Chapter 7

Methods to Assess the Activation of the Alternative (Noncanonical) NF-κB Pathway by Non-death TNF Receptors

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Abstract

The alternative or noncanonical NF-κB pathway regulates the generation of p52-containing NF-κB dimers (e.g., p52/RelB) through a partial degradation (called processing) of the precursor p100 into p52. This pathway is activated by a subset of non-death TNF receptor members, which ultimately activate two kinases: NIK (NF-κB-Inducing Kinase) and IKKα (Inhibitor of κB Kinase alpha). These kinases create a phosphodegron for the E3 ligase SCF-β-TrCP that covalently binds K48-linked polyubiquitin chain onto p100 prior to its proteasomal processing. The resulting p52-containing complexes translocate into the nucleus to activate target genes involved in secondary lymphoid organ development, B cell survival or in osteoclastogenesis.

We describe in this chapter straightforward methods to monitor the activation of the alternative NF-κB pathway. These methods uncover cytosolic and nuclear biochemical modifications of key proteins of the alternative NF-κB pathway required prior to the transcription of NF-κB target genes.

Key words NF-κB, NIK, p100 processing, TRAF, c-IAP1/2 degradation

1 Introduction

The nuclear factor kappa B (NF-κB) is a key transcription factor involved in the transcription of a plethora of genes associated with innate and adaptive immunity, cell survival, or cell proliferation. In human, the NF-κB family contains five proteins named p50, p52, p65, c-Rel, and RelB that form homodimers and heterodimers. In resting cells, NF-κB dimers are kept silent in the cytosol through their binding to members of the Inhibitory kappa B (IκB) family. The latter contains five main proteins that are IκBα, IκBβ, IκBε, p105, and p100. The proteasomal degradation of the IκB inhibitors allows the release of the NF-κB dimers [1]. This process involves the binding of the SCF-β-TrCP E3 ligase to the IκB proteins to build up K48-linked polyubiquitin chains, a posttranslational modification that targets proteins to the proteasome.

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The binding of SCF-β-TrCP to IκB proteins requires the phosphorylation of a conserved motif (degron) DS(G/A)ΦXS to create a phosphodegron [2]. The kinases that phosphorylate the degron DS(G/A)ΦXS within IκBs proteins are activated by two main pathways [3]. First, the classical NF-κB pathway activates the IKK complex in which IKKβ is the main kinase that phosphorylates the two phospho-acceptor Serine within the DSGΦXS degron found in IκBα, IκBβ, IκBε, and p105. The subsequent polyubiquitination of these phosphorylated IκB inhibitors triggers their complete degradation through the proteasome (Fig. 1). Second, the alternative NF-κB pathway activates a kinase called NF-κB-Inducing Kinase (NIK) that together with IKKα phosphorylate the two phospho-acceptor Serine within the atypical DSAYXS degron found in p100 [4]. In that case, the polyubiquitination leads to a partial degradation, called processing, of p100 to generate p52 in association with RelB.

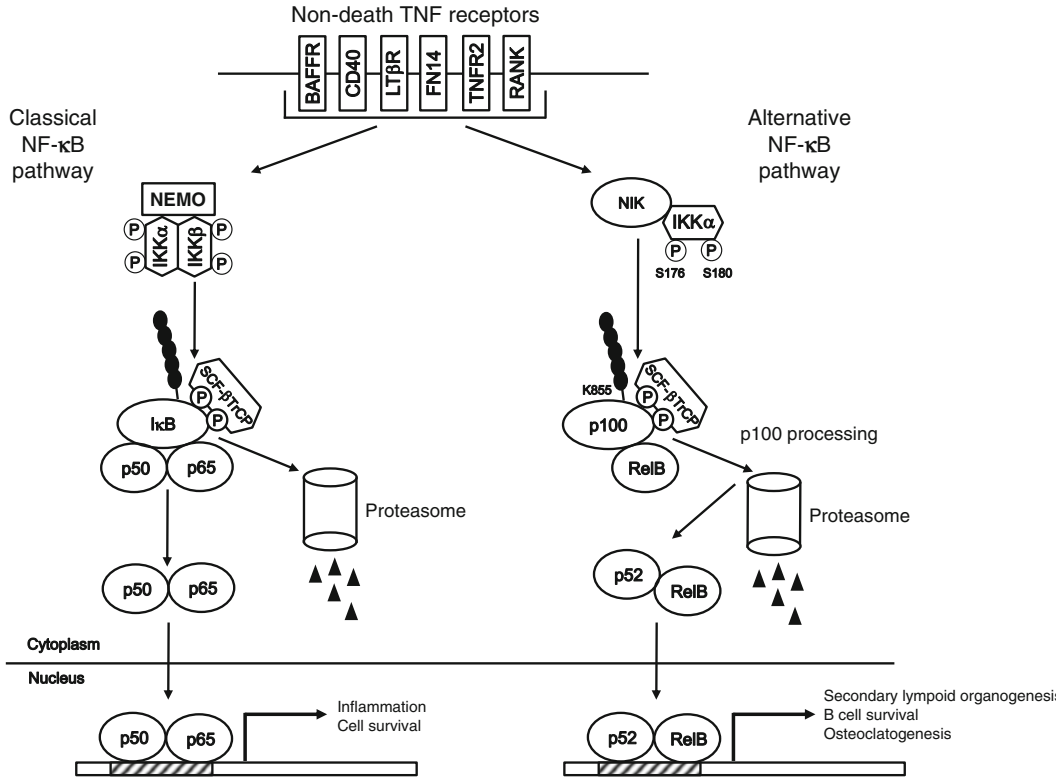

Fig. 1 The classical and the alternative NF-κB signaling pathways are triggered by a subset of TNFR members. The activation of the classical NF-κB pathway relies on the IKK complex containing NEMO/IKKα/IKKβ that phosphorylates IκBs on specific Serine for the binding of the E3 ligase SCF-β-TrCP and the degradation of IκBs by the proteasome leaving p50/p65 free to enter into the nucleus. The alternative NF-κB pathway induces the activity of two kinases, NIK and IKKα that mediate the phosphorylation of p100 prior to its polyubiquitination on K855. These biochemical modifications lead to the processing (partial degradation) of p100/RelB and the release of p52/RelB for its nuclear translocation

The alternative NF-κB pathway is activated by a subset of TNF receptors such as LTβR, BAFFR, CD40, Fn14, TNFR2, and RANK, which altogether activate genes involved in specific immunological processes including secondary lymphoid organ development, B cell survival, and osteoclastogenesis [5–11] (Fig. 1). Once one of these receptors is triggered by its ligand, adaptor proteins such as TRAFs are recruited to the cytoplasmic tail and NIK is activated. NIK, a mitogen-associated protein 3 kinase (MAP3K14), is the central signaling component of the alternative NF-κB pathway. In resting cells, NIK is kept at undetectable levels by a negative regulatory mechanism involving dynamic ubiquitination and proteasomal degradation (Fig. 2a). Indeed, de novo NIK is constantly targeted for degradation by TRAF2/3-c-IAP1/2 complex. TRAF3 physically interacts with NIK and recruits TRAF2, c-IAP1/2. c-IAP1/2 mediates K48-linked polyubiquitination chains onto

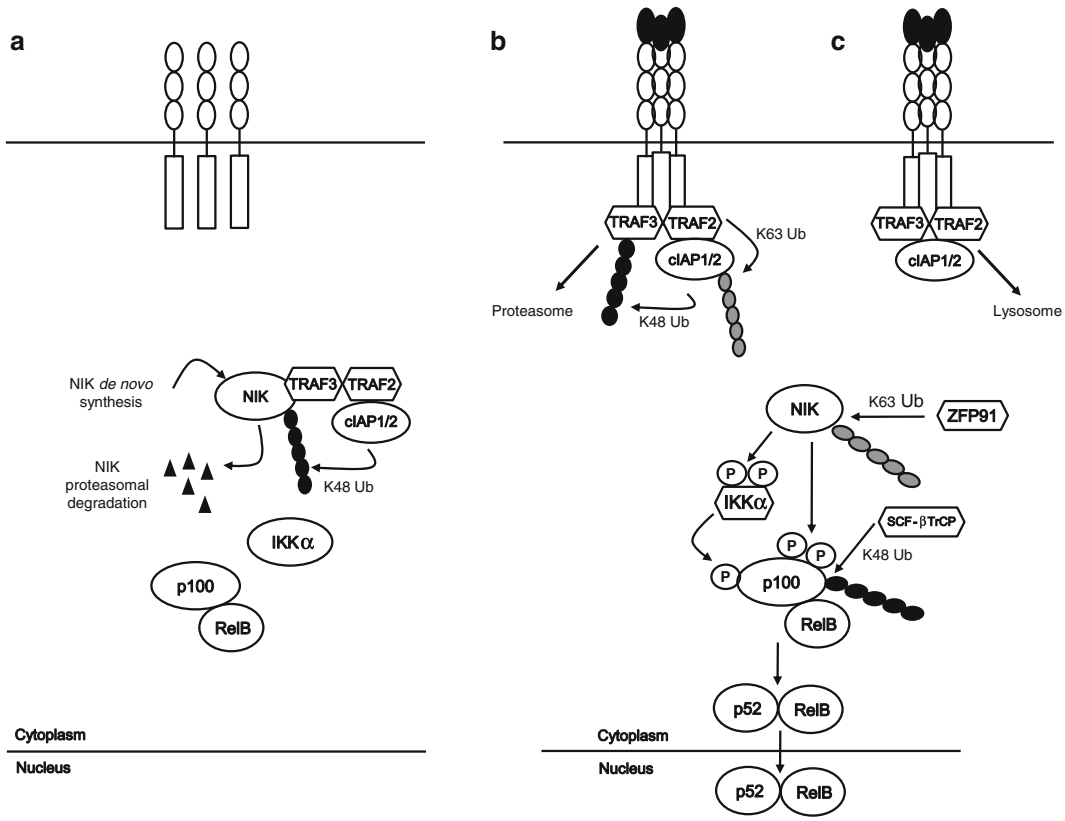

Fig. 2 Model of NIK activation. (**a**) In resting cells, de novo synthesized NIK is constantly targeted by the TRAF2/3-c-IAP1/2 complex for proteasomal degradation. (**b**, **c**) Binding of trimeric ligand to particular TNFR members induce the inhibition of the inhibitory complex TRAF2/3-c-IAP1/2 either through (**b**) proteasomal degradation or (**c**) lysosomal degradation. As a consequence, NIK is ubiquitinated by the E3 ligase ZFP91 for further promoting its stability and participate with IKKα to the phosphorylation of p100. These posttranslational modifications allow the recruitment of the E3 ligase SCF-β-TrCP and the ubiquitination-dependent processing of p100/RelB into p52/RelB

NIK leading to its constant proteasomal degradation [12, 13]. In response to a stimulus such as CD40L, the inhibitory complex is recruited to the receptor where TRAF3 is K48-linked polyubiquitinated by the c-IAP1/2 and degraded by the proteasome (Fig. 2b). Alternatively, some activated receptors like LTβR are internalized, and this recruits the negative regulatory complex TRAF2/3-c-IAP1/2 for its degradation into lysosomes [8] (Fig. 2c).

Thus, TRAF proteins degradation is required for the stabilization of newly synthesized NIK. Yet other posttranslational modifications seem to contribute to the accumulation of NIK. Indeed, a K63-linked polyubiquitination of NIK by the E3 ligase ZFP91 enhances its stability [14]. Recently, two groups crystallized the kinase domain of NIK and found that phosphorylation of the Threonine 559 in the activation loop was not required for its enzymatic activity [15, 16]. NIK structural features are represented in the Fig. 3. Active NIK phosphorylates and activates the kinase IKKα on Ser 176 (*see* Figs. 1 and 4) [17]. However, in resting conditions the processing of p100 is limited due to the presence of a Processing-Inhibitory Domain (PID) and an Ankyrin Repeat Domain (ARD) in its carboxyl-terminal part [4]. However, the inhibitory function of the PID is relieved when Serine 866/870 and Serine 99/108/115/123/872 are phosphorylated by NIK and IKKα, respectively [4, 18]. The phosphorylation of p100 on Serine 866/870 creates the phosphodegron for the E3 ligase

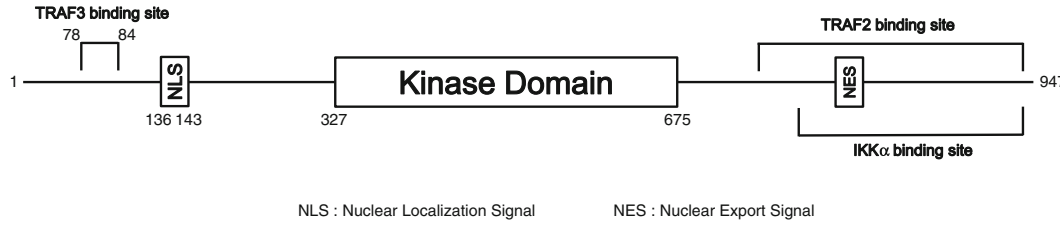

Fig. 3 Regulatory domains of the human NIK protein. Numbers represent the amino acid boundaries of each domain

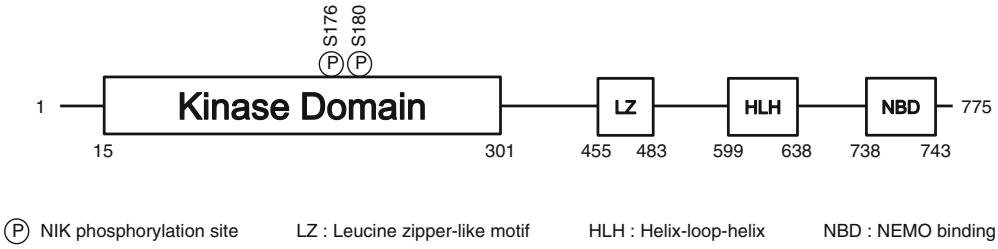

Fig. 4 Regulatory domains of the human IKKα protein. Numbers represent the amino acid boundaries of each domain

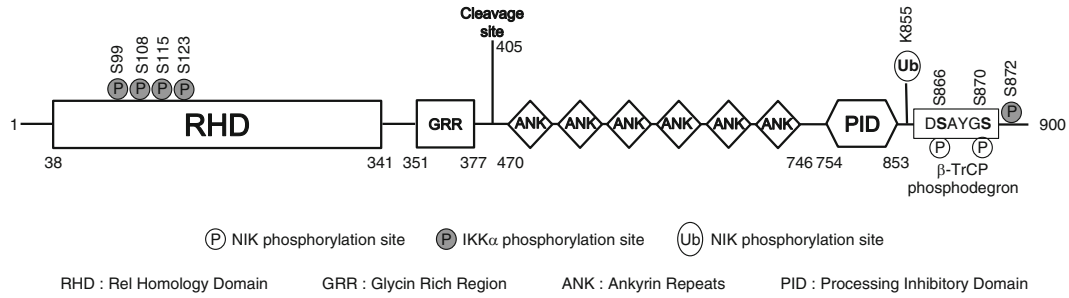

Fig. 5 Regulatory domains of the human p100 protein. Numbers represent the amino acid boundaries of each domain

β-TrCP that promotes the K48-linked polyubiquitination of Lysine 855 (*see* Fig. 5) [19]. The processing of p100 allows the cytoplasmic release of p52 dimerized with RelB.

Once the NF-κB dimers are freed from their IκB inhibitors, they are recognized by transporter proteins called importins, which brings them into the nucleus [20]. The binding of nuclear NF-κB dimers to the chromatin is controlled by many parameters, among which the nucleotide stretch called κB site dictates the affinity towards specific dimers. Whereas the main NF-κB heterodimer of the classical NF-κB pathway (p50/p65) was shown to bind a remarkably loose consensus sequence 5′-GGGRNYYYCC-3′ (R = A or G, Y = T or C) [21], the existence of p52/RelB specific κB sites is still matter of debate. So far, all the κB sites identified for binding p52/ReB displayed comparable affinity for p50/p65 [22]. Interestingly, the X-ray structure of p52/RelB-κB DNA complex revealed that p52/RelB was able to bind a larger spectrum of κB sites than p50/p65 because it was more versatile and less discriminatory in DNA recognition. Arginine 125 of RelB is important to recognize specific κB sites that have more contiguous and centrally located A:T base pairs. Depending on DNA sequence, Arg 125 of RelB allows a switch in the conformation of the dimer in order to interact efficiently with diverse κB sites and thereby activate a broad spectrum of genes involved in inflammation and development [23]. Although much progress is ongoing to refine the specificity of particular NF-kB dimers for the diversity of κB sites in the genome, up to now one can only say that some sites are preferred over others [24, 25].

2 Materials

2.1 Ligands and Agonist Antibodies for TNFR Inducing the Alternative NF-κB Pathway

The ligands and agonist antibodies that we use to activate the alternative pathway are shown in Table 1 along with their species specificities and sources.

Table 1
Ligands and agonist antibodies for TNFR inducing the alternative NF-κB pathway

Name	LTβR		Fn14		CD40		Reference
	Human	Mouse	Human	Mouse	Human	Mouse	
hLT$\alpha_1\beta_2$	+	–					Human lymphotoxin alpha 1 beta 2, R&D Systems (Minneapolis, MN, USA)
Anti-hLTbR	+	–					Human Lymphotoxin βR/ TNFRSF3 Antibody, R&D Systems.
Anti-mLTβR	–	+					Mouse Lymphotoxin beta Receptor antibody (clone 3C8), eBioscience (San Diego, CA, USA)
Flag-hLIGHT	+	+					TWEAK (soluble) (human) (recombinant), Enzo Life Science (Farmingdale, NY, USA)
Flag-hTWEAK			+	+			LIGHT (soluble) (human) (recombinant), Enzo Life Science
Flag-hCD40L					+	–	CD40L (soluble) (human) (recombinant), Enzo Life Science

2.2 Antibodies for Western Blotting, Immunoprecipitation, and Supershift Assays

The ligands antibodies that we use for western blotting, immunoprecipitation, and supershift assays to detect activation of the alternative pathway are shown in Table 2 along with their species specificities and sources.

2.3 Whole-Cell Extracts for Immunoblotting

1. Whole-cell lysis (WCE) buffer: 20 mM HEPES (pH 7.9), 350 mM NaCl, glycerol 20 % (v/v), Igepal 1 % (v/v), 1 mM $MgCl_2$, 0.1 mM EGTA or EDTA with protease and phosphatase inhibitors freshly added (Complete and PhosSTOP, Roche, Madison, WI, USA).
2. SDS lysis buffer: 0.5 % SDS (w/v) with protease and phosphatase inhibitors freshly added (Complete and PhosSTOP, Roche).
3. 10 % SDS-PAGE gel.

2.4 Cell Extracts for Co-immunoprecipitation Experiments

1. TNT buffer: 20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 0.5–1 % (v/v) Triton X-100 with protease and phosphatase inhibitors freshly added (Complete and PhosSTOP, Roche).
2. Protein-A Agarose (Pierce, Waltham, MA, USA).

Table 2
Antibodies for western blotting, immunoprecipitation, and supershift

Name	Human	Mouse	Reference
TRAF2 (H-249)	+	+	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TRAF2 (C-20)	+	+	Santa Cruz Biotechnology
TRAF3 (H-122)	+	–	Santa Cruz Biotechnology
TRAF3 (C-20)	+	+	Santa Cruz Biotechnology
c-IAP1/2	+	+	CycLex, Nagano, Japan
c-IAP1	+	–	R&D Systems
c-IAP2	+	–	R&D Systems
NIK	+	+	Cell Signaling, Danvers, MA, USA
Phospho-NF-kB2 p100 (Ser866/870)	+	+	Cell Signaling
NF-kB p52	+	–	Millipore, Billerica, MA, USA
NF-kB p52 (C-5)	+	+	Santa Cruz Biotechnology
RelB (C-19)	+	+	Santa Cruz Biotechnology
IKKα	+	+	Imgenex, Littleton, CO, USA
IKKα	+	–	BD Pharmingen, Franklin Lakes, NJ, USA
Phospho-IKKα/β (Ser176/180) (16A6)	+	+	Cell Signaling

3. Loading buffer 4×: 6.25 ml of 0.5 M Tris (pH 6.8), 20 ml of glycerol, 4 g of SDS, 10 ml of 1 M β-mercaptoethanol, 2 mg of bromophenol blue.
4. 10 % SDS-PAGE gel.

2.5 Kinase Assays

1. Kinase assay lysis buffer (KLB): 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.2 % (v/v) Igepal, 1 mM EDTA, 10 % (v/v) glycerol with protease and phosphatase inhibitors freshly added (Complete and PhosSTOP, Roche).
2. Kinase assay lysis buffer (KLB) High Salt: 500 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.2 % (v/v) Igepal, 1 mM EDTA, 10 % (v/v) glycerol.
3. Immunoprecipitation materials: NIK antibody (Cell signaling, Danvers, MA, USA), ChromPure Rabbit Ig, whole molecule (Jackson Immunoresearch, West Grove, PA, USA), Protein-A Agarose beads (Pierce).

4. Kinase assay buffer (KAB): 20 mM HEPES (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, with protease and phosphatase inhibitors freshly added (Complete and PhosSTOP, Roche).
5. 1.5 mM ATP.
6. ATP [$\gamma^{32}P$] 10 mCi/ml - 3,000 Ci/mmol 10 mCi/ml (Perkin Elmer, Waltham, MA, USA).
7. 10 % SDS-PAGE gel.

2.6 GST-Fusion Protein Production

1. BL21 (DE3) pLysS Competent bacteria (Promega, Madison, WI, USA).
2. Luria Broth (LB).
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
4. PBS lysis buffer: PBS with 1 % (v/v) Triton X-100 and 1 mM PMSF freshly added.
5. PBS with protease inhibitors (Complete, Roche) freshly added.
6. Glutathione Sepharose 4B (GE Healthcare, Mickleton, NJ, USA).
7. 10 % SDS-PAGE gel.
8. Coomassie blue dye (250 ml): 75 ml of methanol, 25 ml of acetic acid (glacial), 0.25 g of Coomassie blue, 150 ml of H_2O.
9. Destaining solution (500 ml): 150 ml of methanol, 50 ml of acetic acid (glacial), 300 ml of H_2O.
10. 10 % SDS-PAGE gel.

2.7 Cytoplasmic-Nuclear Extracts

1. Cytoplasmic lysis buffer: 0.2 % (v/v) Igepal (for most cells except for MEFs that are more sensitive and require only 0.05 % (v/v) Igepal), 10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA.
2. Nuclear lysis buffer: 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 25 % (v/v) glycerol.
3. Washing buffer: 10 mM HEPES (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

1. Probe for NF-κB mobility shift:
   + Strand: 5′- TTGGAGTTGAGGGGACTTTCCCAGG -3′
   – Strand: 5′- TTGGCCTGGGAAAGTCCCCTCAACT -3′
2. 100 mM NaCl.
3. T4 polynucleotide kinase, 3′-phosphatase free (Roche).
4. Quick Spin Columns for radiolabeled DNA purification (Roche).
5. TBE 10× (1 l): 108 g of Tris base, 55 g of boric acid, 7.5 g of EDTA disodium salt, deionized water.

6. 6 % Native PAGE Gel: 12 ml of acrylamide–bisacrylamide 40 % (29:1), 48 ml of deionized water, 20 ml of TBE 1×, 560 µl of ammonium persulfate (APS) 10 % (w/v), 72 µl of *N*, *N*, *N*′-tetramethyl-ethylenediamine (TEMED).
7. Buffer D: 20 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 10 % (v/v) glycerol, 5 mM DTT (freshly added).
8. Loading buffer: 30 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue in 20 mM HEPES (neutral pH).
9. Nuclear extracts.
10. 1 µg/µl bovine serum albumin (BSA).
11. Poly (dI-dC) (double strand; Sodium salt).

3 Methods

3.1 Induction of the Alternative NF-κB Pathway

Several receptors of the TNF receptor superfamily can activate both the classical and the alternative pathways. In contrast to the classical pathway, the activation of the alternative pathway is slower and can take up to 2–16 h according to the stimulus. Table 3 shows a range of validated stimuli, doses, and timing required to activate the alternative NF-κB pathway.

3.2 TRAF2/3 Recruitment to the Receptor

TRAF2/3 recruitment can be analyzed by co-immunoprecipitation assays (*see* **Note 1**).

1. Grow cells in 6-well plates and treat with appropriate ligands or agonist antibodies for 30 min to 4 h (*see* Table 3).
2. Add the protease and phosphatase inhibitors to the TNT lysis buffer.
3. Discard the medium and wash the cells with ice-cold PBS.
4. Collect the cells by scraping in ice-cold PBS and transfer into tubes.

Table 3
Stimuli, doses, and timing to activate the alternative NF-κB pathway

Stimuli	Type	Receptor	Dose	Time
hLT$\alpha_1\beta_2$	Ligand	hLTβR	1 nM	>2 h
anti-hLTβR	Agonist antibody	hLTβR	0.5–2 µg/ml	>2 h
anti-mLTβR	Agonist antibody	mLTβR	2 µg/ml	>2 h
Flag-hLIGHT	Ligand	h/mLTβR	200 ng/ml	>2 h
Flag-hTWEAK	Ligand	h/mFn14	200 ng/ml	>2 h
Flag-hCD40L	Ligand	hCD40	1 µg/ml	>6 h

5. Pellet the cells by centrifugation at 1,000 × *g* for 1 min at 4 °C and remove the supernatant.
6. Lyse the cells in 100–200 µl (depending on the size of the pellet) of TNT buffer for 10 min on ice.
7. Centrifuge at maximum speed for 10 min at 4 °C to get rid of the insoluble fraction and transfer the supernatant in a new tube.
8. Keep 50 µl of the extract for input. Adjust the volume of the remaining lysate to 500 µl with TNT 1 % and pre-clear for 1 h with 10 µl of protein-A agarose beads (previously washed three times in TNT 1 %) at 4 °C under rotation. Discard the beads by centrifugation for 1 min at maximum speed. Keep the supernatant.
9. Incubate the supernatant overnight with 1 µg of anti-TRAF2 or anti-TRAF3 antibodies at 4 °C under rotation.
10. Add 10 µl of protein-A agarose beads (previously washed three times in TNT 1 %) for an additional hour at 4 °C under rotation.
11. Centrifuge for 1 min at 6,000 × *g*. Discard the supernatant.
12. Wash the beads three times with 500 µl of TNT 1 %. After the final wash, resuspend to 30 µl of immunoprecipitate and add 10 µl of loading buffer 4×. At this stage the samples can be kept at −20 °C.
13. Boil the immunoprecipitate for 10 min, load on an SDS-PAGE 10 % acrylamide gel, and analyze by immunoblotting with antibody for your TNFR of interest (*see* Table 2).

3.3 c-IAP1/2 Degradation (See Note 2)

1. Grow cells in 6-well plates and treat with specific ligands or agonist antibodies for 30 min up to 6 h (*see* Table 3).
2. Add the protease and phosphatase inhibitors to the WCE lysis buffer.
3. Discard the medium and wash the cells with ice-cold PBS.
4. Collect the cells by scraping in ice-cold PBS and transfer into tubes.
5. Pellet the cells by centrifugation at 1,000 × *g* for 1 min at 4 °C and remove the supernatant.
6. Lyse the cells in 100–200 µl (depending on the size of the pellet) of WCE buffer for 10 min on ice.
7. Centrifuge at maximum speed for 10 min at 4 °C to get rid of the insoluble fraction and transfer the supernatant to a new tube.
8. Load 50 µg of protein on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with c-IAP1/2 antibody (*see* Table 2).

3.4 NIK Stabilization and Activation (See Note 3)

1. Grow cells in 6-well plates and treat with specific ligands or agonist antibodies for 2–6 h (*see* Table 3).
2. Perform whole-cell extracts as in Subheading 3.3.
3. Load 80 μg of protein on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with a NIK antibody (*see* Table 2).

3.5 NIK Kinase Activity

Another way to assess NIK kinase activity is to determine its ability to autophosphorylate or to phosphorylate a substrate by performing a kinase assay.

3.5.1 GST-p100 Production

A substrate for NIK is a fusion of GST with p100 spanning from amino acids 660–900 (e.g., cloned in the pGex4T1 vector). This substrate is produced as follows:

1. Inoculate one colony of bacterial strain BL-21 (DE3) expressing the construct GST-p100 into individual 5 ml aliquots of LB broth containing suitable antibiotic selection. Grow overnight at 37 °C with shaking.
2. Inoculate 500 ml of LB containing the antibiotic selection with the 5 ml overnight culture from **step 1**.
3. Grow the cultures at 37 °C to reach an OD_{600} of 0.5–1.0 (this should take 3–4 h).
4. Induce the expression of the GST-p100 by adding IPTG to a final concentration of 1 mM.
5. Incubate the cultures for an additional 4 h at 37 °C with shaking.
6. Centrifuge the bacterial culture at 3,500 × *g* for 10 min at 4 °C and discard the supernatant. At this point, the pellets can be stored frozen at –20 °C if necessary.
7. Resuspend the pellet in 20 ml of PBS lysis buffer and sonicate the bacterial suspension on ice, alternate cycles of 30 s bursts (high frequency) and 30 s of rest on ice. Five cycles of sonication are usually sufficient.
8. Centrifuge the lysate at 12,000 × *g* for 30 min at 4 °C and transfer the supernatant to a fresh tube.
9. Add 500 μl of a 50:50 slurry solution of glutathione-Sepharose beads equilibrated in PBS lysis buffer to the supernatant of bacterial lysate and incubate for 30 min at room temperature, rotating the tube end over end to ensure mixing.
10. Centrifuge the samples at 750 × *g* for 1 min at 4 °C to pellet the beads. Remove the supernatant.
11. Wash the beads in 5 ml of ice-cold PBS with protease inhibitors.
12. Centrifuge the samples at 500 × *g* for 1 min at 4 °C to pellet the beads. Remove the supernatant.

13. Add 5 ml of ice-cold PBS with protease inhibitors. Resuspend the beads by gentle mixing and centrifuge the sample again at 500 × *g* for 1 min at 4 °C to pellet the beads. Remove the supernatant.
14. Resuspend the beads in 250–500 µl of ice-cold PBS with protease inhibitors and store at −80 °C.
15. Run 5–20 µl of the product as well as a range of concentrations of BSA (100 ng up to 1.5 µg) on an SDS-PAGE 10 % acrylamide gel and stain with Coomassie blue dye for 1 h at room temperature with shaking.
16. After de-staining the gel, estimate the concentration of the GST-p100 produced in regard to the titration of BSA.

3.5.2 Kinase Assay of NIK on GST-p100

1. Grow cells in 10 cm plates and treat with specific ligands or agonist antibodies for 2 up to 6 h (*see* Table 3).
2. Add the protease and phosphatase inhibitors as well as the DTT to KLB.
3. Discard the medium and wash the cells twice with ice-cold PBS.
4. Collect the cells by scraping in ice-cold PBS and transfer into tubes.
5. Pellet the cells by centrifugation at 1,000 × *g* for 1 min at 4 °C and remove the supernatant.
6. Lyse the cells in 500 µl of KLB for 10 min on ice.
7. Centrifuge at maximum speed for 10 min at 4 °C and transfer the supernatant in a new tube.
8. Keep 1/10 of the extract for input. Pre-clear the remaining extract with 20 µl of Protein-A agarose beads for 1 h at 4 °C under rotation.
9. Centrifuge for 1 min at maximum speed at 4 °C. Transfer the supernatant to a new tube.
10. Immunoprecipitate with 1 µl of NIK antibody or 1 µg of Rabbit irrelevant Ig (negative control) and incubate overnight at 4 °C under rotation.
11. Add 20 µl of protein-A agarose beads for 2 h at 4 °C under rotation.
12. Centrifuge for 1 min at 6,000 × *g* at 4 °C, discard the supernatant and wash the immunoprecipitate with 500 µl of KLB.
13. Centrifuge again for 1 min at 6,000 × *g* at 4 °C and discard the supernatant. Wash twice with KLB followed by two washes with KLB High Salt and two further washes with KLB have to be performed. Finally, wash the immunoprecipitate with 500 µl KAB with protease and phosphatase inhibitors freshly added.
14. Keep 1/10 of the immunoprecipitate as an immunoprecipitation control.

15. Resuspend the remaining in 27 μl of KAB+ inhibitors.
16. In a radioactive procedure room, prepare a mixture of cold ATP and ATP γ^{32}P (2 μl of cold ATP 1.5 mM and 1 μl of ATP γ^{32}P 10 μCi/μl per condition). Add 3 μl of the mixture to each tube (cold ATP = 100 μM final).
17. Incubate for 30 min with 1 μg of GST-p100 at 30 °C then boil the samples for 10 min to stop the reaction. At this stage the samples can be stored at −20 °C.
18. Load on an SDS-PAGE 10 % acrylamide gel and analyze NIK autophosphorylation and GST-p100 phosphorylation by autoradiography.

3.6 Detecting IKKα Activation

1. Grow cells in 6-well plates and treat with appropriate ligands or agonist antibodies for 2 up to 4 h (*see* Table 3).
2. Perform whole-cell extracts as described in Subheading 3.3.
3. Load 50 μg of protein on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with anti-phospho IKKα Ser 176/180 antibody (*see* Table 2).

3.7 Detecting p100 Phosphorylation on Ser866/870

1. Grow cells in 6-well plates and treat with ligands or agonist antibodies for up to 4 h (*see* Table 3).
2. Perform whole-cell extracts as described in Subheading 3.3.
3. Load 50 μg of protein on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with anti-phospho-NF-κB p100 (Ser866/870) antibody (*see* Table 2).

3.8 Detecting p100 Processing to p52

1. Grow cells in 6-well plates and treat with specific ligands or agonist antibodies for at least 2 h (*see* Table 3).
2. Add the protease and phosphatase inhibitors to the SDS lysis buffer (*see* **Note 4**).
3. Discard the medium and wash the cells with ice-cold PBS.
4. Collect the cells by scraping in ice-cold PBS and transfer into tubes.
5. Pellet the cells by centrifugation at 1,000 × *g* for 1 min at 4 °C and remove the supernatant.
6. Lyse the cells in 100–200 μl (depending on the size of the pellet) of SDS 0.5 %. The extract will probably be viscous due to the extraction of genomic DNA.
7. To render the extract less viscous, either boil it for 10 min at 95 °C, or incubate with DNAse I at 37 °C prior to boiling.
8. Load 30 μg of protein on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with anti-NF-κB p100/p52 antibody (*see* Table 2) (*see* **Note 5**).

3.9 Detecting p52/RelB Translocation into the Nucleus

To analyze p52 and RelB translocation, cytoplasmic and nuclear extracts have to be made (*see* **Note 6**).

1. Grow cells in 10 cm dishes and treat with appropriate ligands or agonist antibodies for at least 2 h (*see* Table 3).
2. Discard the medium and wash the cells twice with ice-cold PBS.
3. Collect the cells by scraping in ice-cold PBS and transfer into tubes.
4. Pellet the cells by centrifugation at 100×*g* for 1 min at 4 °C and remove the supernatant.
5. Lyse the cells in 400 µl of cytoplasmic lysis buffer for 10 min on ice.
6. Centrifuge for 5 min at 100×*g*. Transfer the supernatant (the cytoplasmic fraction) into a new tube.
7. Wash the pellet (nuclei) with 400 µl of washing buffer without resuspending the pellet.
8. Centrifuge at 100×*g* for 1 min at 4 °C and discard the supernatant. Repeat the wash one more time.
9. Add two volumes of nuclear lysis buffer to the pellet. Resuspend the pellet and incubate for 30 min at 4 °C under rotation.
10. Centrifuge at maximum speed for 30 min. Transfer the supernatant (the nuclear fraction) into a new tube.
11. Load 30 µg of protein of cytoplasmic and nuclear fractions on an SDS-PAGE 10 % acrylamide gel and analyze by immunoblotting with anti-NF-κB p52 and anti-RelB antibodies (*see* Table 2).

3.10 Detecting p52/RelB Binding to κB Sites

The activity of binding can be measured by EMSA experiment on nuclear extracts of cells treated with an inducer of the alternative NF-κB pathway (*see* **Note 7**).

3.10.1 Nuclear Extracts

Nuclear extracts for EMSA are made as described in Subheading 3.9, **steps 1–10**

3.10.2 Probe Radiolabeling

1. In a tube, add 2 µl of annealed oligonucleotides (100 ng), 1 µl of T4 polynucleotide kinase PNK 10 U/µl (10 units), 2 µl of buffer PNK 10×, 4 µl of ATP γ^{32}P 10 µCi/µl, and 11 µl of water (final volume = 20 µl).
2. Incubate for 1 h at 37 °C.
3. Purify the radiolabeled probe using the Quick spin columns for radiolabeled DNA purification.
4. Measure the activity of the radiolabeled probe in a scintillation counter.

EMSA

1. Make a 6 % Native PAGE gel.
2. Switch on the cooler and pre-run the gel for 20 min at 300 V.
3. Make up the following reaction mixture for each sample: 5 μg of nuclear protein, 1 μl of Poly(dI-dC) (1 μg/μl), 1 μl of BSA (1 μg/μl), 8 μl of buffer D (add DTT just before use), and radiolabeled probe (100,000 cpm). Adjust the volume with water up to 17 μl.
4. For supershift assays make the following reaction mixture: 5 μg of nuclear proteins, 1 μl of Poly(dI-dC) (1 μg/μl), 1 μl of BSA (1 μg/μl), 8 μl of buffer D (with DTT freshly added), and 1 μg of antibody. Incubate on ice for 20 min then add the radiolabeled probe (100,000 cpm). Adjust the volume with water to 17 μl.
5. Incubate for 30 min at room temperature.
6. Add 3 μl of blue Loading Buffer for EMSA.
7. Load the samples on the gel and run for 2–3 h until the blue dye front reaches ¾ of the way down the gel.
8. Dry the gel for 45 min at 80 °C in a gel dryer.
9. Put the dried gel in an autoradiography cassette.
10. Expose overnight to X-ray film then develop the next day.

4 Notes

1. Once the receptor binds its ligand, the adaptor TRAF proteins are recruited through specific binding sites. Previously, using LTβR as a prototype, we showed that internalization of the receptor and binding of TRAF2 and TRAF3 are a prerequisite for the activation of p100 processing [8].
2. Ligands of the TNF family such as $LT\alpha_1\beta_2$, CD40L, or Tweak were shown to induce c-IAP1/2 depletion through proteasomal and/or lysosomal degradative pathways [8, 26]. Synthetic small molecules mimicking the protein Smac (Smac mimetics) sensitize some cancer cell lines to TNFR1-dependent cell death and can be used as tools to induce c-IAP1/2 depletion [27–29].
3. In resting conditions, NIK is kept at a very low level through a proteasomal-dependent degradation mediated by the TRA2/3-c-IAP1/2 complex. When cells are stimulated by an inducer of the alternative NF-κB pathway, TRAFs are recruited to the activate receptor and c-IAP1/2 are depleted. NIK is no longer targeted for negative regulation and can accumulate in its active form.
4. Although p100 processing is the ultimate step to generate cytoplasmic p52, the latter translocates into the nucleus to

bind enhancer and promoter regions. To analyze correctly the ratio p100/p52, reflecting the degree of activation of the pathway, it is therefore necessary to extract properly the whole p52 content, including p52 associated to the chromatin fraction. A classical lysis buffer with a detergent such as Igepal or Triton X-100 will not be sufficient to extract entirely all the proteins associated to the chromatin. A more stringent extraction can be performed using SDS as detergent in the lysis buffer.

5. The ratio p100/p52 can be quantified with software such as Image J.
6. p52 preferentially binds RelB to form a transcriptionally active dimer (the transactivation domain in this dimer is in RelB) which translocates into the nucleus.
7. No κB sites are exclusively recognized by p52/RelB. Nevertheless, sequences have been described to bind p50/p65 as well as p52/RelB. Nuclear extracts from untreated HT29 cells contained only low levels of NF-κB DNA binding activity. After 6 h of treatment with an agonist anti-LTβR antibody, the binding is increased. The band observed on the gel is supershifted with antibodies against p52, RelA (p65), or RelB [7].

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