

The I κ B Function of NF- κ B2 p100 Controls Stimulated Osteoclastogenesis

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Abstract

The prototranscription factor p100 represents an intersection of the NF- κ B and I κ B families, potentially serving as both the precursor for the active NF- κ B subunit p52 and as an I κ B capable of retaining NF- κ B in the cytoplasm. NF- κ B-inducing kinase (NIK) controls processing of p100 to generate p52, and thus NIK-deficient mice can be used to examine the biological effects of a failure in such processing. We demonstrate that treatment of wild-type osteoclast precursors with the osteoclastogenic cytokine receptor activator of NF- κ B ligand (RANKL) increases both expression of p100 and its conversion to p52, resulting in unchanged net levels of p100. In the absence of NIK, p100 expression is increased by RANKL, but its conversion to p52 is blocked, leading to cytosolic accumulation of p100, which, acting as an I κ B protein, binds NF- κ B complexes and prevents their nuclear translocation. High levels of unprocessed p100 in osteoclast precursors from NIK^{-/-} mice or a nonprocessable form of the protein in wild-type cells impair RANKL-mediated osteoclastogenesis. Conversely, p100-deficient osteoclast precursors show enhanced sensitivity to RANKL. These data demonstrate a novel, biologically relevant means of regulating NF- κ B signaling, with upstream control and kinetics distinct from the classical I κ B α pathway.

Key words: mice knockout • bone remodeling/physiology • MAP kinase kinases • cultured cells/physiology • murine RANKL

Introduction

Osteoclasts (OCs) are multinucleated cells derived from mononuclear myeloid bone marrow progenitors that express receptor activator of NF- κ B (RANK), the receptor for the key osteoclastogenic cytokine RANK ligand (RANKL) (1). Interaction of RANK with RANKL in the presence of macrophage CSF (M-CSF) promotes OC differentiation. Thus, deletion of the RANK, RANKL, or M-CSF genes leads to an absence of OCs, causing osteopetrosis. Like other members of the TNF receptor superfamily, RANK potentially activates the NF- κ B pathway.

In the canonical NF- κ B pathway (for reviews see reference 2, 3), ligation of a transmembrane receptor such as RANK activates the I κ B kinase (IKK) complex, which phosphorylates NF- κ B-associated I κ B α , leading to its ubiquitination and proteosomal degradation. These events release

NF- κ B subunits in the cytosol, allowing them to travel to the nucleus where they enhance transcription of target genes. In mammals, the NF- κ B family has five members: RelA (p65), RelB, c-Rel, NF- κ B1, and NF- κ B2. RelA, RelB, and c-Rel are synthesized as mature proteins, each bearing a transcriptional transactivation domain and a DNA-binding Rel homology domain. In contrast, the active forms of NF- κ B1 and NF- κ B2, p50 and p52, are derived from the full-length products of the *nfkb1* and *nfkb2* genes, p105 and p100, respectively. The generation of the transcriptionally active subunits p50 and p52 is dependent on phosphorylation of their precursors followed by ubiquitination and partial proteosomal degradation of their COOH

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Abbreviations used in this paper: CTR, calcitonin receptor; EMSA, electrophoretic mobility shift assay; IKK, I κ B kinase; M ϕ , bone marrow-derived macrophages; M-CSF, macrophage CSF; MMP9, matrix metalloproteinase 9; NIK, NF- κ B-inducing kinase; OC, osteoclast; RANK, receptor activator of NF- κ B ligand; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase.

termini (4, 5). The NH₂-terminal Rel homology domain of p50 or p52, when cleaved from the COOH terminus, can form active NF- κ B dimers with RelA, RelB, or c-Rel.

The COOH-terminal portions of p105 and p100, before processing to p50 and p52, contain I κ B-like ankyrin repeats. Thus, similar to I κ B, p105 and p100 are capable of retaining proteins with a Rel domain in the cytosol (6, 7). p105 is ubiquitously expressed, and its conversion to p50 appears to be largely constitutive, with only modest increases in levels of p50 in response to NF- κ B activation (2). In contrast, p100 is expressed predominantly in lymphoid organs (8), and its processing is tightly controlled by a kinase cascade. NF- κ B-inducing kinase (NIK) activates IKK α (a subunit of the IKK complex), which phosphorylates p100, targeting it for processing to p52 (9, 10).

The importance of the NIK/IKK α /p100/p52 pathway has been highlighted by several mouse models. The *nfkb2* knockout mouse, which lacks both p100 and p52, exhibits abnormal B cell differentiation and function (11, 12). Radiation chimeras bearing IKK α ^{-/-} bone marrow or mice expressing nonactivatable IKK α have decreased p52 levels and similar defects in B cell maturation (10). Alymphoplasia (aly/aly) mice (13), which carry a mutation in the IKK α interaction domain of NIK (14, 15), also have diminished p52 levels (9, 16) and defective B cell maturation (16). Finally, animals lacking the COOH-terminal I κ B-like domain of NF κ B2, and which thus express unregulated p52, have increased lymphocyte proliferation and gastric mucosal hyperplasia (17). In sum, these models demonstrate that regulated expression of the active NF- κ B subunit p52, via NIK- and IKK α -mediated processing of p100, is biologically relevant.

Despite these findings, the importance of the putative inhibitory effect of unprocessed p100 on global NF- κ B activation, via its I κ B activity, has not been established. In this study, we have used the NIK-deficient mouse (18) to examine the effects of a lack of p100 processing on OC differentiation and NF- κ B signaling. Previous studies of the NIK/IKK α /p100 pathway in a physiological context have only been performed in the B cell lineage, which is dependent on p52 for full maturation (11, 12). The *nfkb2* knockout mouse, which lacks both p100 and p52, has normal basal osteoclastogenesis in vivo (19), indicating that, in contrast to its role in B cell development, p52 itself is dispensable for OC differentiation. The fact that the NIK^{-/-} mouse fails to process p100 to p52 positions us to examine the potential role of p100 in osteoclastogenesis, in the absence of a requirement for p52. Our studies show that when p100 levels are increased, as in NIK-deficient OC precursors, RANKL-stimulated osteoclastogenesis is impaired. Conversely, we find that OC differentiation is enhanced in the absence of p100 in vitro. These results demonstrate that stimulated osteoclastogenesis is regulated by a pathway involving RANKL, NIK, and p100.

Materials and Methods

Mice. NIK^{-/-} mice were generated by homologous recombination as described (18) on a 129Sv/Ev background. Mice were

maintained by heterozygote matings in a specific pathogen-free facility.

Osteoclast Culture. For whole marrow culture, unfractionated bone marrow cells were cultured in the presence of 1,25 dihydroxyvitamin D₃ (2.5 × 10⁻⁸ M) to induce osteoclast differentiation (20). After 8 d, cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker of OC differentiation, according to manufacturer's instructions (Sigma-Aldrich). To obtain bone marrow-derived macrophages (M ϕ), marrow cells were on Petri dishes in the presence of CMG14-12 supernatant containing the equivalent of 100 ng/ml recombinant M-CSF (21) for at least 5 d. Equal numbers of WT and NIK^{-/-} M ϕ s were replated onto tissue culture plates in the presence of partially purified M-CSF (see M-CSF Preparation) equivalent to 20 ng/ml recombinant M-CSF and the indicated dose of GST-RANKL for 6 d and then fixed and stained for TRAP activity. For analysis of resorptive activity, M ϕ s were replated onto multiwell Osteologic slides (BD Biosciences), which are coated with inorganic hydroxyapatite matrix, in the presence of M-CSF and 150 ng/ml GST-RANKL. After 6 d, cells were fixed in neutral buffered formalin and stained with methylene blue.

In Vivo RANKL Treatment. GST-RANKL was prepared as described (22). WT and NIK^{-/-} mice (2-3 mo old) were injected subcutaneously with GST-RANKL (500 μ g per day for 7 d) or PBS (sham). Calvaria were fixed overnight in 10% buffered formalin, decalcified in 14% EDTA, embedded in paraffin, sectioned, and stained for TRAP with a hematoxylin counterstain. The number of OC per 10 fields (100 \times magnification) for five mice in each group was counted. Statistics were performed with the unpaired Student's *t* test.

Antibodies. mAbs to RelA and the NH₂ terminus of NF- κ B2 common to p100 and p52 were from Santa Cruz Biotechnology, Inc., as were the polyclonal antiserum directed against I κ B α and RelB and the agarose conjugated anti-RelA polyclonal antibody used for immunoprecipitation.

Semiquantitative RT-PCR. Total RNA was extracted using RNeasy (QIAGEN), and snf cDNA was generated with AMV-RT (Fisher Scientific) according to manufacturers' instructions using 1 μ g total RNA and oligo-dT primers diluted to a final volume of 100 μ l. Oligonucleotide primers used for PCR were: *nfkb2* sense, 5'-TGGCTGGTGGGACTTGG-3'; *nfkb2* antisense, 5'-ACCCACCGAGATGTGGGC-3'; GAPDH sense, 5'-ACTTTGTCAAGCTCATTTC-3'; GAPDH antisense, 5'-TGCAGCGAAGCTTTATTGATG-3'; calcitonin receptor (CTR) sense 5'-CATTCCTGTACTTGGTTGGC-3'; CTR antisense, 5'-AGCAATCGACAAGGAGTGAC-3'; matrix metalloproteinase 9 (MMP9) sense, 5'-CCTGTGTGTTCCGTTTCATCT-3'; MMP9 antisense, 5'-CGCTGGGAATGATCTAAGCCCA-3'; β 3 integrin sense, 5'-TTACCCCGTGGACATCTACTA-3'; and β 3 integrin antisense, 5'-AGTCTTCCATCCAGGGCAATA-3'. For each reaction, 1-2 μ l cDNA was used for the following PCR cycle numbers: *nfkb2*-24; GAPDH-24; CTR-34; MMP9-24; and β 3 integrin-30.

M-CSF Preparation. Supernatant was harvested from CMG14-12 cells grown in phenol red-free media, diluted into triethanolamine buffer, and loaded onto a Q sepharose Fast-Flow column (Amersham Biosciences). M-CSF was eluted first with a NaCl gradient (0-1.0 M), and then eluted from a second Q column by pH gradient (pH 7 to 4). Peak fractions were dialyzed against PBS and sterile filtered. Bioactivity was compared with recombinant murine M-CSF (R&D Systems) by assaying proliferation of M-NFS-60, an M-CSF-dependent cell line (21).

Subcellular Fractionation. Cells were harvested by treatment with 10 mM EDTA in PBS for 15–30 min at 4°C followed by scraping. Nuclear and cytoplasmic fractions were isolated using the NuCLEAR kit (Sigma-Aldrich). Extracts were quantitated by a modified Coomassie method (Pierce Chemical Co.). For immunoblots, 5 µg of nuclear extract or 25 µg of cytoplasmic extract was used, and equivalent loading was verified by Ponceau red staining after transfer to PVDF membrane.

NF-κB DNA Binding Assays. For electrophoretic mobility shift assay (EMSA), nuclear extracts (2 µg) were incubated with labeled double stranded κB3 oligonucleotide (5'-AAA-CAGGGGCTTCCCTCCTC-3') at 4°C for ³²P-labeled probes (see Figs. 4 and 5) or at room temperature for biotin-labeled probes (see Fig. 8). Protein–DNA complexes were resolved on nondenaturing 4–20% polyacrylamide gels (Invitrogen) in 0.5 × TBE. Radiolabeled gels were dried and subjected to autoradiography. For nonradioactive detection, the LightShift chemiluminescent system (Pierce Chemical Co.) was used, according to manufacturer's instructions, using a biotinylated version of the same oligo (Invitrogen). For RelA supershift, 2 µg polyclonal anti-RelA (Santa Cruz Biotechnology, Inc.) was added after formation of DNA–protein complexes at room temperature for 15 min. An ELISA-based NF-κB assay, the EZDetect p65 ELISA (Pierce Chemical Co.) was performed according to manufacturer instructions, using 2 µg nuclear extract per well, performed in triplicate. Background (extraction buffer alone, averaged for three wells) was subtracted from each sample. In this assay, nuclear extract is applied to wells precoated with specific NF-κB oligos followed by anti-p65 (RelA) antibody and an HRP-conjugated secondary. The assay is developed with chemiluminescent substrates and read on a luminometer/plate reader (SpectraFluor Plus; Tecan).

Retroviral Transduction. Mφ were transduced with retrovirus containing vector alone (pMX-puro) (23) or a noncleavable form of p100 (pMX-p100ΔGRR) for 24 h in the presence of CMG14–12 supernatant equivalent to 200 ng/ml rM-CSF and 4 µg/ml polybrene (Sigma-Aldrich) and then selected with puromycin for 3 d. Surviving Mφ were passaged on Petri dishes and grown in M-CSF overnight before the preparation of nuclear extracts, or passaged into 96-well plates, cultured in M-CSF (20 ng/ml) and GST-RANKL (150 ng/ml) for 5 d, and then fixed and stained for TRAP activity.

Results

Stimulated Osteoclastogenesis Is Blunted in *NIK*^{-/-} Mice.

To determine if p100 processing has a role in OC differentiation, we placed *NIK*^{-/-} (18) bone marrow cells in osteoclastogenic conditions and found they fail to generate multinucleated cells expressing TRAP, a marker of the OC phenotype (Fig. 1 A). In these whole marrow cultures, stromal cells provide the M-CSF and RANKL required for osteoclastogenesis. OCs can also be generated in vitro from pure populations of Mφs treated with soluble recombinant M-CSF and RANKL. As shown in Fig. 1 B, WT Mφs exposed to these cytokines form confluent sheets of large, spread OCs and exhibit exuberant resorptive activity (Fig. 1 C). In contrast, *NIK*^{-/-} cultures, although rich in Mφs, contain only rare, poorly spread TRAP-positive cells (Fig. 1 B) with no detectable resorptive capacity (Fig. 1 C). The lack of osteoclastogenesis in *NIK*^{-/-} cultures cannot be

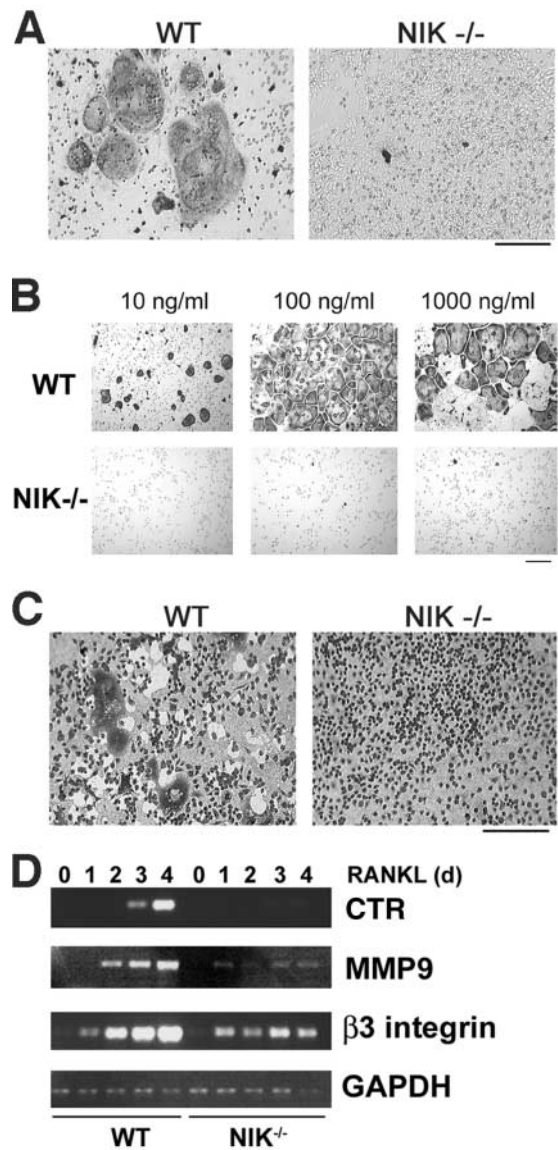


Figure 1. *NIK*^{-/-} mice have defective RANKL-mediated osteoclastogenesis in vitro. (A) Equal numbers of WT and *NIK*^{-/-} cells from unfractionated bone marrow were cultured for 8 d in the presence of vitamin D3 and then fixed and stained for TRAP, a marker of OC differentiation. Under these conditions, both stromal cells and OC precursors are present. *NIK*^{-/-} cultures fail to form large, spread, TRAP-positive OCs. Bar, 300 µm. (B) Equal numbers of Mφs (expanded from bone marrow in high doses of M-CSF) from WT and *NIK*^{-/-} mice were cultured in the presence of M-CSF and the indicated dose of RANKL for 6 d and then fixed and stained for TRAP. Very few, poorly spread TRAP-positive cells are seen in the *NIK*^{-/-} cultures. Bar, 100 µm. (C) Osteoclastogenic cultures were generated as in B on an artificial hydroxyapatite matrix (Osteologic slides). On day 6, cultures were stained with methylene blue at basic pH. Resorbed matrix is seen as a clear white area around dark cells. *NIK*^{-/-} cultures have negligible matrix resorption. Bar, 300 µm. (D) WT and *NIK*^{-/-} Mφs were treated with RANKL for 0–4 d and then harvested for RNA extraction. Semiquantitative RT-PCR was performed for CTR, MMP9, and β3 integrin, all markers of OC differentiation, with GAPDH as control. Although β3 integrin levels are comparable at day 1, all three markers show a failure in induction from days 2–4 in *NIK*^{-/-} cultures, confirming the morphological lack of differentiation.

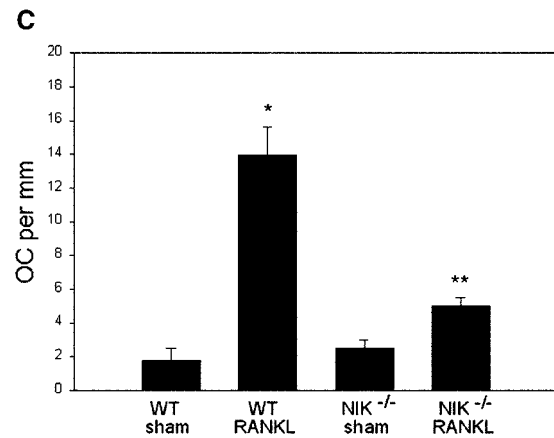
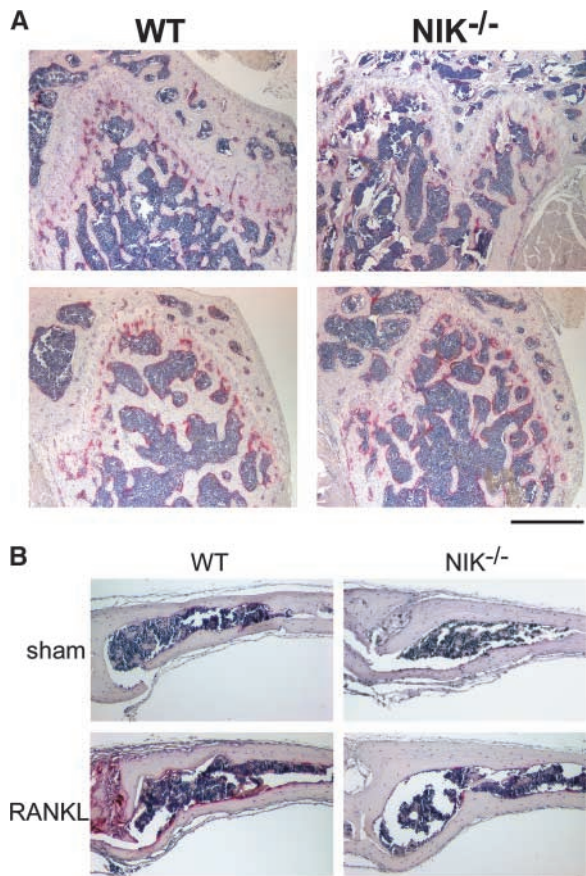


Figure 2. NIK^{-/-} mice have normal basal osteoclastogenesis and a blunted response to RANKL in vivo. (A) TRAP-stained sections of femurs from unmanipulated 3–4-mo-old WT and NIK^{-/-} mice show no difference in trabecular architecture or OC number red-stained cells. Bar, 600 μ m. (B) WT and NIK^{-/-} mice were injected with 500 μ g GST-RANKL or PBS daily for 7 d and killed on the 8th day. TRAP-stained coronal sections of calvaria adjacent to the sagittal suture show a dramatic increase in OCs along sutures and sinusoids only in WT RANKL-treated animals. Bar, 150 μ m. (C) Quantitation of osteoclasts along inner (sinusoidal) calvarial surfaces for animals described in B confirms the significant difference in the response of WT and NIK^{-/-} mice to RANKL injection. Error bars indicate SEM. * $P < 0.0001$ compared with WT sham. ** $P < 0.001$ compared with NIK^{-/-} sham and WT RANKL.

rescued by increasing RANKL by amounts up to 10-fold the optimal dose for WT cultures (Fig. 1 B, 1,000 ng/ml RANKL) or by doubling the time in culture (unpublished data). Given that these experiments were performed using only M ϕ s, the osteoclastogenic defect in NIK^{-/-} whole marrow cultures resides in OC precursors and not in cytokine-producing stromal cells. The lack of morphological changes in NIK^{-/-} cultures is accompanied by a failure, beyond day 1, to up-regulate transcripts characteristic of OCs, including β 3 integrin, matrix metalloproteinase 9, and CTR (Fig. 1 D). However, NIK^{-/-} cultures are not completely unresponsive to RANKL. At day 1, β 3 integrin mRNA is induced in NIK^{-/-} cultures at levels similar to WT. Additionally, NIK^{-/-} M ϕ s show normal MAPK activation (unpublished data).

Interestingly, in vivo basal osteoclastogenesis is unaltered in NIK^{-/-} mice. Femurs of unmanipulated 3–4-mo-old WT and NIK^{-/-} mice have similar cortical thickness, trabecular size and distribution, and number of osteoclasts (Fig. 2 A). On the other hand, and consistent with our in vitro observations, these mutants exhibit a blunted response to osteoclastogenic stimuli. Whereas WT mice generate a robust sevenfold increase in OC number along calvarial sinuses after 1 wk of RANKL treatment, NIK^{-/-} mice show only a twofold rise (Fig. 2, B and C). Similarly, NIK^{-/-} mice fail to show a significant osteoclastogenic response to a 3-d course of parathyroid hormone injection (unpublished data).

RANKL Induces Processing of p100 in WT but not NIK^{-/-}, OC Precursors. Because NIK has been shown to control p100 processing in response to other TNF family cytokines, we next assessed the effect of RANKL on p100 and p52 levels in WT and NIK^{-/-} cultures. In unstimulated WT M ϕ s (Fig. 3 A, 0 h), p100 but not p52 is readily detected. Exposure to RANKL yields accumulation of p52 beginning at 4 h, with no change in p100 levels. In contrast, RANKL exposure for as long as 48 h fails to induce the appearance of p52 in NIK^{-/-} OC precursors. Moreover, whereas basal p100 levels are comparable in WT and NIK^{-/-} M ϕ s (0 h for WT and NIK^{-/-} in Fig. 3 A), p100 is substantially enhanced (2.5–4-fold) in mutant cells by 8 h of RANKL exposure, and this increase persists for at least 48 h. This effect is specific for RANKL, since exposure of WT M ϕ to TNF α does not induce production of p52, although it does increase p100 levels (Fig. 3 B).

Because the *nfkb2* gene is itself regulated by NF- κ B (24), we asked if increased transcription contributes to the accumulation of p100 in NIK-deficient cells. Semiquantitative RT-PCR shows that RANKL treatment up-regulates *nfkb2* mRNA fourfold by 6 h in both WT and NIK^{-/-} M ϕ cultures (Fig. 3 C). Interestingly, *nfkb2* mRNA levels are substantially lower in NIK^{-/-} cells at 24 and 48 h. Because NF- κ B transactivates the *nfkb2* gene, this observation is consistent with a possible decrease in NF- κ B signaling at these later times.

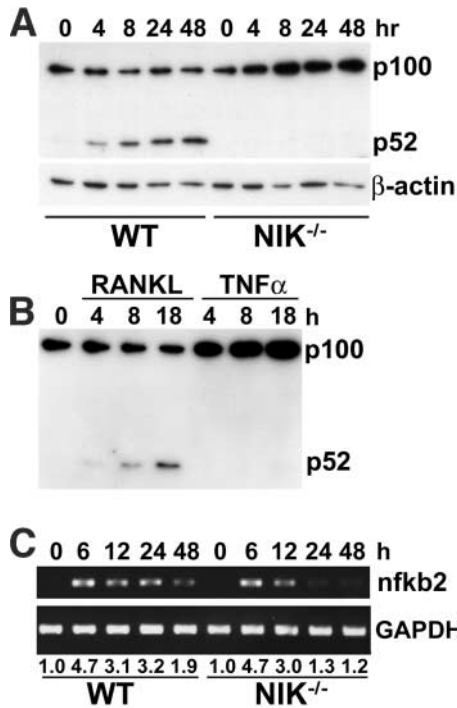


Figure 3. RANKL-induced p100 processing does not occur in $NIK^{-/-}$ OC precursors despite normal induction of *nfk2* transcription. (A) WT and $NIK^{-/-}$ Mφs were treated with RANKL for increasing times, and total lysates were analyzed by immunoblot to detect changes in p100 and p52 levels, using a monoclonal antibody recognizing the NH₂ terminus common to p100 and p52. The blot was stripped and reprobed with anti-β actin as a loading control (bottom). In WT cultures, p100 levels remain steady, whereas p52 levels are increased by RANKL treatment. Parallel $NIK^{-/-}$ cultures fail to generate p52 at any time point and accumulate p100. (B) WT Mφs were treated with RANKL or TNFα for the indicated times, and p100/p52 levels were assessed by immunoblot as in A. p100 processing does not occur in response to TNFα. (C) WT and $NIK^{-/-}$ Mφs were treated with RANKL for increasing times, and RNA was harvested. Levels of *nfk2* mRNA were analyzed by semiquantitative RT-PCR using GAPDH as a control. Numbers beneath lanes indicate fold increase over WT 0 h baseline. Although WT and $NIK^{-/-}$ cultures show equivalent induction of *nfk2* at 6–12 h, levels are significantly lower in $NIK^{-/-}$ cultures thereafter.

NF-κB Signaling Is Intact in $NIK^{-/-}$ Mφs but Blunted in $NIK^{-/-}$ preOCs. To determine whether the differences in p100 processing between WT and $NIK^{-/-}$ cultures impacts NF-κB signaling, we assessed NF-κB activation in Mφs (time 0 in Fig. 3 A in which WT and $NIK^{-/-}$ cultures have equivalent p100 levels) and preOCs (48 h in Fig. 3 A in which $NIK^{-/-}$ cultures have more p100 and no p52). Acute stimulation of Mφs with RANKL led to normal degradation of IκBα, nuclear translocation of RelA, NF-κB DNA binding activity, and resynthesis of IκBα (an NF-κB-dependent event) in $NIK^{-/-}$ Mφ cultures (Fig. 4, A and B) consistent with the findings of Yin et al. (18). In contrast, RANKL stimulation of preOCs (generated by exposing Mφs to RANKL for 48 h, then starving of serum and cytokines for 3 h) has very different results in WT and $NIK^{-/-}$ cultures. Similar to its effects in Mφs (Fig. 4), RANKL stimulation of WT preOCs leads to IκBα degradation, nuclear translocation of RelA, and increased NF-κB DNA

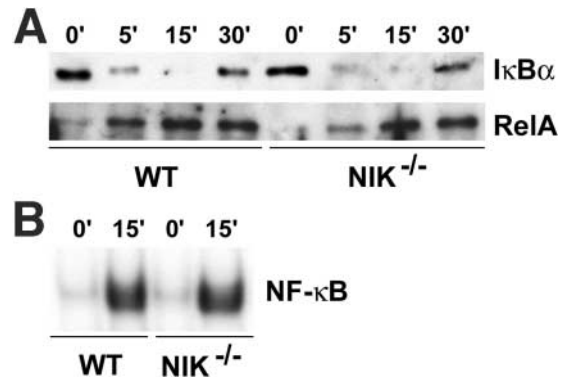


Figure 4. NF-κB signaling in $NIK^{-/-}$ Mφs is intact. (A) Mφs were acutely stimulated with RANKL for the indicated times, and cytoplasmic (top) and nuclear (bottom) fractions were analyzed by immunoblot for IκBα and RelA, respectively. (B) NF-κB DNA binding activity was assessed at 0 and 15 min by EMSA using a κB3 oligonucleotide probe and nuclear extracts from A. NF-κB signaling is comparable in WT and $NIK^{-/-}$ Mφ cultures in response to RANKL.

binding capacity (Fig. 5, A–C). $NIK^{-/-}$ preOCs degrade IκBα in a manner similar to that of WT cells (Fig. 5 A), although basal levels are a bit lower than WT. On the other hand, RelA does not appear in nuclear extracts of the mutant cells (Fig. 5 A). Concordantly, both basal and stimulated NF-κB DNA binding activity is decreased as assessed by EMSA (Fig. 5 B) and an ELISA-based chemiluminescent assay for RelA-specific NF-κB activation (Fig. 5 C). In addition, resynthesis of IκBα is blunted in the absence of NIK (Fig. 5 A, 60 min).

To determine if the nuclear translocation of other NF-κB subunits is affected in $NIK^{-/-}$ cultures, we analyzed nuclear extracts of WT and $NIK^{-/-}$ Mφs and preOCs by immunoblot (Fig. 6). As expected based on Fig. 3 A, within 60 min of RANKL stimulation, neither WT nor $NIK^{-/-}$ Mφs have nuclear p52. Only WT preOCs contain nuclear p52, and this is only slightly responsive to RANKL stimulation. Additionally, RelB is present at high levels in WT preOCs, at very low levels in WT Mφs, and absent in $NIK^{-/-}$ Mφs and preOCs.

These data indicate that, as in other cytokine signaling pathways, NIK is not involved in the degradation of IκBα in response to RANKL. NIK does, however, play a permissive role in NF-κB nuclear translocation in committed preOCs, suggesting increased activity of another IκB-like protein in NIK-deficient preOCs but not Mφ.

p100 Functions as an IκB Protein in $NIK^{-/-}$ preOCs. To determine if the enhanced p100 appearing in RANKL-treated $NIK^{-/-}$ preOCs functions as an IκB, RelA was immunoprecipitated from resting or RANKL-stimulated preOCs, and its association with p100 and IκBα was assessed by immunoblot (Fig. 7). As expected, RelA levels are similar in WT and $NIK^{-/-}$ cells, and association of the subunit with IκBα is decreased by RANKL treatment in both circumstances. On the other hand, although p100 does not associate with RelA in WT cells, it is readily detected in resting $NIK^{-/-}$ RelA immunoprecipitates, and this association increases in response to RANKL. These data suggest

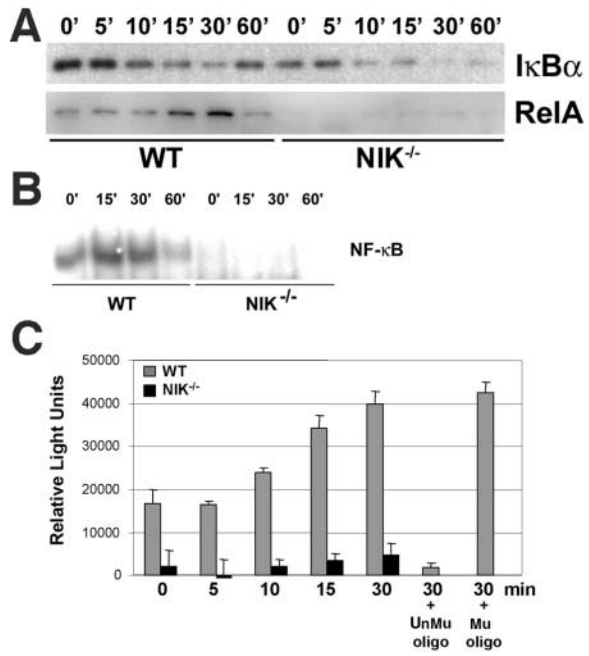


Figure 5. NF- κ B does not accumulate in the nucleus of NIK^{-/-} preOCs. (A) PreOCs, generated by treating M ϕ s with RANKL for 48 h followed by a 3 h starvation, were stimulated with RANKL for the indicated times, and cytoplasmic (top) and nuclear (bottom) fractions were isolated and analyzed by immunoblot for I κ B α and RelA, respectively. Although I κ B α gets degraded in both WT and NIK^{-/-} cultures, RelA does not accumulate in the nucleus in the absence of NIK. (B) Nuclear extracts prepared as in A were analyzed for NF- κ B DNA binding activity by EMSA. Whereas WT preOC cultures have detectable NF- κ B activity at baseline and further induction by RANKL, NIK^{-/-} preOC cultures show little binding activity with or without RANKL. (C) RelA-specific NF- κ B DNA binding activity was assessed by ELISA (EZ Detect kit; Pierce Chemical Co.) using the same preOC nuclear extracts as in A. In this assay, NF- κ B-specific oligos are bound to a plate. Nuclear extract is then applied followed by anti-p65 (RelA) antibody and an HRP-conjugated secondary. The assay is developed with chemiluminescent reagents and read on a luminometer/plate reader. Each sample was run in triplicate. Unmutated (UnMu) and mutant (Mu) soluble oligos were added to the WT 30-min sample to confirm specificity. The results of this assay mirror the EMSA in B and confirm lower baseline NF- κ B activity and lack of RANKL-mediated induction in NIK^{-/-} preOCs. Binding activity is abolished by addition of the unmutated oligo and unaffected by the mutant oligo (last two bars).

that, in NIK^{-/-} OC precursors, exposure to RANKL results in accumulation of p100 because it cannot be processed to p52. Accumulated p100 would then bind NF- κ B subunits as they are released from I κ B α , preventing their nuclear translocation and the subsequent induction of gene transcription. This model predicts that expression of a non-processable form of p100 in WT OC precursors would inhibit osteoclastogenesis.

To test this hypothesis, we used a p100 construct, p100 Δ GRR, which lacks the glycine-rich region determining the site of proteosomal cleavage and hence cannot be processed to p52 (5). Using the pMX-puro retroviral vector (23), we expressed p100 Δ GRR or vector alone as a control in WT M ϕ s (Fig. 8 A). Cells transduced with pMX or pMX-p100 Δ GRR were stimulated with RANKL for 15 min, and NF- κ B DNA binding activity was assessed by

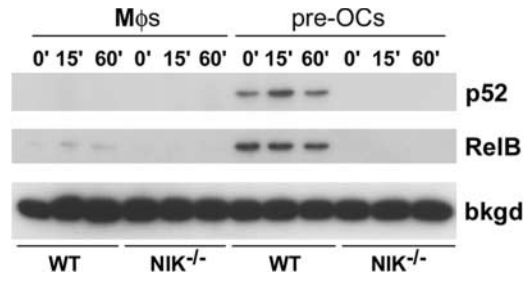


Figure 6. RelB and p52 fail to accumulate in the nucleus in NIK^{-/-} preOCs. Immunoblot of nuclear extracts from M ϕ and preOC cultures treated acutely with RANKL shows accumulation of p52 only in WT preOCs (top). Levels of nuclear RelB are highest in WT preOCs, with minimal acute induction by RANKL. WT M ϕ s show low levels of nuclear RelB, whereas NIK^{-/-} M ϕ s and preOCs lack this NF- κ B subunit. A background band (bkgd) in RelB immunoblots shows equivalent signal in all lanes.

EMSA compared with unstimulated controls. Expression of p100 Δ GRR markedly inhibits induction of NF- κ B activity (Fig. 8 B). Transduced M ϕ s were also cultured in osteoclastogenic concentrations of RANKL plus M-CSF. Consistent with the role of unprocessed p100 as an NF- κ B inhibitory protein, expression of p100 Δ GRR inhibits formation of mature OCs (Fig. 8 C).

Finally, if, as we propose, p100 blunts RANKL-stimulated osteoclastogenesis, OC precursors lacking p100 should show enhanced sensitivity to RANKL. In fact, *nfk2b2*^{-/-} M ϕ s (which lack both p100 and p52) form OCs at lower doses of RANKL and with shorter exposure to the cytokine compared with WT controls (Fig. 9). These data confirm that the *nfk2b2* gene product p100 has an inhibitory effect on RANKL-stimulated osteoclastogenesis, although p52, which potentially contributes to total NF- κ B signaling, is dispensable.

Discussion

OCs are uniquely capable of degrading both the organic and inorganic components of bone, and in concert with bone-building osteoblasts are required for maintenance of skeletal integrity and mineral homeostasis (1). Stimulated OC recruitment prompts the skeletal lesions of postmenopausal osteoporosis, rheumatoid arthritis, and osteolytic metastasis of malignant tumors. Unlike the mesenchyme-

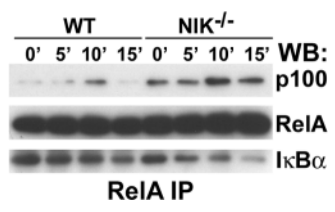


Figure 7. Association of RelA and p100 is increased in NIK^{-/-} preOCs. PreOCs (generated as in Fig. 5) were starved for 3 h and restimulated with RANKL for the indicated times before lysis. Total lysates were immunoprecipitated with anti-RelA antibody and analyzed by immunoblot for p100, RelA, and I κ B α . In both WT and NIK^{-/-} cultures, association of RelA and I κ B α were reduced upon RANKL treatment. In WT cells, RelA associates with p100 only transiently. In contrast, RelA is bound to p100 in NIK^{-/-} cultures before addition of RANKL, and this association is increased with RANKL treatment as I κ B α is degraded.

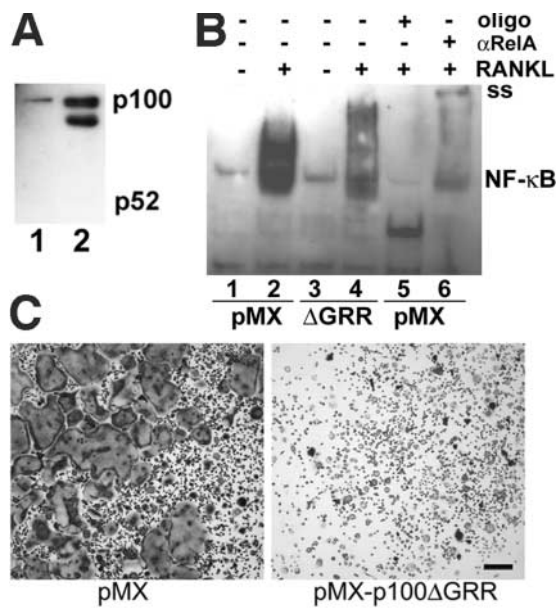


Figure 8. Expression of a noncleavable form of p100 in WT Mφs inhibits NF-κB signaling and osteoclastogenesis. (A) WT Mφs were transduced with retrovirus bearing empty pMX or pMX-p100ΔGRR, a noncleavable form of p100, overnight and selected in puromycin for 3 d before lysis. Immunoblots for p100/p52 demonstrate expression of the retroviral driven p100ΔGRR (lane 2) at levels three to fourfold above endogenous p100 (lane 1), similar to that seen in RANKL-treated NIK^{-/-} Mφs. (B) NF-κB activity was assessed by EMSA on Mφs transduced with pMX (control) or pMX-p100ΔGRR as above. Lanes 1–2, cells transduced with pMX, unstimulated (lane 1), or treated with RANKL for 15 min (lane 2); lanes 3–4, cells transduced with pMX-p100ΔGRR, unstimulated (lane 3), or treated with RANKL for 15 min (lane 4); lane 5, RANKL-stimulated vector control, with unlabeled probe at 50-fold excess; and lane 6, RANKL-stimulated vector control supershifted with polyclonal anti-RelA antibody. The supershifted band is indicated (ss). Expression of p100ΔGRR in Mφs dramatically decreases NF-κB signaling by RelA in response to RANKL. (C) After the 3 d puromycin selection, retrovirally transduced Mφs were cultured in RANKL for 5 d and then stained for TRAP. Expression of p100ΔGRR inhibits osteoclastogenesis in these WT cultures. Bar, 300 μm.

derived osteoblasts that survive for several months, bone marrow-derived OCs are short-lived with a lifespan of 1–2 wk (25). Therefore, tight control of OC differentiation is as critical as control of OC activity for skeletal homeostasis.

NF-κB is potently induced by RANKL in OC precursors (26), and the occurrence of osteopetrosis, with an early block in osteoclastogenesis, in *nfk1/nfk2* double knockout mice established the importance of this signal (19, 27). Individual ablation of either *nfk1* or *nfk2* does not impact the skeleton of unmanipulated mice, indicating mutual compensation between p50 and p52. In vitro osteoclastogenesis, by coculture with osteoblasts, is also not impaired in either *nfk1*- or *nfk2*-deficient mice (19). *nfk1*^{-/-} and *nfk2*^{-/-} mice lack both the precursor (p105 or p100) and the active NF-κB subunit (p50 or p52). Due to the potential opposing functions of the precursors (as IκBs) and the products (as active NF-κBs), loss of both precursor and product may mask the function of either one. Thus, the impact of individual subunits on specific NF-κB targets, or on a physiological process such as osteoclastogenesis, is unresolved.

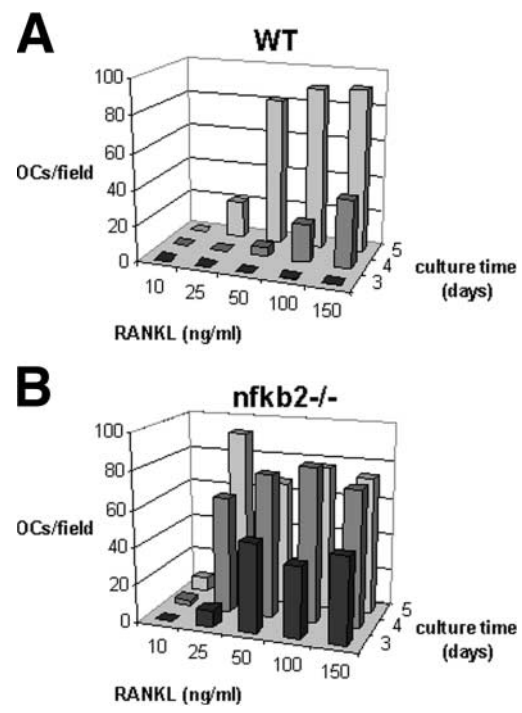


Figure 9. RANKL-mediated osteoclastogenesis in vitro is enhanced in the absence of p100. Mφs from WT (A) and *nfk2*^{-/-} (B) mice were cultured with increasing doses of GST-RANKL for 3, 4, or 5 d and then fixed and stained for TRAP. The number of mature OCs was counted for one 4× field per well, 4 wells per condition. At high doses of RANKL (50–150 ng/ml), WT OCs begin to form on day 4 and are confluent on day 5. In contrast, *nfk2*^{-/-} OCs begin to form on day 3 with as little as 25 ng/ml RANKL and are confluent on day 4. The peak numbers of OCs are slightly lower for *nfk2*^{-/-} cultures than for WT cultures because the *nfk2*^{-/-} OCs are larger. This is also the case for the *nfk2*^{-/-} cultures at 25 ng/ml RANKL on day 5 in which the OCs are smaller than at the higher doses. Thus, *nfk2*^{-/-} cultures, which lack both p100 and p52, show accelerated osteoclastogenesis at lower doses of RANKL compared with WT controls.

The conversion of p105 to p50 is predominantly constitutive, and no specific regulators of the process are in hand (4). In contrast, processing of p100 to p52 is tightly controlled by a known signaling pathway involving NIK (9, 10). Therefore, by manipulating this pathway it is possible to substantially alter the ratios of these two proteins. For instance, in the absence of processing, increased transcription of the *nfk2* gene can enhance p100 levels. When both synthesis and processing of p100 are stimulated, p52 levels rise while p100 levels remain stable, greatly increasing the ratio of p52 to p100. We have used the NIK^{-/-} system in which p100 accumulates due to an absence of processing and have uncovered a requirement for the processing of p100 for RANKL-stimulated osteoclastogenesis.

NIK directly interacts with TRAFs (28), a family of adaptors that mediate NF-κB and JNK activation downstream of TNF receptor family members, including RANK, CD40, and LtβR. Although overexpression studies suggested that NIK is the kinase bridging this receptor/adaptor family and the classical NF-κB cascade (via its direct interaction with IKKs) (29), this role is not supported

by data from the alymphoplasia and $NIK^{-/-}$ mice, which do not exhibit a global defect in NF- κ B signaling (14, 18, 30). Furthermore, NIK, which binds to and activates $IKK\alpha$, is specifically required for the processing of p100 to p52 (9). The similar defects in $NIK^{-/-}$, $IKK\alpha^{-/-}$, and $nfkb2^{-/-}$ mice indicate that the p100 to p52 pathway is critical for B cell maturation (10–12, 16, 18). BAFF, a TNF family member, has been shown recently to activate this pathway in B cells (31).

In the OC lineage, we find that p100 processing is initiated by RANKL and promotes osteoclastogenesis. Other RANKL-mediated signaling events, including activation of JNK and ERK and degradation of $I\kappa B\alpha$, occur within 5–15 min of cytokine–receptor interactions (32). NIK, $IKK\alpha$, and p100 are all present in unstimulated OC precursors,

but p100 processing is not detectable before 4–6 h after exposure to RANKL. Blockade of protein translation with cyclohexamide also prevents p100 processing (33, 34 and unpublished data). It is therefore likely that several intermediary events, including protein translation, occur between RANK activation and NIK-mediated p52 expression, leading to a several hour delay.

We and others find that induction of p100 processing is restricted to a subset of TNF family cytokines, including RANKL, BAFF, and CD40L but not $TNF\alpha$ or $IL-1\beta$ (31, 33, 34 and this article). Although $TNF\alpha$ can synergize with RANKL to enhance osteoclastogenesis when added after 48 h or RANKL exposure, it inhibits OC differentiation if added simultaneously (35). The $TNF\alpha$ -mediated increase in p100 levels, without generation of p52, seen in

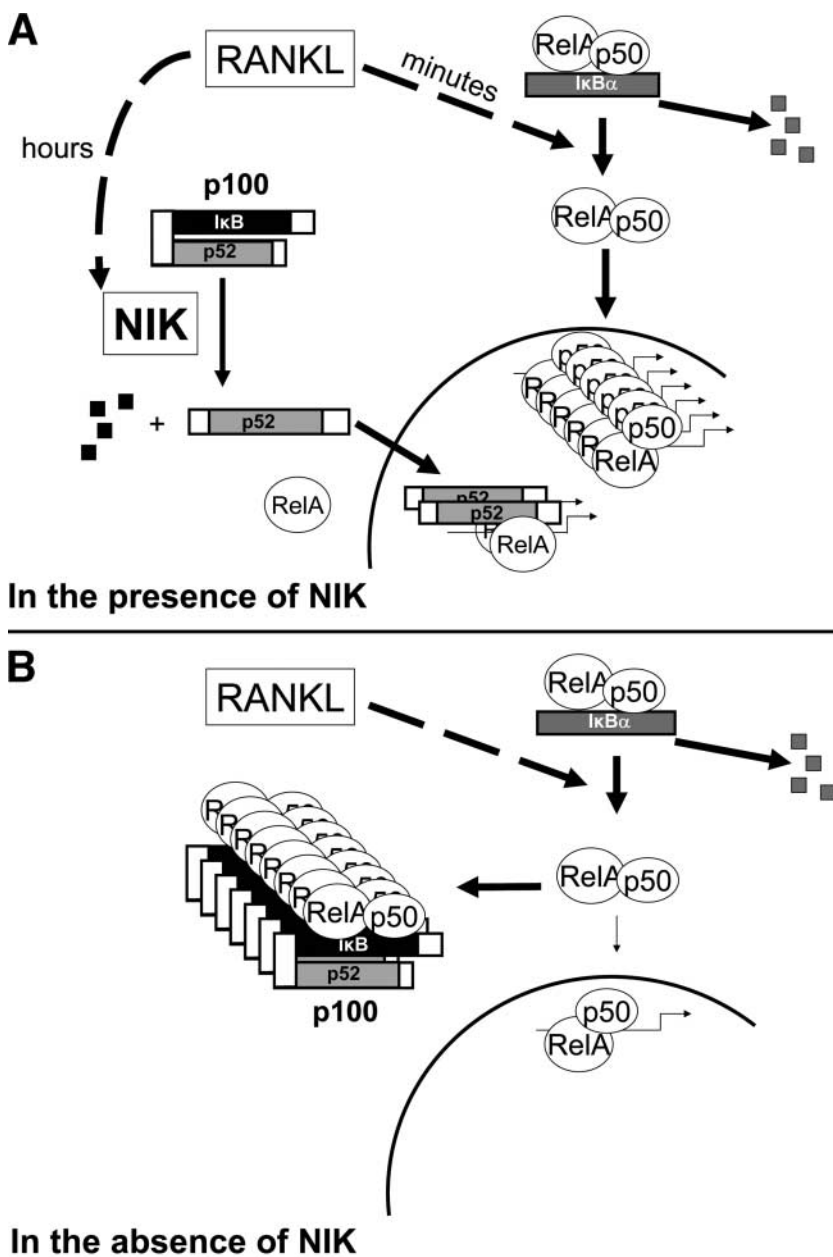


Figure 10. Model for p100-mediated blockade of NF- κ B signaling in NIK-deficient preOCs. See Discussion.

M ϕ (Fig. 3 B) may explain the initial inhibitory effect of TNF at early stages of osteoclastogenesis. The clear stimulatory effect of TNF α on OCs in vivo is not easily explained. However, other factors present in inflammatory conditions may counteract the p100 effect, allowing the many signals common to TNF α and RANKL, such as activation of the classical NF- κ B pathway, MAPKs, and PI3K, to act unopposed.

The features of the NIK/IKK α /p100 pathway in the OC lineage distinguish it from all previous descriptions of the NF- κ B pathway. We propose (Fig. 10) that RANKL induces degradation of I κ B α , releasing NF- κ B, predominantly as RelA/p50 dimers, to rapidly accumulate in the nucleus. After several hours, RANKL also stimulates NIK to process p100, thus releasing p52, which can then complex with RelA or with RelB and travel to the nucleus (36, 37). This transcriptionally active pool of nuclear NF- κ B stimulates osteoclastogenesis. In the absence of NIK, NF- κ B signaling in OC precursors is initially intact. However, after 1–2 d of RANKL stimulation sufficient p100 accumulates in the cytosol to bind RelA/p50 dimers as they are released from I κ B α . This cytoplasmic retention of NF- κ B combined with the lack of p52 reduces NF- κ B-mediated transcription in the nucleus and inhibits osteoclastogenesis.

In vitro, the defect in RANKL-mediated osteoclastogenesis in NIK $^{-/-}$ cultures is profound. In vivo, NIK-deficient mice are resistant to stimulated osteoclastogenesis in response to injection of RANKL or parathyroid hormone but show no bone phenotype in the basal state. A lymphoplasia mice, like NIK $^{-/-}$ mice, have normal bone architecture and a striking defect in osteoclastogenesis in vitro (unpublished data). Mice lacking p100 (*nfk2b2* $^{-/-}$) also show no basal bone phenotype, despite a pronounced enhancement of RANKL-mediated osteoclastogenesis in vitro. Two general possibilities present themselves to explain the apparent paradox in the in vivo and in vitro phenotypes. First, culture conditions may mirror stimulated but not basal osteoclastogenesis. Second, because of compensatory mechanisms operating in vivo, many knockout mice exhibit no phenotype until stressed. As regards to the present situation, although M-CSF and RANKL are necessary for osteoclastogenesis in vivo and sufficient for differentiation of pure OC precursors in vitro, other cytokines modulate osteoclastogenesis within the bone microenvironment. For example, TNF α , in the context of permissive levels of RANKL, enhances osteoclastogenesis in many inflammatory conditions (35, 38–40) and also supports a normal level of basal osteoclastogenesis via the p55 TNF receptor (41). TGF β can also support RANKL-mediated osteoclastogenesis (42, 43). In fact, a combination of RANKL, TNF α , and TGF β rescues the NIK $^{-/-}$ defect in osteoclastogenesis in vitro (unpublished data). We suspect that the presence of these factors, and perhaps others in the bone microenvironment, allows OC differentiation to occur at a normal basal rate in NIK $^{-/-}$ mice but cannot compensate for the inhibitory influence of accumulated p100 induced by exogenous RANKL or PTH, which exerts its osteoclastogenic effect through RANKL.

Our studies uncover a novel NF- κ B pathway using RANKL-induced processing of p100 to control the pool of inhibitory I κ B proteins. Whereas p100 processing is also regulated in B cells by NIK, it is increased p52 and not decreased p100 that is critical for B cell maturation (10, 17). Interestingly, p52 itself is dispensable for osteoclastogenesis, since the *nfk2b2*-deficient mouse, which lacks both p100 and p52, has normal OC numbers in vivo (19) and increased OC numbers in vitro (Fig. 9). In addition, regulation of NF- κ B signaling by p100 is distinct from the classical I κ B pathway in both kinetics (hours versus minutes) and upstream regulation (IKK complex versus NIK/IKK α). Thus, these two distinct mechanisms for NF- κ B control (I κ B α degradation and p100 processing) allow for cell type and receptor-specific responses within the NF- κ B pathway.

NIK $^{-/-}$ osteoclastogenesis in vivo is not impaired in the basal state but only in conditions of excess RANKL such as accompanies administration of PTH. This observation leads us to propose that the modulating effect of NIK/p100 on osteoclastogenesis is important in clinical conditions of increased osteoclastogenesis, such as osteoporosis, rheumatoid arthritis, and osteolytic bone metastasis, in which RANKL expression is enhanced. Most importantly, the fact that p100 arrests stimulated osteoclastogenesis makes it a potential therapeutic target in diseases of accelerated bone loss.

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