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Methods to detect NF- κ B Acetylation and Methylation

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Summary

Post-translational modifications of NF- κ B, including acetylation and methylation, have emerged as an important regulatory mechanism for determining the duration and strength of NF- κ B nuclear activity as well as its transcriptional output. Within the seven NF- κ B family proteins, the RelA subunit of NF- κ B is the most studied for its regulation by lysine acetylation and methylation. Acetylation or methylation at different lysine residues modulates distinct functions of NF- κ B, including DNA binding and transcription activity, protein stability, and its interaction with NF- κ B modulators. Here, we describe the experimental methods to monitor the *in vitro* and *in vivo* acetylated or methylated forms of NF- κ B. These methods include radiolabeling the acetyl- or methyl- groups and immunoblotting with pan or site-specific acetyl- or methyl-lysine antibodies. Radiolabeling is useful in the initial validation of the modifications. Immunoblotting with antibodies provides a rapid and powerful approach to detect and analyze the functions of these modifications *in vitro* and *in vivo*.

1. Introduction

Post-translational modifications (PTMs) play crucial roles in modulating the activation and termination of NF- κ B signaling. These modifications, including ubiquitination, phosphorylation, acetylation and methylation, target various cytoplasmic or nuclear proteins and modulate the strength and duration of the transcriptional outcomes in response to various extracellular signals. It is well known that PTMs (e.g., phosphorylation and ubiquitination) in the cytoplasm are essential for the activation of I κ B kinases (IKKs) and the subsequent degradation of I κ B α , allowing the rapid translocation of NF- κ B into the nucleus, where NF- κ B binds to the κ B enhancer and stimulates the expression of hundreds of NF- κ B target genes involved in different biological processes, including inflammation, proliferation and cell survival (1).

Accumulating evidence indicates that PTMs within the nucleus are also important in determining the transcriptional outcome of NF- κ B signaling (2, 3). Within the nucleus, the RelA subunit of NF- κ B undergoes a variety of post-translational modifications and these modifications regulate the activity of nuclear NF- κ B and fine-tune the expression of NF- κ B target genes, adding another layer of complexity to the transcriptional regulation of NF- κ B (3, 4).

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Lysine acetylation and methylation have recently emerged as important modifications for the regulation of nuclear NF- κ B function. Acetylation is a reversible event mediated by histone acetyltransferases (HAT) and histone deacetylases. These enzymes mediate the addition or removal of the acetyl group to or from lysine residues. Reversible acetylation of RelA regulates diverse functions of NF- κ B, including DNA binding activity, transcriptional activity, and its ability to associate with other proteins, and plays important roles in the NF- κ B-mediated inflammatory response and cancer (3, 4). Seven lysines, acetylated either by p300/CBP or PCAF, have been identified within RelA. These lysines include lysines (K) 122, 123, 218, 221, 310, 314 and 315 (5–7). Acetylation of these different lysines modulates distinct functions of NF- κ B. For example, acetylation of K221 enhances the DNA binding of NF- κ B and, together with acetylation at K218, impairs its association with IB (6). Acetylation of K122 and K123 by p300/CBP or PCAF seems to negatively regulate NF- κ B-mediated transcription by reducing RelA binding to the κ B enhancer (5). Whereas, acetylation of K314 and K315 by p300 affects neither NF- κ B shuttling, DNA binding, nor the induction of anti-apoptotic genes, but differentially regulates the expression of specific sets of NF- κ B target genes (7, 8). K310 is acetylated by p300/CBP and is deacetylated by SIRT1(9). Acetylation of K310 is required for the full transcriptional potential of NF- κ B (6). Acetylation of K310 also regulates the stability of RelA by preventing its methylation at lysine 314/315 (10). Recent studies also indicate an important role of K310 acetylation in maintaining the sustained NF- κ B activity in cancer cells (11–13).

Like lysine acetylation, lysine methylation has been extensively studied in recent years for its ability to control the nuclear NF- κ B activity. The functional consequence of methylation depends on both position and state of the methylation site, since lysine can be mono-, di-, or tri-methylated. Several methyltransferases have been identified to methylate RelA at different lysines, including K37, K218, K221, K310, K314, and K315 (14–17). Methylation of these lysines regulates different properties of NF- κ B. For example, TNF- α - or LPS-induced mono-methylation of RelA by Set9 negatively regulates the function of NF- κ B by inducing the ubiquitination and degradation of promoter-bound RelA (18). However, methylation of K37 by Set9 appears to be important for the activation of a subset of NF- κ B target genes by stabilizing the binding of NF- κ B to its enhancers (15). RelA is also methylated by nuclear receptor-binding Set domain-containing protein1 (NSD1) at K218 and K221 (16). NSD1-mediated monomethylation of K218 and dimethylation of K221 enhances the transcriptional activity of NF- κ B and the expression of NF- κ B target genes. These methylated lysines are demethylated by F-box and leucine-rich repeat protein 11 (FBXL11) (16). Demethylation of RelA by FBXL11 negatively regulates the transcriptional activity of NF- κ B and decreases cell proliferation and colony formation of HT29 cancer cells (16).

Due to the essential role of lysine acetylation and methylation of RelA in fine-tuning the nuclear activity of NF- κ B, it is important to develop sensitive and specific assays to detect these modifications. A variety of assays have been used to successfully detect the acetylation or methylation of RelA. These assays include radiolabeling the acetyl- or methyl- groups, immunoblotting with pan or site-specific acetyl- or methyl-lysine antibodies, and mass spectrometry (6, 7, 16, 18, 19). Radioactive labeled acetyl-CoA or S-

adenosyl methionine (SAM) is widely used in *in vitro* assays utilizing the recombinant proteins. In these assays, the recombinant enzymes transfer radiolabeled acetyl- or methyl-groups from acetyl-CoA or SAM to the lysines in recombinant RelA (20). With the availability of antibodies against acetylated or methylated lysines, immunoblotting has become an easy and powerful tool. These commercially available antibodies are able to detect modified RelA only when RelA is over-expressed with the enzymes (6, 18). However, these antibodies cannot precisely determine the site and status of a modification and are not suitable for endogenous RelA. Several site-specific anti-acetylated or methylated RelA antibodies have been developed and used to detect the modifications of endogenous RelA in response to various stimuli (15–18, 21).

2. Materials

2.1 Cell lines

HEK293T and A549 cells purchased from ATCC are cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), Penicillin-Streptomycin 100 U/mL-100 µg/mL and 2 mM L-Glutamine.

2.2 Antibodies

Anti-pan acetylated lysine antibodies are from Cell Signaling (Cat. No.: 9441). Site-specific anti-acetylated lysine-310 antibodies are from Cell Signaling (Cat. No.: 3045) and from Abcam (Cat. No.: ab52175). Anti-pan methylated lysine antibodies are from Abcam (Cat. No.: ab7315). Polyclonal antibodies against monomethylated lysines 314/315 RelA were generated by New England Peptide with a synthesized peptide corresponding to amino acids 308–320 of RelA (NH₂-TFKSIMK[Me]K[Me]SPFSGC-COOH) as the antigen (18). Anti-NF-κB/p65 antibodies (F-6) are from Santa Cruz (Cat. No.: sc-8008).

2.3 Transient transfection with calcium phosphate

1. 2.5 M calcium chloride (CaCl₂) solution.
2. 2× HBS buffer: 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄·2H₂O, 12 mM dextrose, and 50 mM Hepes. Adjust pH to 7.05 using HCl and sterilize with 0.45 µm filters. Buffer can be aliquoted and stored at –80°C.

2.4 *In vitro* acetylation assay

1. Recombinant RelA: The recombinant RelA is commercially available and can be purchased from vendors such as Abnova (*see* Note 1).
2. p300 histone acetyltransferase (HAT): HA-tagged p300 immunoprecipitated from transiently transfected HEK293T cells has high HAT activity and can be used in *in vitro* acetylation assay.
3. [¹⁴C]-Acetyl-CoA and unlabeled Acetyl-CoA are from PerkinElmer (*see* Note 2).

¹Recombinant full-length RelA is difficult to obtain in the *Escherichia coli* (*E. coli*) cells due to the premature translation termination or partial proteolyses. However, under denaturing conditions full-length RelA protein can be obtained from *E. coli* as described (23). Recombinant RelA can also be purified using the baculovirus-insect cell expression system.

4. 5× HAT assay buffer: 250 mM Tris-HCl (pH 8.0), 50% glycerol, 0.5 mM EDTA, and 5 mM dithiothreitol (DTT).
5. Amersham Amplify Fluorographic Reagent is from GE Healthcare (Cat. No.: NAMP100)

2.5 *In vitro* methylation assay

1. Recombinant RelA: the same as in 2.4.1.
2. Methyltransferase Set9: Recombinant Set9 can be purchased from Millipore or purified from *E. coli*.
3. [³H]-S-adenosyl-L-methionine ([³H]-SAM) is from GE Healthcare; unlabeled S-adenosyl-L-methionine is from NEB (*see* Note 3).
4. 5× Methylation buffer: 750 mM NaCl, 100 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% Triton.

2.6 Immunoprecipitation assay

1. Immunoprecipitation (IP) buffer: 50 mM Hepes (pH 7.4), 250 mM NaCl, 0.1% NP-40, 1 mM EDTA (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), and cOmplete protease inhibitor (Roche).
2. Preparation of Protein-G-agarose (Roche) (50% slurry): centrifuge 500 µl protein-G-agarose beads at 1,000 × g for 1 min at 4°C, carefully remove the supernatant without disturbing the beads. Wash beads three times with 1 ml IP buffer. Drain the beads with a syringe and needle and measure the volume of beads. Add same volume of IP buffer to the beads to make 50% slurry. Store the beads at 4°C.
3. Preparation of anti-HA or anti-Flag antibody-conjugated agarose (Sigma): the anti-HA or anti-Flag antibody-conjugated agarose beads are prepared as described for protein-G-agarose beads. Store the beads at 4°C.
4. Anti-T7 antibody-conjugated agaroses are from Novagen and are ready for use.
5. Syringe (1 ml, Becton Dickinson) and needle (26G1/2, Becton Dickinson).
6. 6× SDS loading buffer: 0.375 M Tris-HCl (pH 6.8), 12% SDS, 60% glycerol, 9% 2-Mercaptoethanol, 0.06% bromophenol blue and 1mM DDT.

2.7 Chromatin Immunoprecipitation assays (ChIPs)

1. 1% formaldehyde: Dilute 37% formaldehyde to 1% with PBS.
2. Salmon Sperm DNA/Protein A agarose is from Millipore (Cat. No.: 16–157)
3. Low salt wash buffer: 0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl.

²Since Acetyl CoA is unstable, it is usually prepared fresh from powder. Aqueous solutions stored in aliquots at –20°C are stable for no longer than 2 weeks. Solutions can be stored at –80°C for up to 6 months.

³SAM is stored at –20°C as a 32 mM solution dissolved in 0.005 M H₂SO₄ and 10% ethanol (pH 7.5). Under this condition, SAM is stable for up to 6 months. SAM is unstable at 37°C and should be replenished in reactions if the reactions run longer than 4 hours.

4. High salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl.
5. LiCl wash buffer: 0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)
6. TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
7. Elution buffer: 25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS
8. PCR primers: human E-selectin: forward: 5'-AAGGCATGGACAAAGGTGAAG-3'; reverse: 5'-TGTCCACATCCAGTAAAGAGGAAAT-3'); human TNF- α : forward: 5'-CGCTTCCTCCAGATGAGCTC-3'; reverse: 5'-TGCTGTCCTTGCTGAGGGA-3').

3. Methods

3.1 Acetylation of RelA

Although time-consuming, radioactive labeling with [^3H]-acetate provided the first direct evidence that RelA is acetylated *in vivo*, the weak signal from [^3H] limits its further application to study the kinetics or stoichiometry of RelA acetylation. As many antibodies that react with acetylated histone or non-histone proteins are now commercially available, immunoblotting has become a popular method to detect the acetylation of specific proteins. The anti-acetylated lysine antibodies (for example, anti-acetylated lysine antibodies from Cell Signaling and Upstate Biotechnology) recognize acetylated lysine residues in a sequence-independent manner and thus can be used for immunoblotting of acetylated non-histone proteins. We have successfully used the antibody from Cell Signaling to detect the acetylation of over-expressed RelA in the presence of co-expressed p300 (6). Both polyclonal and monoclonal anti-acetylated lysine antibodies from Cell Signaling (#9441 and #9681) recognize *in vitro* acetylated recombinant RelA or *in vivo* over-expressed RelA when co-expressed with p300.

3.1.1 *in vitro* acetylation assay

3.1.1.1 preparation of p300 for *in vitro* acetylation: GST-p300 HAT domain fusion recombinant proteins have been shown to acetylate a variety of histone or non-histone proteins and can be purchased from several vendors. However, we found that it barely acetylated recombinant RelA *in vitro*. We have been using p300 immunoprecipitated from transfected HEK293T cells as HAT for *in vitro* acetylation assay.

1. Seed 293T cells ($2 \times 10^5/\text{ml}$) in 100 mm dishes.
2. When the cells reach 60–80% confluency 16 to 24 hr later, transfect each dish with 15 μg of HA-tagged p300 plasmid DNA with the calcium phosphate procedure (*see* Note 4).

⁴We typically use calcium phosphate method for transient transfection of HEK293T cells. This method is cost-effective and displays excellent transfection efficiency in HEK293T cells. Other commercially available transfection reagents (e.g., Lipofectamine or FuGENE) can also be used.

3. 36 hr after transfection, aspirate the medium and add 1 ml of IP buffer to cells and lyse the cells at 4°C for 10–15 min by leaving the dishes on a rocker.
4. Centrifuge the cell lysates at 12,000 rpm for 10 min at 4°C and then collect the supernatants.
5. To pre-clear the supernatants, add 20 µl of protein-G-agarose bead slurry (50% slurry) to the supernatants and incubate for 30 min at 4°C on a rotator (*see* Note 5).
6. Remove the agarose beads by centrifugation at 5,000 rpm for 5 min at 4°C. Transfer the supernatant to a fresh centrifuge tube.
7. Immunoprecipitate HA-p300 from the pre-cleared lysate with anti-HA antibody-conjugated agarose beads (60 µl of 50% slurry for 1 ml of cell lysates) for 2 hr at 4°C.
8. After immunoprecipitation, collect the agarose beads by centrifugation at 12,000 rpm for 30–60 s and wash the beads twice with ice-cold IP buffer.
9. Wash the beads once in 1× HAT assay buffer (diluted from 5× HAT assay buffer). Drain the wash buffer completely using a 1 ml syringe and a needle (26G1/2).
10. Add 200 µl of 1× HAT buffer to the beads and aliquot them into 1.5-ml Eppendorf tubes with 20 µl in each tube and store at –80°C for later use (*see* Note 6).
11. Test the activity of p300 immunoprecipitates in an *in vitro* acetylation assay using histone H3 or H4 as substrates.

3.1.1.2 *In vitro* acetylation assay

1. Thaw one tube of frozen p300 immunoprecipitates aliquot on ice and spin the tube at 4°C. Empty 1× HAT buffer completely using a syringe and needle.
2. Add 4 µl of 5× HAT assay buffer, 1 µg of recombinant RelA protein, and 2 µl of [¹⁴C]-acetyl-CoA or acetyl-CoA to the beads in the tube. Add distilled water to a total volume of 20 µl (*see* Note 7).
3. Spin the tube and mix the contents of the tube by tapping the bottom of the tube. Incubate the tube at 30°C for 1 hr with occasionally shaking (*see* Note 8).
4. Collect the supernatants by spinning the tube and transfer the supernatant to a new 1.5-ml Eppendorf tube. Stop the reaction by adding 4 µl of 6× SDS loading buffer to the supernatant and boil for 5 min.
5. Run the samples by SDS-PAGE. When using [¹⁴C]-acetyl-CoA as the acetyl-group donor, fix the gel using a fixing solution (isopropanol:water:acetic acid (25:65:10, v/v) for approximately 30 min.

⁵Pre-clearing cell lysates reduces non-specific binding of proteins to agarose beads, and improves the quality of p300 immunoprecipitates.

⁶The freshly made p300 is ready for use. One 20 µl of aliquot is good for one *in vitro* acetylation reaction. Since the 1× HAT buffer contains 10% glycerol, HA-p300 immunoprecipitates can be stored in –80°C for later use (up to several months) without losing much of the HAT activity.

⁷Non-radioactive acetyl-CoA is used when acetylated RelA is detected using anti-acetylated antibodies.

⁸Gently tap the bottom of the reaction tube to occasionally mix the beads with substrates for a more efficient acetylation reaction.

6. Soak the gel in Amersham Amplify Fluorographic Reagent (sufficient for the gel to be free floating) with agitation for 15–30 minutes.
7. Dry the gel and hold the gel in close contact with an appropriate X-ray film and at -70°C to -80°C (*see* Note 9).
8. Detect the radioactive signal by autoradiography.
9. When non-radioactive acetyl-CoA is used as the acetyl-group donor, gel can be directly transferred to a nitrocellulose membrane, and the acetylation signal of RelA can be detected by immunoblotting with an anti-acetylated lysine antibody (*see* Note 10).

3.1.2 *In vivo* acetylation assay—*In vivo* acetylated RelA can be detected by radiolabeling using radiolabeled sodium acetate or by immunoblotting using anti-acetylated lysine antibodies. Before the antibodies for acetylated RelA became available, radiolabeling using [^3H]-acetate was used to demonstrate the acetylated RelA in cultured cells (19). However, due to the limited amount of proteins that can be labeled in the cells and the weak radioactive signals from [^3H], it would take several weeks before the acetylation signal can be seen from the X-ray film. *In vivo* labeling the acetylated RelA using radioisotope has been described previously (22). Here, we focus on how to detect the acetylated RelA *in vivo* using anti-acetylated lysine antibodies.

3.1.2.1 Detection of acetylation of over-expressed RelA

1. Seed 2 ml of HEK293T cells ($2 \times 10^5/\text{ml}$) in each well of a six-well plate and culture overnight at 37°C .
2. Use two wells for each sample. Make a master mix for the transfection mixture. Dilute 1 μg of T7-tagged RelA and 4 μg of p300 expression vector DNA in 230 μl of distilled water in a 1.5-ml Eppendorf tube.
3. Add 20 μl of 2.5 M CaCl_2 to the mixture and mix evenly by pipetting up and down.
4. Slowly add 250 μl of $2\times$ HBS buffer to the diluted DNA and mix gently by pipetting up and down. Leave the mixture sit at room temperature for 15 min.
5. Slowly add 250 μl of transfection mix to each well of the duplicates. Swirl the dish to ensure even distribution.
6. Thirty-six hours after transfection, aspirate the medium completely and add 350 μl of IP buffer to each well and leave the plate on a rocker at 4°C for 10–15 min to promote cell lysis.
7. Collect supernatants from the cell lysates of two wells by centrifugation at 12,000 rpm for 10 min.

⁹Exposure time varies with the acetylation signal. It usually takes one to seven days. Phosphoimaging might also be used to help reduce the exposure time.

¹⁰Anti-pan acetylated lysine antibodies from Cell Signaling (Cat. No.: 9441) or anti-acetylated lysine-310 antibodies from Cell Signaling (Cat. No.: 3045) or from Abcam (Cat. No.: ab52175) can be used in the immunoblotting. Incubation with these antibodies overnight is required for the detection of acetylated RelA.

8. Pre-clear the cell lysates by adding 20 μ l of protein-G-agarose beads (50% slurry) to the supernatants and incubate for 30 min at 4°C on a rotator.
9. Remove the agarose beads by centrifugation at 5,000 rpm for 5 min at 4°C. Transfer the supernatant to a fresh Eppendorf tube.
10. Add 20 μ l of anti-T7 antibody-conjugated agarose beads (50% slurry) to the supernatant. Gently mix the cell lysates and agarose beads for 2 h at 4°C on a rotator.
11. Spin the Eppendorf tube for 30 sec in the microcentrifuge at 12,000 rpm, then discard the supernatant.
12. Wash the beads three times with 500 μ l of ice-cold IP buffer and use a syringe and needle to drain the wash buffer completely.
13. Add 50 μ l of 2 $\times$ SDS sample buffer into the beads and boil the agarose beads for 5 min. Spin the Eppendorf tube and use the supernatant for SDS-PAGE.
14. Transfer the gel to a nitrocellulose membrane.
15. Incubate the membrane in a blocking solution for 30 min at room temperature with agitation.
16. Rinse the membrane briefly with wash buffer PBS-T twice.
17. Incubate the membrane with polyclonal anti-pan acetylated lysine antibodies (1:1,000 dilution, Cell Signaling) overnight with agitation.
18. Wash the membrane three times with wash buffer for 10 min each.
19. Incubate the membrane with diluted (1:5,000) secondary antibodies (anti-rabbit IgG antibody conjugated to horseradish peroxidase) for 30 min at room temperature with agitation.
20. Wash the membrane three times with wash buffer for 10 min each.
21. Add ECL detection reagents to the membrane following the manufacturer's instructions and detect the signal by using a ChemiDoc imaging system or using an X-ray film developer.

3.1.2.2 Detection of acetylation of endogenous RelA by immunoblotting and

immunoprecipitation: The anti-pan acetylated lysine antibodies from Cell Signaling are effective in detecting the acetylated RelA when RelA is co-expressed with p300 (6). However, this antibody is not specific enough to detect acetylated endogenous RelA. Instead, site-specific anti-acetylated RelA antibodies could be used. For example, the anti-acetylated lysine-310 antibodies from Cell Signaling and Abcam are able to recognize acetylated endogenous RelA. For detection of acetylation of endogenous RelA, it is also important to treat the cells with a stimulus (e.g, TNF- α and LPS) to allow the translocation of RelA into nucleus, where most HATs localize. The acetylated RelA could be detected by directly immunoblotting the whole-cell-extracts with anti-acetylated RelA antibodies (21). But the levels of acetylated RelA might vary among different cells. Alternatively,

immunoprecipitation of the acetylated RelA with anti-acetylated RelA antibodies would concentrate the acetylated RelA and enhance the detectable acetylated RelA signals.

1. Seed 2 ml A549 cells (5×10^5) in each well of a six-well plate and culture overnight at 37°C. Prepare two wells for each sample.
2. When the cells reach 90% confluency, treat the cells with TNF- α (20 ng/ml) for the desired time and wash twice with cold PBS.
3. Add 350 μ l of IP buffer to each well and lyse the cells for 30 min with agitation at 4°C.
4. Use a cell scraper to collect the cell lysates from the two wells into 1.5-ml Eppendorf tubes.
5. Centrifuge the sample for 10 min at 12,000 rpm. Transfer the supernatant to a new 1.5-ml Eppendorf tube.
6. Pre-clear the cell lysates by adding 20 μ l of protein-G-agarose bead slurry (50%) and incubate for 30 min at 4°C on a rotator.
7. Remove the agarose beads by centrifugation at 5,000 rpm for 5 min at 4°C. Transfer the supernatant to a fresh Eppendorf tube.
8. Add 5 μ l of anti-NF- κ B/p65 (acetyl K310) antibodies to the supernatant. Incubate at 4°C for 3 h with gentle agitation.
9. Add 25 μ l of Protein-G agarose beads (50% slurry in IP buffer) to cell lysates. Gently mix the cell lysates and agarose beads over night at 4°C.
10. Collect the agarose beads by pulse centrifugation at 12,000 rpm for 30 s. Discard the supernatant fraction. Wash the beads three times with 500 μ l of ice-cold IP buffer and use a syringe and needle to drain the wash buffer completely.
11. Resuspend the agarose beads in 30 μ l of 2 $\times$ SDS sample buffer and mix gently. Boil the agarose beads for 5 min. Collect the beads by centrifugation and use the supernatant for SDS-PAGE.
12. Resolve the samples on a 10% SDS-PAGE gel and transfer the gel to a nitrocellulose membrane.
13. Immunoblot the membrane with diluted anti-RelA antibody (Santa Cruz, F-6, 1:500) overnight at 4°C. Overnight incubation of the antibody is required for detection of a significant signal.

3.1.2.3 Detection of chromatin-associated acetylated endogenous RelA by ChIPs

1. Seed A549 cells (2×10^6) in 100 mm dishes and culture overnight at 37°C.
2. When cells reach 90% confluency, treat the cells with TNF- α (20 ng/ml) for indicated time points.

3. Wash the cells once with PBS to remove the TNF- α and cross link proteins to DNA by adding 2 ml of 1% formaldehyde to the cells and incubate for 10 min at room temperature.
4. Stop the fixation with 125 μ M glycine for 5 min.
5. Use a cell scraper to collect the cells to a conical tube and pellet cells for 5 min, at 2000 rpm at 4°C.
6. Wash the cells once with cold PBS and resuspend the cell pellets in 500 μ l of solution containing 1% SDS, 50 mM Tris (pH 8.1) and 10 mM EDTA. (5×10^5 cells/100 μ l solution)
7. Lyse the cell with sonication under conditions optimized to generate DNA fragments with an average size of 200–1000 base pairs.
8. Centrifuge samples for 10 minutes at 13,000 rpm at 4°C, and transfer 200 μ l of the sonicated cell supernatant to a new Eppendorf tube. The remaining supernatants can be stored at –80°C for IP with a different antibody.
9. Dilute the supernatant by adding 1.8 ml of dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl.
10. Add 20 μ l of Salmon Sperm DNA/Protein A agarose beads to the cell lysates and rotate for 30 min at 4°C to pre-clear the cell lysates.
11. Pellet the agaroses by spinning the tubes and transfer the supernatant into a new Eppendorf tube.
12. Add 5 to 10 μ l of anti-acetylated lysine-310 RelA and incubate for 4 h at 4°C with agitation, followed by addition of 50 μ l of Salmon Sperm DNA/Protein A agarose beads and incubate for overnight at 4°C with agitation. An immunoprecipitation without adding any antibodies is used as a negative control.
13. Pellet the agarose beads by centrifugation at 1,000 rpm for 1 min at 4°C. Discard the supernatant.
 1. Wash the beads for 5 min on a rotating platform with 1 ml of each of the following buffers once in the order as given below: low salt wash buffer, high salt wash buffer and LiCl wash buffer. Followed by 1 $\times$ TE buffer twice.
 2. Add 250 μ l of the elution buffer to the agarose beads. Vortex briefly to mix and incubate at room temperature for 15 minutes with rotation. Spin down agarose, and carefully transfer the supernatant fraction to another tube and repeat elution. Combine supernatants (total volume $\approx$ 500 μ l).
 3. De-crosslink the protein and DNA crosslinks by adding 20 μ l of 5 M NaCl and incubate the samples at 65°C O/N. Digest the samples with pronase for 1 h at 42°C.
14. Purify the decrosslinked DNA using Qiagen QuickSpin Purification kit and elute in 50 μ l DNA elution buffer.

15. Use 1–5 µl DNA eluates as template and 40 cycles of PCR amplification with Taq polymerase and proper primers for desired genes (e.g., E-selectin and TNF-α). The PCR products are analyzed on 2.5% agarose gels (see Note 11).

3.2 Methylation of RelA

The approaches to assess RelA methylation are quite similar to those approaches used to assess RelA acetylation. [³H]-labeled SAM is often used in the *in vitro* methylation assay using recombinant RelA and methyltransferases. Immunoblotting with anti-pan methylated lysine antibodies or site-specific methylated lysine antibodies allows the rapid detection of *in vitro* or *in vivo* methylated RelA. We have used anti-pan methylated lysine antibodies from Abcam to detect Set9-mediated methylation of recombinant RelA or over-expressed RelA in cultured cells. It is not clear whether these antibodies will recognize other forms of methylated RelA mediated by methyltransferase rather than Set9. Several site-specific methylated RelA antibodies are used in the immunoblotting or ChIP assays to detect the stimulus-coupled methylation of endogenous RelA (10, 15–17). Due to the complexity of lysine methylation, mass spectrometry has been successfully used to map the methylation sites and status (16–18).

3.2.1 *In vitro* methylation assay

1. Prepare the following reaction in an Eppendorf tube: 1 µg of recombinant 6×His-tagged RelA or GST-RelA fusion proteins, 1 µg of recombinant Set9, 6 µl of 5× methylation buffer. Depending on the reaction, use either 1 µM [³H]-labeled SAM or 40 µM non-radioactive SAM. Add H₂O to reach a final volume of 30 µl.
2. Spin the tube by centrifugation then mix the tube by slightly tapping the bottom of the tube.
3. Incubate the samples at 30°C for 60 min.
4. Stop the reaction by adding 6 µl of 6× SDS protein sample loading buffer and heat at 95°C for 5 min.
5. Take 15 µl of the reaction products and resolve the samples on a 10% SDS-PAGE gel.
6. When using [³H]-SAM as the methyl-group donor, fix the gel using a fixing solution (isopropanol:water:acetic acid (25:65:10, v/v)) for 30 min.
7. Soak the gel in Amersham Amplify Fluorographic Reagent (sufficient for the gel to be free floating) with agitation for 15–30 min.
8. Dry the gel and hold the gel in close contact with an appropriate X-ray film and at –70°C to –80°C overnight.
9. Detect the radioactive signal by autoradiography.
10. When non-radioactive SAM is used as the methyl-group donor, gel can be directly transferred to a nitrocellulose membrane, and the methylation signal of RelA can be

¹¹Quantitative real-time PCR can also be used to determine the amount of DNA in the eluates.

detected by immunoblotting with an anti-methylated lysine antibodies (*see* Note 12).

3.2.2. Methylation of RelA *in vivo*

3.2.2.1 Detection of methylation of over-expressed RelA: The detection of methylation of over-expressed RelA is similar to that of acetylation (*see* Subheading 3.1.2.1).

1. Seed 2 ml of HEK293T cells ($2 \times 10^5/\text{ml}$) in each well of a six-well plate and culture overnight at 37°C. Use two wells for each sample.
2. Transfect HEK293T cells with 0.5 µg of T7-tagged RelA and 2 µg of Set9 expression plasmids using calcium phosphate method into each well.
3. Thirty-six hours after transfection, lyse the cells with IP buffer.
4. Immunoprecipitate T7-RelA and methylation levels can be assessed by immunoblotting of immunoprecipitates with anti-pan-methyl-lysine antibodies (Abcam) or anti-methylated K314/315 antibodies.

3.2.2.2 Detection of methylation of endogenous RelA: The detection of methylation of endogenous RelA is similar to that of acetylation (*see* Subheading 3.1.2.2). Anti-methylated lysine 314/315 antibodies have been used to detect the TNF-α-induced methylation of endogenous RelA (18). However, these antibodies are not suitable for straight immunoblotting with the cell lysates. This might be due to the low reactivity of the antibodies. Another possible reason is that *in vivo* methylated RelA is chromatin-bound and the amount of methylated RelA in the whole cell extracts is relatively low. However, if these antibodies are used to immunoprecipitate methylated RelA, a significant amount of methylated RelA can be detected either from the TNF-α-treated whole cell extracts or from chromatin.

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¹²Anti-pan methylated lysine antibodies are from Abcam (Cat. No.: ab7315). Incubation with these antibodies overnight is required for the detection of methylation signals.

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