 ng/ml TNF-α for 1 h as indicated. Anti-NEMO immunoprecipitation was performed with 500 μg of whole-cell extract (lanes 1 to 4), 1 mg of cytosolic fraction (lanes 5 and 6), or 200 μg of nuclear extract (lanes 7 and 8), and the indicated immunoprecipitates were incubated with 1 μg of GST-IκB and[γ-^{32}P]ATP at room temperature. After 30 min, the reaction mixture was resolved by SDS-PAGE and processed for autoradiography. (I) Chondrogenic lineage cells exhibit augmented steady-state nuclear IKK activity. Nuclear extracts isolated from vector or Nkx3.2 stable ATDC5 cells were treated with 5 ng/ml TNF-α for 1 h as indicated. Anti-NEMO immunoprecipitation was performed with 200 μg of nuclear extract isolated from HPC or ATDC5, C3H10T1/2, or NIH 3T3 cells, and the indicated immunoprecipitates were processed for *in vitro* IKK assays. (J) Nkx3.2 can be associated with IKK activity. ATDC5 cells were transfected with an empty vector (lane 1) or the Nkx3.2-3HA expression plasmid (lanes 2 and 3). Forty-eight hours after transfection, anti-HA immunoprecipitation was carried out with 500 μg of whole-cell extracts. The indicated immunoprecipitates were preincubated with dimethyl sulfoxide (DMSO) (lanes 1 and 2) or 5 μM IKK2 inhibitor-IV for 5 min prior to the addition of GST-IκB and [γ-^{32}P]ATP. The reaction mixture was analyzed by SDS-PAGE and autoradiography.

in both vector and Nkx3.2 stable cells (Fig. 4H, lanes 3 and 4). However, interestingly, nuclear IKK activity was remarkably elevated by stable overexpression of Nkx3.2 in the absence of exogenous IKK activation signals (Fig. 4H, lanes 7 and 8). To further substantiate these results, we determined the levels of steady-state nuclear IKK activity between chondrogenic and nonchondrogenic lineage cells. Consistent with the results shown in Fig. 4H, significantly greater IKK activities were measured from the nuclear extracts prepared from chondrogenic human polydactyl chondrocytes (HPC) and ATDC5 cells

than from nonchondrogenic C3H10T1/2 and NIH 3T3 cells (Fig. 4I). Therefore, we consider that the results shown in Fig. 4H and I together may indicate a reasonable connection between Nkx3.2 expression levels and nuclear IKK activity levels. Finally, we were able to isolate substantial IKK activity by immunoprecipitation of ectopically expressed Nkx3.2 in ATDC5, and this Nkx3.2-associated IKK activity was effectively inhibited by an IKKβ-specific inhibitor termed IKK2 inhibitor-IV (Fig. 4J). Thus, these results together indicate that Nkx3.2 can establish a functional IKK complex in the nucleus, leaving the activation status of cytosolic IKK relatively unaltered.

IκB-α phosphorylation at Ser32 and Ser36 is not required for Nkx3.2 to induce IκB-α ubiquitination and NF-κB activation. IκB phosphorylation in its N terminus by IKKβ has been shown to be essential for subsequent IκB degradation (3, 13, 36). Since we have found that IKKβ is required for Nkx3.2-mediated NF-κB activation, we asked whether IκB-α phosphorylation at Ser32 and Ser36 is required for Nkx3.2-mediated NF-κB activation, using a phosphorylation-defective IκB-α mutant termed IκB(S32/36A) ($I\kappa B^{SA}$) (3, 36). Consistent with the previous reports, functional activation of NF-κB by an exogenous stimulation, such as exposure to TNF-α, was effectively attenuated by wild-type IκB ($I\kappa B^{WT}$) and completely abolished by $I\kappa B^{SA}$ (Fig. 5A). However, surprisingly, Nkx3.2-mediated NF-κB activation was not affected by the presence of either $I\kappa B^{WT}$ or $I\kappa B^{SA}$, suggesting that IκB phosphorylation at Ser32 and Ser36 may not be an essential event for Nkx3.2 to cause NF-κB activation (Fig. 5B). These results prompted us to examine whether IκB phosphorylation is necessary for Nkx3.2 to induce IκB ubiquitination. As would be expected, TNF-α treatment augmented ubiquitination of $I\kappa B^{WT}$ but not of $I\kappa B^{SA}$ (Fig. 5C). On the contrary, Nkx3.2 efficiently increased ubiquitination of both $I\kappa B^{WT}$ and $I\kappa B^{SA}$ (Fig. 5D). Therefore, these results clearly demonstrate that Nkx3.2 is capable of inducing IκB-α ubiquitination in the absence of its phosphorylation at Ser32 and Ser36.

Since Nkx3.2 is required to form a complex with IκB-α in the nucleus to render RelA activation (28), we next examined whether IκB-α phosphorylation status would alter the interaction between Nkx3.2 and IκB-α in the nucleus. To this end, we employed a bimolecular fluorescence complementation (BiFC) assay (14). In brief, two nonfluorescent fragments of yellow fluorescent protein (YFP) can reestablish fluorescence when they are brought together by interactions between proteins fused to each fragment of YFP. Using this YFP-based BiFC assay, we confirmed that Nkx3.2 can directly interact with $I\kappa B^{SA}$ as well as $I\kappa B^{WT}$ in the nucleus (Fig. 5E). Besides, co-IP assays also demonstrated that Nkx3.2 can stably interact with both $I\kappa B^{WT}$ and $I\kappa B^{SA}$, and immunocytochemistry assays further confirmed that Nkx3.2 can cause nuclear translocation of both $I\kappa B^{WT}$ and $I\kappa B^{SA}$ (data not shown). Taken together, these results indicate that Nkx3.2 is able to trap IκB in the nucleus regardless of its phosphorylation status at Ser32 and Ser36.

As shown in Fig. 2, IKKβ is required for Nkx3.2-mediated NF-κB activation. On the other hand, it is evident from the results shown in Fig. 5 that IκB phosphorylation at Ser32 and Ser36 presumably mediated by IKKβ is dispensable for Nkx3.2 to induce IκB ubiquitination and NF-κB activation. Thus, to unambiguously rule out the possibility that IKKβ may function via IκB phosphorylation, we next examined the effects of $IKK\beta^{KM}$ on Nkx3.2-induced ubiquitination of $I\kappa B^{SA}$, which carries mutations at IKKβ phosphorylation sites (3, 36). Consistent with the results shown in Fig. 5D, Nkx3.2 was capable of inducing ubiquitination of $I\kappa B^{SA}$ (Fig. 5F, top panel, lane 2). However, interestingly, Nkx3.2 failed to cause $I\kappa B^{SA}$ ubiquitination in the presence of $IKK\beta^{KM}$ (Fig. 5F, top panel, lane 3), while the interaction between Nkx3.2 and $I\kappa B^{SA}$ was not modulated by $IKK\beta^{KM}$ (Fig. 5F, second panel). Therefore, these results demonstrate that IKKβ has a crucial role in Nkx3.2-mediated IκB-α ubiquitination other than IκB-α phosphorylation at Ser32 and Ser36.

IKKβ-dependent Nkx3.2 phosphorylation plays a role in Nkx3.2-mediated NF-κB activation subsequent to Nkx3.2-NEMO-IKKβ complex formation. So far in this work, we have found that IKKβ is necessary for Nkx3.2-mediated NF-κB activation despite the fact that IKKβ-mediated IκB-α phosphorylation at Ser32 and Ser36 is not required and that Nkx3.2 is able to functionally activate IKKβ in the nucleus in association with NEMO. Hence, we decided to next explore the possibility that IKKβ may phosphorylate Nkx3.2 and regulate its function. Interestingly, *in vitro* kinase assays revealed that, at least *in vitro*, Nkx3.2 can be a direct substrate of IKKβ but not of IKKα (Fig. 6A). In addition, mass spectrometric analyses indentified that the Nkx3.2 residues Ser146 (or Ser148) and Ser168 are specifically phosphorylated *in vivo* in response to IKKβ coexpression (Fig. 6B). Therefore, we generated and characterized Nkx3.2 mutants, including those with S146A, S148A, S168A, S148/168A, and S146/168A mutations, and found that the S148/168A mutation was most effective in eliminating the ability of Nkx3.2 to activate NF-κB in association with IKKβ (data not shown). Consistent with these findings, we found that, unlike $Nkx3.2^{WT}$, Nkx3.2(S148/168A) ($Nkx3.2^{SA}$) was unable to induce IκB-α ubiquitination (Fig. 6C). Furthermore, stable overexpression of $Nkx3.2^{SA}$, in contrast to that of $Nkx3.2^{WT}$, failed to delay chondrocyte apoptosis induced by NF-κB inhibition (Fig. 6D). However, interestingly, despite their functional differences in terms of causing IκB ubiquitination (Fig. 6C) and delaying programmed cell death (Fig. 6D), $Nkx3.2^{WT}$ and $Nkx3.2^{SA}$ were equally competent in forming complexes with IKKβ and NEMO (Fig. 6E). Thus, these results suggest that IKKβ-induced Nkx3.2 phosphorylation becomes necessary for steps subsequent to Nkx3.2-IKKβ-NEMO complex formation.

βTrCP can function in Nkx3.2-mediated IκB ubiquitination in the nucleus. Our results to this point led us to hypothesize that IKKβ-induced Nkx3.2 phosphorylation may enhance the recruitment of a ubiquitin ligase complex responsible for Nkx3.2-induced IκB ubiquitination. It has been well documented for the classical NF-κB activation pathways that βTrCP is responsible for signal-inducible IκB ubiquitination in the cytoplasm (12, 32). However, to the best of our knowledge, no ubiquitin ligases associated with nuclear ubiquitination of IκB have been characterized.

To identify an E3 ligase engaged in Nkx3.2-mediated IκB ubiquitination, we first investigated whether the IκB-α residues Lys21 and Lys22 are associated with this process. As can be seen in Fig. 7A, when the IκB mutant IκB(K21/22R) ($I\kappa B^{KR}$), which carries mutations at sites of ubiquitination mediated by

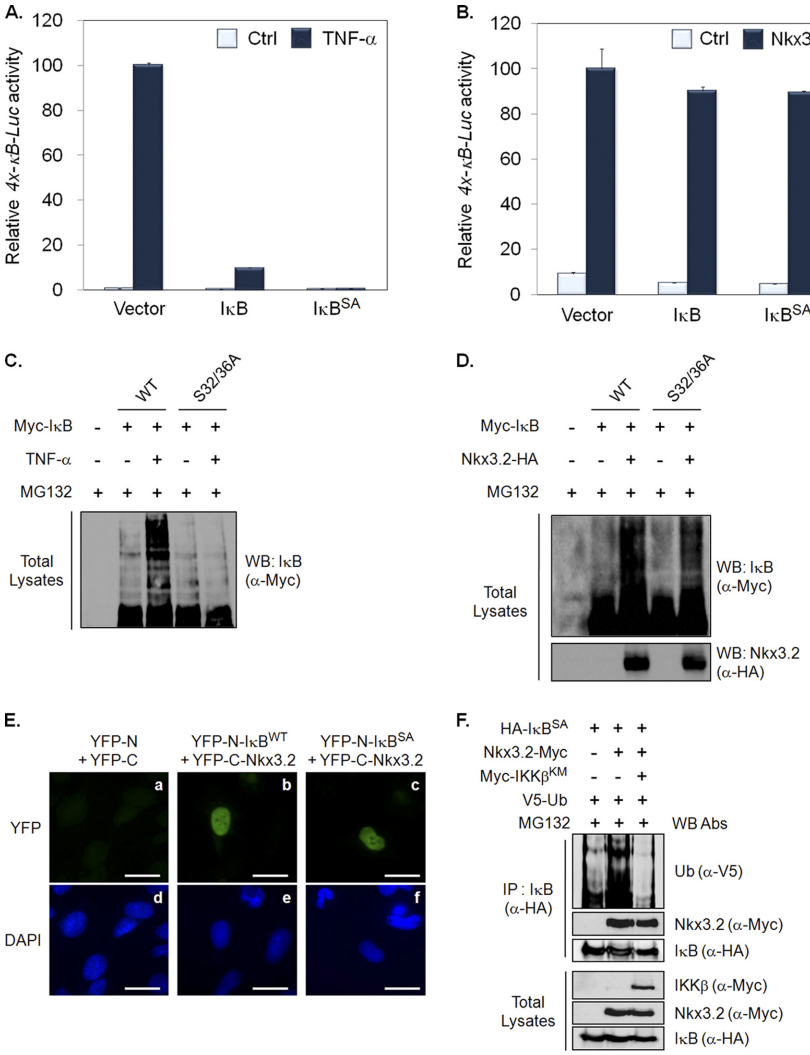

FIG. 5. IκB phosphorylation is not required for Nkx3.2 to induce IκB ubiquitination. (A) IκB phosphorylation at Ser32 and Ser36 is essential for NF-κB activation by TNF-α. 293T cells were transfected with the 4× κB-Luc reporter with an empty vector or the 6Myc-tagged $I\kappa B^{WT}$ or $I\kappa B^{SA}$ expression plasmid. A subset of transfected cells was treated with 10 ng/ml TNF-α for the final 6 h of transfection. Luciferase activity was measured from total cell extracts. (B) $I\kappa B^{SA}$ is not able to suppress NF-κB activation mediated by Nkx3.2. 293T cells were transfected with the 4× κB-Luc reporter constructs with an empty vector, 6Myc-$I\kappa B^{WT}$, or 6Myc- $I\kappa B^{SA}$ expression plasmid in the absence or presence of the Nkx3.2-3HA expression vector. After 30 h, luciferase activity was measured from total cell extracts. (C) IκB phosphorylation is required for TNF-α-induced IκB ubiquitination. 293T cells were transfected with the 6Myc-tagged $I\kappa B^{WT}$ or $I\kappa B^{SA}$ expression plasmid for 24 h. Transfected cells were pretreated with MG132 for 30 min before a final 1 h of incubation with 10 ng/ml TNF-α. Total lysates were analyzed by Western blotting. (D) IκB phosphorylation is dispensable for IκB ubiquitination mediated by Nkx3.2. Expression vehicles for 6Myc-tagged $I\kappa B^{WT}$ or $I\kappa B^{SA}$ were transfected into 293T cells in the absence or presence of the Nkx3.2-3HA expression plasmid for 24 h. Transfected cells were treated with MG132 for the final 6 h of incubation. Total lysates were analyzed by Western blotting. (E) Nuclear interaction of Nkx3.2 and IκB-α is not affected by the S32/36A mutations of IκB-α. ATDC5 cells were cotransfected with expression vectors encoding N-terminal YFP (YFP-N) (a and d), YFP-N–$I\kappa B^{WT}$ (b and e), or YFP-N–$I\kappa B^{SA}$ (c and f) with C-terminal YFP (YFP-C) (a and d) or the YFP-C–Nkx3.2 expression plasmid (b, c, e, and f) for 48 h. Transfected cells were exposed to MG132 for the final 6 h of incubation. YFP fluorescence (a, b, and c) and DAPI staining (d, e, and f) were detected. The scale bars represent 25 μm. (F) IKKβ is required for Nkx3.2-mediated $I\kappa B^{SA}$ ubiquitination. Expression constructs for 3HA-$I\kappa B^{SA}$ and 2V5-ubiquitin were transfected into 293T cells in the absence or presence of the Nkx3.2-3Myc or 6Myc-$IKK\beta^{KM}$ expression plasmid for 24 h. Transfected cells were treated with MG132 for the final 6 h of incubation. Anti-HA immunoprecipitates and total lysates were analyzed by Western blotting.

βTrCP, was tested, Nkx3.2 failed to enhance ubiquitination of $I\kappa B^{KR}$ (Fig. 7A, lane 7), while both $I\kappa B^{WT}$ and $I\kappa B^{SA}$ displayed elevated levels of ubiquitination in the presence of Nkx3.2 (Fig. 7A, lanes 3 and 5). Since these results indicate that βTrCP-mediated IκB-α ubiquitination and Nkx3.2-induced IκB-α ubiquitination employ the same lysine residues *per se*, we next examined the possibility that βTrCP may play a role in Nkx3.2-induced IκB ubiquitination. Interestingly, βTrCP siRNA effectively attenuated Nkx3.2-mediated IκB-α ubiquitination (Fig. 7B, top). Consistent with these results, we also found that NF-κB activation induced by Nkx3.2 can be diminished by βTrCP siRNA (Fig. 7C). As additional evidence in support of the involvement of βTrCP, we observed that dominant negative βTrCP significantly reduced the ability of

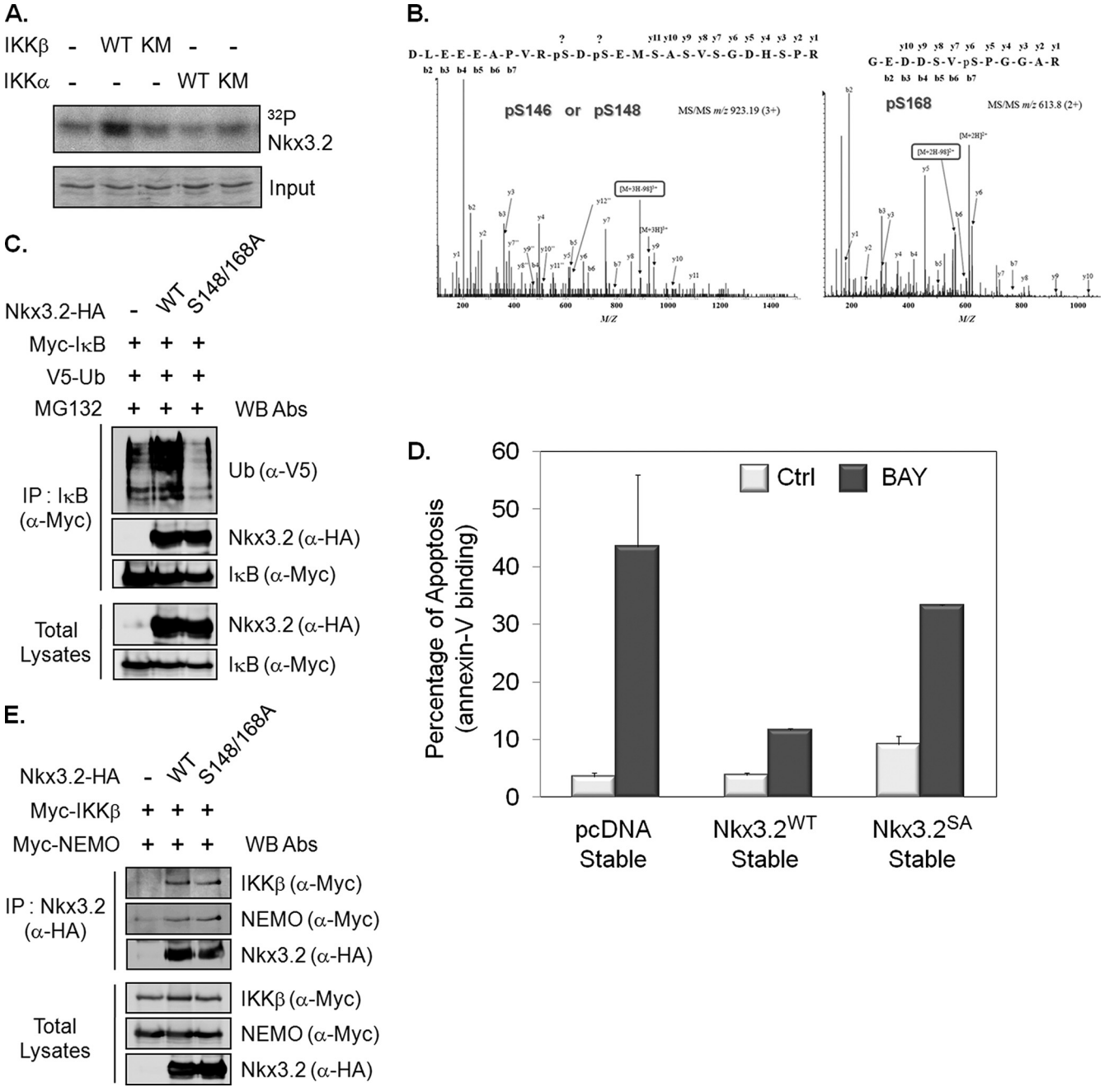

FIG. 6. Nkx3.2 phosphorylation induced by IKKβ is required for Nkx3.2-mediated NF-κB activation subsequent to Nkx3.2-NEMO-IKKβ complex formation. (A) Nkx3.2 can be phosphorylated by IKKβ *in vitro*. Plasmids encoding 6Myc-tagged IKKβ^{WT}, IKKβ^{KM}, IKKα^{WT}, or IKKα^{KM} were transfected into 293T cells. Forty-eight hours after transfection, immunoprecipitation was performed using anti-Myc antibodies, followed by incubation with [γ-^{32}P]ATP and 0.5 μg of recombinant Nkx3.2 proteins as substrates. The reaction mixture was resolved by SDS-PAGE, stained with Coomassie blue, and processed for autoradiography. (B) IKKβ-induced *in vivo* phosphorylation sites of Nkx3.2. 293T cells were transfected with the Nkx3.2-3Flag expression plasmid with or without the 6Myc-IKKβ expression vector. After 48 h, anti-Flag immunoprecipitates were resolved by SDS-PAGE and processed for mass spectrometry analyses. (C) Nkx3.2^{SA} is not able to induce IκB ubiquitination. Expression constructs encoding 6Myc-IκB and 2V5-ubiquitin were transfected into 293T cells in the absence or presence of the 3HA-tagged Nkx3.2^{WT} or Nkx3.2^{SA} expression plasmid. After 24 h of transfection, which included a final 6 h of MG132 incubation, the cells were harvested. Anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. (D) Nkx3.2^{SA} is not capable of inhibiting chondrocyte apoptosis. Vector, Nkx3.2^{WT}, or Nkx3.2^{SA} stable ATDC5 cells were treated with 10 μM BAY. The percentage of apoptotic cells was estimated by three independently repeated FACS analyses, and the average values are presented. (E) S148/168A mutation of Nkx3.2 does not affect its ability to form a complex with IKKβ and NEMO. 293T cells were transfected with 6Myc-NEMO and 6Myc-IKKβ expression plasmids in the absence or presence of the 3HA-tagged Nkx3.2^{WT} or Nkx3.2^{SA} expression construct. After 24 h, transfected cells were harvested. Anti-HA immunoprecipitates and total lysates were analyzed by Western blotting.

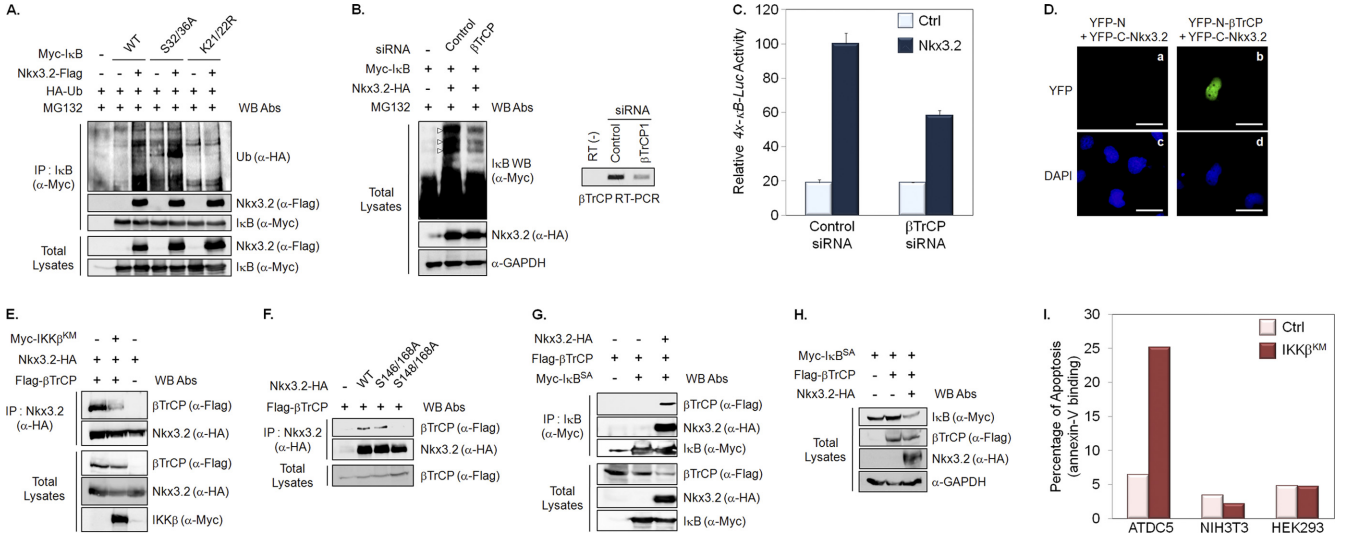

FIG. 7. Nkx3.2 recruits βTrCP to induce IκB phosphorylation-independent IκB ubiquitination. (A) Lys21 and Lys22 of IκB-α are employed for Nkx3.2-mediated IκB-α ubiquitination. Expression constructs encoding 6Myc-tagged $IκB^{WT}$, $IκB^{SA}$, or $IκB^{KR}$ were transfected with the 3HA-ubiquitin expression vector in the absence or presence of the Nkx3.2-3Flag expression plasmid. After 24 h of transfection, which included a final 6 h of MG132 incubation, the cells were harvested. Anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. (B) βTrCP siRNA reduces Nkx3.2-mediated IκB ubiquitination. Plasmids encoding 6Myc-IκB and Nkx3.2-3HA were cotransfected with control or βTrCP siRNA into ATDC5 cells. After 24 h, a second transfection of control or βTrCP siRNA was performed for an additional 18 h, which included a final 6 h of MG132 incubation. Total lysates were then analyzed by Western blotting. (Top) Open triangles indicate polyubiquitinated forms of IκB. (Bottom) The level of βTrCP mRNA was assessed by RT-PCR. (C) βTrCP RNA knockdown attenuates Nkx3.2-mediated NF-κB activation. ATDC5 cells were transfected with control or βTrCP siRNA. Thirty hours later, cells were transfected with 4× κB-Luc reporter in the absence or presence of the Nkx3.2-3HA expression construct. After an additional 30 h, luciferase assays were performed. (D) Nkx3.2 can form a complex with βTrCP in the nucleus. ATDC5 cells were cotransfected with expression vectors encoding YFP-N (a and c), YFP-N–βTrCP (b and d), and YFP-C–Nkx3.2 (a to d). After 40 h of transfection, which included a final 6 h of MG132 incubation, YFP fluorescence (a and b) and DAPI staining (c and d) were detected. The scale bars represent 25 μm. (E) Dominant negative IKKβ abolishes the interaction between Nkx3.2 and βTrCP. Expression vectors encoding Nkx3.2-3HA and 3Flag-βTrCP were transfected into 293T cells in the absence or presence of the 6Myc-$IKKβ^{KM}$ expression plasmid for 24 h. Anti-HA immunoprecipitates and total lysates were analyzed by Western blotting. (F) IKKβ-induced phosphorylation of Nkx3.2 is required for the interaction between Nkx3.2 and βTrCP. 293T cells were transfected with the 3Flag-βTrCP expression construct in the absence or presence of the 3HA-tagged $Nkx3.2^{WT}$, $Nkx3.2^{SA}$, or $Nkx3.2^{SA}$ expression plasmid. After 24 h, cells were harvested, and anti-HA immunoprecipitates and total lysates were analyzed by Western blotting. (G) Nkx3.2 can target βTrCP to $IκB^{SA}$. Expression plasmids for 6Myc-$IκB^{SA}$ and 3Flag-βTrCP were transfected into 293T cells in the absence or presence of Nkx3.2-3HA. After 24 h, anti-Myc immunoprecipitates and total lysates were analyzed by Western blotting. (H) Nkx3.2 enables βTrCP to induce $IκB^{SA}$ degradation. Expression constructs encoding 6Myc-$IκB^{SA}$ and 3Flag-βTrCP were transfected into 293T cells in the absence or presence of the Nkx3.2-3HA expression plasmid for 48 h. Total lysates were analyzed by Western blotting. (I) IKKβ function is required to be intact for chondrocyte viability maintenance. ATDC5, NIH 3T3, or HEK293 cells were infected with vector- or $IKKβ^{KM}$-encoding lentivirus. The percentage of apoptotic cells was estimated by FACS.

Nkx3.2 to induce IκB ubiquitination and activate NF-κB (data not shown). Furthermore, YFP-based BiFC assays verified that Nkx3.2 was able to establish direct protein-protein interactions with βTrCP in the nucleus, where it mediates the degradation of IκB (Fig. 7D). Taken together, these results demonstrate that Nkx3.2 can recruit βTrCP into the nucleus and render βTrCP-mediated nuclear IκB ubiquitination.

IKKβ-induced Nkx3.2 phosphorylation can employ βTrCP for IκB-α degradation in the absence of IκB-α phosphorylation at Ser32 and Ser36. Since we have found that βTrCP can function in Nkx3.2-mediated IκB ubiquitination and form a complex with Nkx3.2 in the nucleus (Fig. 7A to D), we next asked whether IKKβ-dependent Nkx3.2 phosphorylation can regulate the interaction between Nkx3.2 and βTrCP. Co-IP assays revealed that Nkx3.2 is able to form a complex with βTrCP, and this interaction is remarkably reduced by $IKKβ^{KM}$ coexpression (Fig. 7E, top panel). In addition, we demonstrated that $Nkx3.2^{SA}$, which contains mutations at IKKβ-induced phosphorylation sites, is unable to interact with βTrCP, unlike $Nkx3.2^{WT}$ and Nkx3.2(S146/168A) (Fig. 7F, top panel) [Nkx3.2(S146/168A) was included as an internal negative control for S-to-A mutations.] These results clearly indicate that IKKβ-induced Nkx3.2 phosphorylation at Ser148 and Ser168 is necessary for Nkx3.2 to form a stable complex with βTrCP.

It has been previously demonstrated that IκB phosphorylation at Ser32 and Ser36 is essential for both βTrCP–IκB-α interaction and βTrCP-mediated IκB-α ubiquitination (33, 42). In contrast, our results revealed that Nkx3.2 can employ βTrCP to cause IκB-α ubiquitination regardless of IκB-α phosphorylation at Ser32 and Ser36. Thus, we next attempted to determine whether Nkx3.2 is indeed capable of inducing βTrCP–IκB-α interaction in the absence of IκB-α phosphorylation at Ser32 and Ser36 (i.e., βTrCP-$IκB^{SA}$ interaction). In agreement with the previous reports (33, 42), βTrCP alone failed to interact with $IκB^{SA}$; however, interestingly, βTrCP was competent to form a complex with $IκB^{SA}$ in the presence of Nkx3.2 (Fig. 7G, top panel). Consistent with these results, βTrCP was able to induce $IκB^{SA}$ degradation in the presence but not in the absence of Nkx3.2 (Fig. 7H, top panel). Therefore, these results together demonstrate that Nkx3.2 is capable of recruiting βTrCP to the complex to cause IκB degradation in the absence of IκB phosphorylation at Ser32 and Ser36.

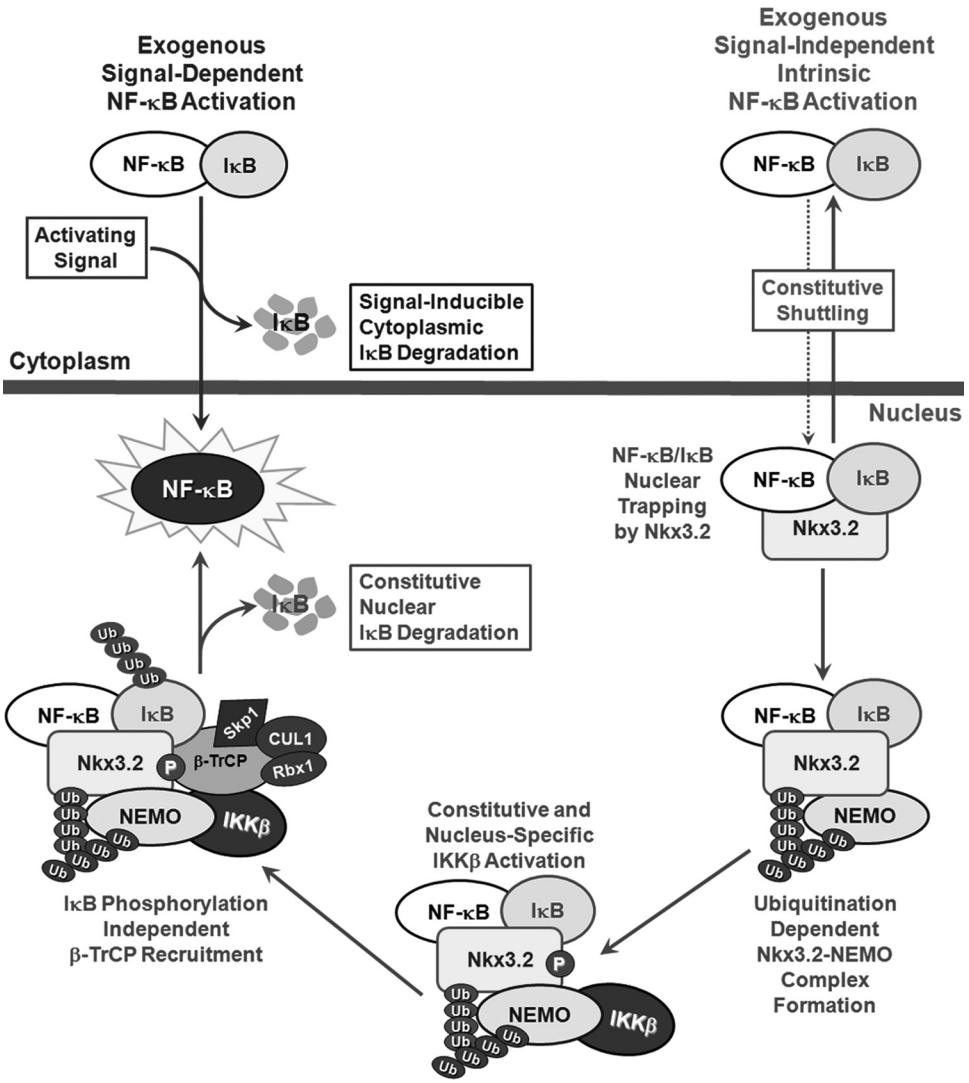

FIG. 8. Schematic presentation of molecular mechanisms for exogenous signal-independent intrinsic NF-κB activation mediated by Nkx3.2.

As we have characterized a precise role of IKKβ in Nkx3.2-mediated NF-κB activation, we finally sought to determine whether the enzymatic activity of IKKβ is indeed required for chondrocyte survival. Interestingly, lentiviral infection of $IKK\beta^{KM}$ alone was sufficient to cause significant cell death in chondrogenic ATDC5 cells in the absence of any additional apoptotic stimuli (Fig. 7I, second bar). On the other hand, this was not the case in other nonchondrogenic cell types such as NIH 3T3 and HEK293 cells (Fig. 7I, fourth and sixth bars). Therefore, analogous to the findings with NEMO, these results indicate that IKKβ is a critical component for cell type-specific viability maintenance in chondrocytes.

DISCUSSION

While many analyses of IκB degradation have focused on cytoplasmic degradation mechanisms functioning in response to NF-κB-activating signals, it is unclear how IκB degradation can occur to allow intrinsic NF-κB activation in the absence of exogenous signals. Here we describe that polyubiquitin chain-dependent but exogenous signal-independent Nkx3.2-NEMO interactions establish constitutive IKKβ activation in the nucleus, and subsequently, IKKβ-dependent phosphorylation of Nkx3.2 allows βTrCP recruitment to this nuclear complex to induce IκB-α ubiquitination independently of its phosphorylation at Ser32 and Ser36 (Fig. 8). Therefore, our results provide a precise molecular explanation as to how an intracellular factor can function to accomplish intrinsic NF-κB activation without a requirement of external signals.

In fact, our findings suggest an intriguing role for cell type-specific intrinsic NF-κB activation. By employing the mechanisms presented in this work, an intracellular factor expressed in a particular group of cells would be able to accomplish autonomous NF-κB activation to fulfill a physiological need without a requirement for external signals. In our case, these mechanisms enable Nkx3.2 to cause persistent NF-κB activation, which, in turn, supports chondrocyte survival. In addition, we interestingly observed that functionally intact IKKβ and NEMO are required for viability maintenance in multiple

chondrogenic lineage cells, including ATDC5 cells and various human primary chondrocytes, but not in other cell types we tested, such as fibroblasts, mesenchymal cells, and epithelial cells. Therefore, it appears that IKKβ and NEMO play a critical role for a subset of cell types requiring persistent antiapoptotic NF-κB signaling for their survival. Although an antiapoptotic role for NF-κB during embryogenesis has been suggested (2, 10), our understanding relevant to cartilage development is relatively limited (16, 17, 28, 43). While these previous studies did not address molecular mechanisms responsible for constitutive IKK and/or NF-κB activation in chondrocytes, our current study may provide a molecular explanation as to how a nuclear factor such as Nkx3.2 can enable steady-state IKK activation leading to constitutive NF-κB activation, which can be associated with cell viability maintenance in chondrocytes (28, 43). The results shown in this work may enhance our mechanistic understanding as to how the NF-κB pathway functions during bone development.

As would be expected, to demonstrate that Nkx3.2 is capable of inducing constitutive nuclear IKKβ activation and IκB degradation, the use of exogenous NF-κB-activating reagents was not necessary. However, it is still possible that Nkx3.2 might have established an autocrine loop for NF-κB activation by inducing the expression of NF-κB-activating ligands. Nevertheless, the involvement of additional NF-κB-activating ligands induced by Nkx3.2 during Nkx3.2-mediated NF-κB activation process is quite unlikely, since we found that dominant negative IκB (i.e., $I\kappa B^{SA}$), which is a potent suppressor against ligand-induced NF-κB activation, did not affect Nkx3.2-mediated NF-κB activation (Fig. 5). In addition, it has been shown that overexpression of various components in the NF-κB pathway, such as the TNF receptor and RIP, can cause NF-κB activation in the absence of exogenous stimulation. However, it should be pointed out that NF-κB activation under these circumstances clearly mimics signal-dependent processes such cytoplasmic IKKβ activation and Ser32/Ser36 phosphorylation-dependent IκB degradation. In clear contrast, Nkx3.2 overexpression causes nucleus-specific IKK activation and triggers Ser32/Ser36 phosphorylation-independent degradation of IκB in the nucleus. Furthermore, NF-κB activation induced by TNF receptor or RIP overexpression can be completely abolished by dominant negative IκB (i.e., $I\kappa B^{SA}$), whereas Nkx3.2-induced NF-κB activation is not obstructed by $I\kappa B^{SA}$. Thus, these lines of information indicate that Nkx3.2-mediated NF-κB activation mechanisms are fundamentally distinct from the classical ligand-dependent NF-κB activation processes.

It has been demonstrated that polyubiquitin chain-dependent complex formation between NEMO and RIP1 permits signal-dependent IKK activation (9, 41). In this regard, the role of Nkx3.2 is quite similar to that of RIP in the classical NF-κB pathways. However, a critical difference between the actions of RIP and Nkx3.2 is the fact that the RIP-NEMO interaction takes place in the cytoplasm and is induced in response to NF-κB-activating signals, while the Nkx3.2-NEMO interaction in chondrocytes occurs in the nucleus and is exogenous signal independent (i.e., constitutive). Furthermore, our results show that polyubiquitin chain-dependent Nkx3.2-NEMO interactions give rise to functional activation of IKKβ in the nucleus but not in the cytoplasm.

While both Nkx3.2 and NEMO are found to be polyubiquitinated in the complex of Nkx3.2 and NEMO, our results indicate that sensing of the polyubiquitinated forms of Nkx3.2 by NEMO is possibly more critical in stabilizing the interactions between Nkx3.2 and NEMO than vice versa. For instance, Nkx3.2 maintains lower but still detectable interactions with $NEMO^{D304N}$ (Fig. 4A), while NEMO shows negligible affinity to $Nkx3.2^{KR}$ (Fig. 3D). Thus, we speculate that the ability of NEMO to recognize the polyubiquitinated forms of Nkx3.2, which constitutively exist in the nucleus, is most important to enable persistent nuclear IKK activation in chondrocytes. However, the presence of polyubiquitin chains may not be an absolute requirement for the initial contacts between Nkx3.2 and NEMO, as we found both nonubiquitinated and polyubiquitinated forms of each in the complex. Nevertheless, multiple experiments shown in this work demonstrate that both $NEMO^{D304N}$ (defective in polyubiquitin chain sensing) and $Nkx3.2^{KR}$ (defective in being polyubiquitinated) are functionally impaired. Therefore, it is most likely that the presence of polyubiquitinated forms of NEMO and Nkx3.2 is necessary for subsequent IKKβ recruitment and that this additional stabilization is necessary, in practice, for the Nkx3.2-NEMO complex to be capable of inducing functional activation of NF-κB.

We demonstrate that Nkx3.2 directly interacts with βTrCP in the nucleus and that this interaction between Nkx3.2 and βTrCP requires IKKβ-induced Nkx3.2 phosphorylation at Ser148 and Ser168. In theory, these phosphorylation sites may directly serve as a motif for βTrCP contacts, or Ser148/Ser168 phosphorylation may induce critical conformational changes in Nkx3.2 to permit stable interactions between Nkx3.2 and βTrCP. Since we have not yet determined whether the IKKβ-dependent phosphorylation sites on Nkx3.2 are indeed the sites of contact with βTrCP, either of these two distinct mechanisms would be possible. However, in any scenario, it is apparent that the presence of Nkx3.2 in the complex allows βTrCP to trigger IκB-α ubiquitination regardless of IκB-α phosphorylation at Ser32 and Ser36. Thus, one can envision that IκB-α and βTrCP, once complexed with Nkx3.2 in the nucleus, may establish an interface distinct from the cytoplasmic IκB degradation complex.

ACKNOWLEDGMENTS

We thank Michael Karin and Jin Mo Park for kindly providing IKK knockout (KO) cells. Various human primary chondrocytes were generously gifted to us by Byoung-Hyun Min and Byung Hyune Choi.

This work was supported by grants (2010-0018860, FPR08B1-260, 2011-0001029, and 2010-0020535) funded by the Korean Ministry of Education, Science and Technology.

REFERENCES

1. **Adli, M., E. Merkhofer, P. Cogswell, and A. S. Baldwin.** 2010. IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway. PLoS One **5:**e9428
2. **Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore.** 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature **376:**167–170.
3. **Chen, Z., et al.** 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev. **9:**1586–1597.
4. **Chen, Z. J.** 2005. Ubiquitin signalling in the NF-kappaB pathway. Nat. Cell Biol. **7:**758–765.
5. **Chen, Z. J., L. Parent, and T. Maniatis.** 1996. Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell **84:**853–862.
6. **DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin.** 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature **388:**548–554.

7. **Doffinger, R., et al.** 2001. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. Nat. Genet. **27:**277–285.
8. **Dull, T., et al.** 1998. A third-generation lentivirus vector with a conditional packaging system. J. Virol. **72:**8463–8471.
9. **Ea, C. K., L. Deng, Z. P. Xia, G. Pineda, and Z. J. Chen.** 2006. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell **22:**245–257.
10. **Gerondakis, S., M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont.** 1999. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. Oncogene **18:**6888–6895.
11. **Hacker, H., and M. Karin.** 2006. Regulation and function of IKK and IKK-related kinases. Sci. STKE **2006:**re13.
12. **Hatakeyama, S., et al.** 1999. Ubiquitin-dependent degradation of IkappaB-alpha is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Proc. Natl. Acad. Sci. U. S. A. **96:**3859–3863.
13. **Hayden, M. S., and S. Ghosh.** 2004. Signaling to NF-kappaB. Genes Dev. **18:**2195–2224.
14. **Hu, C. D., Y. Chinenov, and T. K. Kerppola.** 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell **9:**789–798.
15. **Hu, Y., et al.** 1999. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science **284:**316–320.
16. **Janssen, R., et al.** 2004. The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes. J. Exp. Med. **200:**559–568.
17. **Kanegae, Y., A. T. Tavares, J. C. Izpisua Belmonte, and I. M. Verma.** 1998. Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb. Nature **392:**611–614.
18. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. **18:**621–663.
19. **Kim, D. W., H. Kempf, R. E. Chen, and A. B. Lassar.** 2003. Characterization of Nkx3.2 DNA binding specificity and its requirement for somitic chondrogenesis. J. Biol. Chem. **278:**27532–27539.
20. **Kim, D. W., and A. B. Lassar.** 2003. Smad-dependent recruitment of a histone deacetylase/Sin3A complex modulates the bone morphogenetic protein-dependent transcriptional repressor activity of Nkx3.2. Mol. Cell. Biol. **23:**8704–8717.
21. **Li, Q., S. Withoff, and I. M. Verma.** 2005. Inflammation-associated cancer: NF-kappaB is the lynchpin. Trends Immunol. **26:**318–325.
22. **Li, Z. W., et al.** 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J. Exp. Med. **189:**1839–1845.
23. **Meffert, M. K., and D. Baltimore.** 2005. Physiological functions for brain NF-kappaB. Trends Neurosci. **28:**37–43.
24. **Mercurio, F., et al.** 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science **278:**860–866.
25. **Murtaugh, L. C., L. Zeng, J. H. Chyung, and A. B. Lassar.** 2001. The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. Dev. Cell **1:**411–422.
26. **Oeckinghaus, A., and S. Ghosh.** 2009. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. **1:**a000034.
27. **Oshima, S., et al.** 2009. ABIN-1 is a ubiquitin sensor that restricts cell death and sustains embryonic development. Nature **457:**906–909.
28. **Park, M., et al.** 2007. Constitutive RelA activation mediated by Nkx3.2 controls chondrocyte viability. Nat. Cell Biol. **9:**287–298.
29. **Provot, S., et al.** 2006. Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. Development **133:**651–662.
30. **Rothwarf, D. M., and M. Karin.** 1999. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci. STKE **1999:**RE1.
31. **Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin.** 1998. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature **395:**297–300.
32. **Scherer, D. C., J. A. Brockman, Z. Chen, T. Maniatis, and D. W. Ballard.** 1995. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc. Natl. Acad. Sci. U. S. A. **92:**11259–11263.
33. **Skowyra, D., K. L. Craig, M. Tyers, S. J. Elledge, and J. W. Harper.** 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91:**209–219.
34. **Tang, E. D., C. Y. Wang, Y. Xiong, and K. L. Guan.** 2003. A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. J. Biol. Chem. **278:**37297–37305.
35. **Tokunaga, F., et al.** 2009. Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat. Cell Biol. **11:**123–132.
36. **Traenckner, E. B., et al.** 1995. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J. **14:**2876–2883.
37. **Tribioli, C., and T. Lufkin.** 1997. Molecular cloning, chromosomal mapping and developmental expression of BAPX1, a novel human homeobox-containing gene homologous to Drosophila bagpipe. Gene **203:**225–233.
38. **Tsukahara, T., et al.** 1999. Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax. J. Virol. **73:**7981–7987.
39. **Vallabhapurapu, S., and M. Karin.** 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu. Rev. Immunol. **27:**693–733.
40. **Windheim, M., M. Stafford, M. Peggie, and P. Cohen.** 2008. Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. Mol. Cell. Biol. **28:**1783–1791.
41. **Wu, C. J., D. B. Conze, T. Li, S. M. Srinivasula, and J. D. Ashwell.** 2006. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat. Cell Biol. **8:**398–406.
42. **Wu, G., et al.** 2003. Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. Mol. Cell **11:**1445–1456.
43. **Wu, S., J. K. Flint, G. Rezvani, and F. De Luca.** 2007. Nuclear factor-kappaB p65 facilitates longitudinal bone growth by inducing growth plate chondrocyte proliferation and differentiation and by preventing apoptosis. J. Biol. Chem. **282:**33698–33706.
44. **Yamaoka, S., et al.** 1998. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell **93:**1231–1240.
45. **Zaffran, S., and M. Frasch.** 2005. The homeodomain of Tinman mediates homo- and heterodimerization of NK proteins. Biochem. Biophys. Res. Commun. **334:**361–369.
46. **Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin.** 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell **91:**243–252.
47. **Zeng, L., H. Kempf, L. C. Murtaugh, M. E. Sato, and A. B. Lassar.** 2002. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. Genes Dev. **16:**1990–2005.
48. **Zhou, H., et al.** 2004. Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. Nature **427:**167–171.