Chapter 4

Molecular and Functional Characterization of Histone Deacetylase 4 (HDAC4)

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Abstract

Histone deacetylases (HDACs) regulate various nuclear and cytoplasmic processes. In mammals, these enzymes are divided into four classes, with class II further divided into two subclasses: IIa (HDAC4, HDAC5, HDAC7, HDAC9) and IIb (HDAC6 and HDAC10). While HDAC6 is mainly cytoplasmic and HDAC10 is pancellular, class IIa HDACs are dynamically shuttled between the nucleus and cytoplasm in a signal-dependent manner, indicating that they are unique signal transducers able to transduce signals from the cytoplasm to chromatin in the nucleus. Once inside the nucleus, class IIa HDACs interact with MEF2 and other transcription factors, mainly acting as transcriptional corepressors. Although class IIa HDACs share many molecular properties in vitro, they play quite distinct roles in vivo. This chapter lists methods that we have used for molecular and biochemical characterization of HDAC4, including development of regular and phospho-specific antibodies, deacetylase activity determination, reporter gene assays, analysis of subcellular localization, and determination of interaction with 14-3-3 and MEF2. Although described specifically for HDAC4, the protocols should be adaptable for analysis to the other three class IIa members, HDAC5, HDAC7, and HDAC9, as well as for other proteins with related properties.

Key words Lysine acetylation, Histone deacetylase, HDAC4, 14-3-3, MEF2, Transcriptional repression, Nucleocytoplasmic trafficking

1 Introduction

Lysine acetylation has emerged as a major posttranslational modification for histones and nonhistone proteins [1, 2]. This is a reversible modification process that is controlled by lysine acetyltransferases and deacetylases. Traditionally, the deacetylases have been referred to as histone deacetylases (HDACs), even though it is now known that they also target nonhistone proteins. In mammals, 18 HDACs have been identified. Based on sequence homology to the yeast deacetylases Rpd3, HDA1, and Sir2, the 18 mammalian HDACs are divided into four classes, namely class I (HDAC1, -2, -3, -8), class II (HDAC4, -5, -6, -7, -9, and -10), class III (containing seven sirtuins), and class IV (HDAC11) [3, 4]. Class II HDACs have been further classified into class IIa

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(HDAC4, -5, -7, and -9) and class IIb (HDAC6 and 10) [4]. In the deacetylase superfamily, class IIa HDACs are unique due to their roles as novel signal transducers able to transmit signal information from the cytoplasm to the nucleus [4]. For serving in this role, they contain intrinsic nuclear import and export signals for dynamic shuttling between the cytoplasm and nucleus (Fig. 1) [5]. Moreover, this dynamic trafficking is actively regulated by phosphorylation-dependent interaction with 14-3-3 proteins, as well as by association with other proteins such as the MEF2 family of transcription factors (Fig. 1) [5]. Different kinases then phosphorylate class IIa HDACs at three or four sites, to promote 14-3-3 binding and subsequent retention of the deacetylases in the cytoplasm [4]. Once in the nucleus, class IIa HDACs interact with transcription factors such as MEF2 to repress transcription [5]. Although class IIa HDACs share many molecular properties, they play quite distinct roles in vivo. HDAC4 and HDAC7 are important for brain and vasculature development, respectively, whereas HDAC5 and HDAC9 play critical roles in regulating skeletal and cardiac muscle development [6]. Here we list methods used for molecular and biochemical analyses of HDAC4. These methods are also applicable to characterization of HDAC5, -7, and -9, as well as for other proteins with related properties.

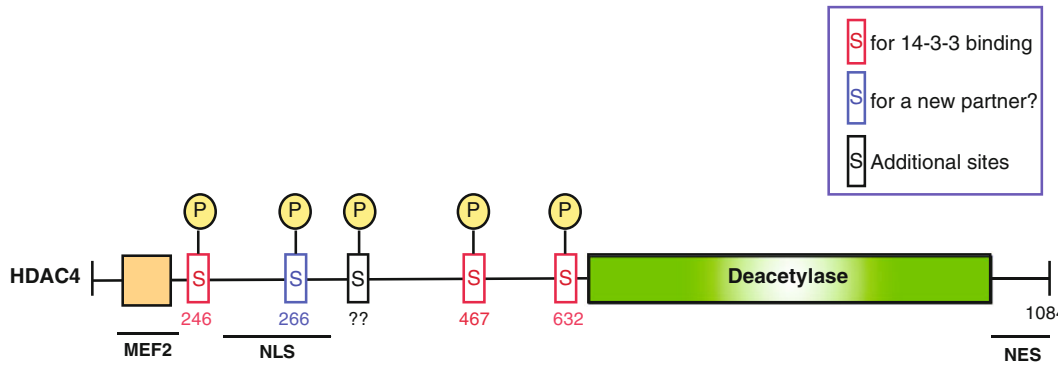

Fig. 1 Schematic illustration of HDAC4. This deacetylase contains a nuclear localization signal (NLS) in the N-terminal part, a highly conserved deacetylase domain, and a nuclear export signal (NES) at the C-terminal end [26]. Moreover, the N-terminal part of HDAC4 possesses not only an MEF2-binding site conserved from *C. elegans* to humans, but also three 14-3-3-binding sites, S246, S467, and S632, the first of which is conserved from *C. elegans* to humans. These three sites need to be phosphorylated for mediating interaction with 14-3-3 proteins. S266 is a fourth phosphorylation site and is located within the NLS, so its phosphorylation impedes nuclear localization of HDAC4 [8]. There are other phosphorylation sites [27–29], but their functions are less clear. HDAC5, HDAC7, and HDAC9 have very similar domain organizations [5]. Not illustrated here is an N-terminal PxLPxI motif conserved among HDAC4, HDAC5, and HDAC9, but not HDAC7. This motif is important for interaction with the ankyrin-repeat protein ANKRA2 [28, 30–32]. In addition, HDAC4 possesses an efficient sumoylation site within the N-terminal part [33] and is able to promote sumoylation of binding partners such as MEF2 [34–36]. HDAC4 is also subject to ubiquitination, degradation, and specific cleavage [37, 38]

2 Materials

2.1 Cell Lines and Animals

1. NIH3T3, SKN, HEK293, and 293T cells, which are cultured in DMEM media supplemented with 10 % FBS and 100 U penicillin and streptomycin in a 37 °C incubator at 5 % CO_2.
2. Rabbits (young rabbits 10–16 weeks of age, 2.5–3.0 kg).

2.2 Reagents and Materials

1. Peptides and proteins: For generation of a regular polyclonal HDAC4 antibody, an N-terminal fragment of human HDAC4 protein was expressed and affinity-purified as an MBP (maltose-binding protein) fusion protein; for producing an anti-phospho-Ser-246 HDAC4 antibody, the peptide LRKTApSEPNLKC was synthesized; FLAG peptide (DYKDDDDK); bovine serum albumin (BSA).
2. Plasmids: pBluescript KSII (+) and mammalian expression vectors for GFP-HDAC4, HA-14-3-3β, the yeast transcription factor Gal4 (residues 1-147), Gal4 (resides 1-147)-HDAC4, and β-galactosidase, all under the control of the CMV promoter.
3. Antibodies: Anti-HDAC4 polyclonal antibodies (anti-rabbit, produced in our lab), anti-HA monoclonal antibody, anti-14-3-3 antibodies.
4. Freund's adjuvant, complete and incomplete.
5. Anesthetic and disinfectant: Acepromazine, butorphanol, EMLA cream, and chlorhexidine.
6. Affinity resins and beads: CNBr-activated Sepharose 4B, anti-Flag M2 agarose beads, immobilized protein A/G beads.
7. Disposable column, tips, and Eppendorf tubes.
8. Sulfhydryl Immobilization Kit (which includes Sulfhydryl coupling resin (6 % cross-linked beaded agarose supplied as a 50 % slurry in the storage buffer (10 mM EDTA–Na, 0.05 % NaN_3, 50 % glycerol)); sample preparation buffer: 0.1 M sodium phosphate, 5 mM EDTA–Na, pH 6.0; coupling buffer: 50 mM Tris, 5 mM EDTA–Na, pH 8.5; wash solution: 1.0 M NaCl and 0.05 % NaN_3; L-cysteine·HCl; polyethylene porous discs: five discs, to add to the top to the gel bed after coupling to prevent the column from running dry).
9. mcKLH (mariculture keyhole limpet hemocyanin).
10. Cell culture dishes and plates.
11. Transfection reagents.
12. Drugs: 5 μg/ml leptomycin B in ethanol, 3 μM trichostatin A in DMSO.

13. Chemicals: [^{3}H] acetate (2.4 Ci/mmol), [^{3}H] acetylcoenzyme A (4.7 Ci/mmol), D-(−)-luciferin, acetone.

2.3 Solutions and Buffers

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 (pH 7.4 or pH 8.5 adjusted with NaOH for the latter).
2. Buffer A: 50 mM Tris–HCl pH 8.0, 10 % glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA, and 10 mM sodium butyrate.
3. Buffer B: 20 mM Tris–HCl pH 8.0, 10 % glycerol, 5 mM MgCl2, 0.1 % NP-40, protease inhibitors, and KCl (0.15 or 0.5 M).
4. Buffer N: 10 mM Tris–HCl [pH 8.0], 250 mM sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors.
5. Buffer H: 50 mM Tris–HCl pH 8.0, 10 % glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, and 1 mM PMSF.
6. Hypotonic lysis buffer: 20 mM HEPES pH 7.6, 20 % glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 % Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM dithiothreitol, and protease inhibitors.
7. 1 and 2 mM HCl.
8. 0.1 M Ethanolamine in PBS.
9. 6 M Guanidine in PBS.
10. 0.1 M Glycine–HCl (pH 2.5).
11. 0.1 M Tris–HCl (pH 8.0).
12. Elution buffer (0.1 M glycine–HCl pH 2.7).
13. 3× Sodium dodecyl sulfate (SDS) sample buffer: 150 mM Tris–HCl pH 6.8, 30 % glycerol, 6 % SDS, 0.3 % bromophenol blue, and 300 mM DTT.
14. 0.4 N H_2SO_4.
15. 5 M NaCl.
16. 0.1 M HCl-0.16 M acetic acid.

2.4 Equipment

1. Sterile needles (22 G) and syringes.
2. Dialyzer.
3. Flow-through UV monitor.
4. Pipettes.
5. Cell scrapper.
6. Fluorescence microscope.
7. Luminometer plate reader.
8. Benchtop centrifuge.

3 Methods

3.1 Development of Antibodies Specific to Regular and Phosphorylated HDAC4

To study the function and regulation of HDAC4, regular and phospho-specific antibodies were developed as described [7–9]. For the regular antibody, an HDAC4 fragment was expressed as a MBP-tagged fusion protein (Subheadings 3.1.1 and 3.1.2). Protocols for bacterial expression and protein purification can be found elsewhere [10, 11]. For the phospho-specific antibody, a phosphopeptide (LRKTApSEPNLKC), corresponding to the sequence of Ser-246 and its surrounding residues of human HDAC4, was synthesized commercially (Subheading 3.1.3).

3.1.1 Rabbit Immunization for Antiserum Production

Rabbit immunization and antiserum production were carried out at McGill Animal Resource Center (https://www.mcgill.ca/cmarc/home), and the following protocol was modified from a standard operating procedure (#406) developed by the Center.

1. Choose two young adult female rabbits (2–2.5 kg; specific pathogen free) for immunization.
2. Tranquilize the rabbits by injecting intramuscularly with mixed acepromazine (0.5 mg/kg) and butorphanol (0.2 mg/kg).
3. Apply EMLA cream over the ear at the blood collection site 15 min before puncture to collect 1 ml of blood using a 22 G needle from the ear artery. Centrifuge and collect the supernatant as the preimmune serum. Aliquot and keep it at −80 °C.
4. For primary immunization, combine 0.5 ml antigen (200–500 μg of MBP-HDAC4 in 500 μl of sterile PBS) with 0.5 ml of Freud's complete adjuvant (FCA) and emulsify (*see* **Note 1**).
5. Rinse the injection site with chlorhexidine. Inject the 1 ml sample subcutaneously into ten sites, 0.1 ml per site, bilaterally along the thoracic-lumbar region of the spine. Before removing the needle, withdraw on plunger slightly to prevent the leakage of adjuvant into the dermal layer (*see* **Note 2**).
6. Wait for 3–4 weeks for the rabbits to build up a primary immunological response.
7. For secondary immunization, inject in the vicinity of the initial sites as described except that Freud's incomplete adjuvant (FIA) is used (*see* **Note 3**).
8. Wait for another 3–4 weeks after secondary immunization.
9. Collect 1 ml blood sample and determine the titer by indirect ELISA if the titer is not sufficient; repeat **steps 8** and **9**. In most cases, the antibody titer reaches an acceptable level after two boosters.
10. When the antibody titer is sufficient, inject 0.5 mg of antigen (in 0.5 ml sterile PBS, without any adjuvant) into the ear artery.

11. If the rabbits are read for bleeding out, exsanguinate via cardiac puncture under general anesthesia; otherwise, collect 8.5 ml/kg of blood every 4 weeks. Do not exceed 6 months after initial immunization.
12. About 50 ml of antiserum can be collected per rabbit. The antiserum can be used directly or for affinity purification to enrich the antibody.

3.1.2 Antibody Affinity Purification on CNBr-Activated Sepharose

This procedure is adapted from a published method [12].

1. Dialyze the antigen at least twice with PBS (pH8.5) to remove any trace amount of Tris (*see* **Note 4**).
2. Centrifuge and collect the supernatant (if there are precipitates).
3. Determine the protein concentration (0.5–2 mg is needed).
4. Swell 0.17 g of CNBr-activated Sepharose 4B in a chromatography column (0.8 × 4 cm) containing 12 ml HCl (1 mM) for 10 min at room temperature.
5. Wash the resin three times quickly with 1 mM HCl (~12 ml each).
6. Wash the resin once with 5 ml PBS (pH 8.5).
7. Add the antigen prepared above (**step 2**) to the resin and rotate the suspension at room temperature for at least 2 h.
8. Check protein concentration of the supernatant to determine the cross-linking efficiency.
9. Wash the resin three times with regular PBS if the coupling efficiency is good (i.e., more than a half of the input has disappeared from the supernatant).
10. Incubate the resin in 10 ml PBS/0.1 M ethanolamine for 1 h at room temperature to inactivate the resin.
11. Wash the resin sequentially with PBS/6 M guanidine, PBS, and 0.1 M glycine–HCl pH 2.5 (10 ml/each).
12. Repeat the wash cycle once.
13. Wash the resin once with 10 ml PBS/6 M guanidine and twice with 10 ml PBS.
14. Suspend the resin in 0.5 ml PBS and store it at 4 °C.
15. Mix 0.5 ml resin prepared above with 10 ml serum in a disposable plastic column such as poly-prep chromatography columns (0.8 × 4 cm from Bio-Rad, with caps at both ends). Cap the column at both ends, put it inside a 50 ml Falcon tube (to prevent the cap from falling off), and rotate the column at room temperature for at least 2 h.
16. Centrifuge the column for ~2 min inside the 50 ml Falcon tube at ~3,000 × *g*, suspend the resin in 10 ml PBS, and incubate at room temperature for ~2 min.

17. Repeat step 16 four times.
18. Add 0.5 ml 0.1 M glycine–HCl pH 2.5.
19. Incubate at room temperature for 5 min (important: no longer than this) and transfer the column quickly to a 15 ml Falcon tube containing 0.5 ml 0.1 M Tris–HCl pH 8.0 so that the eluate drains directly to the Tris buffer for immediate neutralization.
20. Add 0.5 ml 0.1 M Tris–HCl pH 8.0 to resin and allow the eluate to drain directly to the eluate from the above step. The resulting mixture contains the affinity-purified antibody. Procced immediately to step 21. The resin is washed later with PBS and stored at 4 °C for further purification of additional antisera.
21. Take a small drop to estimate the pH value with a pH paper. It should be 7.0–8.0; if not, add more 0.1 M Tris–HCl pH 8.0 to neutralize the solution. Flash-freeze the purified antibody in aliquots on dry ice and keep it at –80 °C.

3.1.3 Antibody Affinity Purification on Sulfhydryl Coupling Resin

A rabbit polyclonal antibody specific to phospho-Ser246 of HDAC4 was prepared by use of a phosphopeptide antigen as the affinity tag. For the anti-phospho-Ser246 HDAC4 antibody, 10–20 mg of the phosphopeptide LRKTApSEPNLKC (where pS is phospho-serine, corresponding to residues 241-251 of HDAC4) was synthesized and HPLC-purified to 85 % purity in a commercial vendor; this amount was sufficient for both immunization of two rabbits and subsequent affinity purification. Two rabbits were immunized with the phosphopeptide [9]. Similarly, an anti-phospho-Ser266 antibody was developed [8]. The C-terminal Cys is not from HDAC4, but was added for antigen preparation by conjugation of the peptide to mariculture keyhole limpet hemocyanin (mcKLH), according to the manufacturer's instruction. The conjugated antigens were used for serum production as described in Subheading 3.1.1. After serum production, the same phosphopeptides were used for conjugation to sulfhydryl coupling resin, via the extra Cys residue, to prepare affinity gel for purification of the antibody. The following protocol is adapted from the manufacturer's manual (Pierce Manual 20402). It is based on a gel bed volume of 1 ml; for column with other gel bed volumes, adjust all solution (e.g., sample, wash, elution) volumes accordingly.

1. Equilibrate the sulfhydryl coupling resin to room temperature and add an appropriate volume of gel slurry to a disposable poly-prep column (*see* Subheading 3.1.1). For example, add 2 ml of gel slurry to obtain a gel bed of 1 ml.
2. Equilibrate the resin with four column volumes of coupling buffer (*see* **Note 5**).

3. Dissolve 2 mg of sulfhydryl-containing peptide in 1 ml coupling buffer and transfer it to the column prepared from the above step. Retain a small aliquot of the peptide solution for determination of the coupling efficiency later.
4. Cap the column at both ends, put it inside a 50 ml Falcon tube (to prevent the caps from falling off), and rotate the column at room temperature by end-over-end mixing at room temperature for 15 min.
5. Incubate the column at room temperature for additional 30 min without mixing.
6. Sequentially remove top and bottom column caps and allow the solution to drain from the column into a clean tube.
7. Place the column over a new collection tube and wash column with 3 column volumes of coupling buffer.
8. Determine the coupling efficiency by measuring and comparing absorbance (at 280 nm) of the unbound fraction (**step 6**) and the initial peptide solution (**step 3**).
9. Install the bottom cap onto the column (*see* **Note 6**).
10. Prepare 50 mM L-cysteine·HCl in coupling buffer.
11. Apply 1 ml of 50 mM L-cysteine to the column and install the top cap.
12. Put the capped column inside a 50 ml Falcon tube (to prevent the cap from falling off) and rotate the column at room temperature by end-over-end mixing at room temperature for 15 min. Then incubate for additional 30 min, without mixing.
13. Sequentially remove the top and bottom caps, allow the buffer to drain from the column, and discard the drainage.
14. Wash the column with at least six column volumes of wash solution. The resin can be used for antibody purification in the same manner as described in **steps 15–21** of Subheading 3.1.2.
15. If not used immediately, wash the column with two column volumes of the storage buffer. Install the bottom cap and add 2 ml storage buffer. The column can be capped and kept at 4 °C for storage (*see* **Note 7**).

3.2 HDAC4 Shuttling Between the Cytoplasm and Nucleus

3.2.1 Analysis of Subcellular Localization of HDAC4 by Fractionation

1. Seed NIH3T3 cells in a 6-well plate (3×10^5 cells per well).
2. Wash cells twice with PBS when they reach ~90 % confluency.
3. Add 1 ml of ice-cold hypotonic lysis buffer. Put the plate on ice for 5 min, with occasional shaking.
4. Harvest the cells with a scrapper and transfer the cell suspension into a 1.5 ml tube.

5. Centrifuge at 1,300 × *g* and 4 °C for 5 min. Transfer the supernatant into a clean tube and leave the pellet in the original tube.
6. Centrifuge the supernatant at 16,000 × *g* and 4 °C for 10 min, and collect the supernatant as the cytoplasmic fraction.
7. Suspend pellet from **step 5** in 0.2 ml of the hypotonic lysis buffer containing 0.5 M NaCl, and rotate the lysate at 4 °C for 20 min.
8. Centrifuge the nuclear lysate at 16,000 × *g* and 4 °C for 10 min, and collect the supernatant as the nuclear extract.
9. Analyze cytoplasmic and nuclear extracts by Western blotting with regular and phospho-specific anti-HDAC4 antibodies.

Endogenous HDAC4 in NIH3T3 cells is mainly localized to the cytoplasm, but there is also a small fraction in the nucleus [7]. Similarly, ectopic expression of GFP-HDAC4 in NIH3T3 showed that GFP-HDAC4 mainly resides in the cytoplasm. For HEK293 cells, GFP-HDAC4 exists in the cytoplasm in a majority of them, but in a very small proportion, GFP-HDAC4 forms dot-like structure in the nucleus [7].

3.2.2 Analysis of Subcellular Localization of HDAC4 by Nuclear Export Inhibition

In NIH3T3, HDAC4 is mainly in cytoplasm, but in other cell lines such as HeLa, HDAC4 and its paralogs are mainly in the nucleus [13–15]. Also, an HDAC4 mutant lacking the N-terminal 117 residues was first reported to be actively exported to the cytoplasm [16], implying that nucleocytoplasmic shuttling is a regulatory mechanism for HDAC4. To study whether HDAC4 is actively shuttled between the cytoplasm and the nucleus, we treated cells with leptomycin B [7], a specific inhibitor of CRM1-mediated nuclear export [17, 18]. The following protocol is modified from the published studies [17, 18].

1. Seed NIH3T3 and other cells onto three wells of a 12-well plate.
2. On the next day, 0.1 μg of GFP-HDAC4-expressing plasmid is transfected into the cells by use of a transfection reagent such as lipofactamine 2000 and Superfect according to the manufacturer's instructions.
3. About 24 h later, examine live GFP fluorescence under a fluorescence microscope.
4. To the three wells, add leptomycin B to a final concentration of 10 ng/ml at three different time points, 0, 15, and 40 min. Alternatively, use one well and examine live GFP fluorescence under a fluorescence microscope at different time points.
5. Examine live GFP fluorescence under a fluorescence microscope. As shown [7], GFP-HDAC4 is relocated from the cytoplasm to the nucleus.

3.3 Interaction of 14-3-3 Proteins with HDAC4

HDAC4 is actively exported to the cytoplasm in a CRM1-dependent manner [7]. Protein shuttling often involves anchor proteins. 14-3-3 proteins are such anchors. For example, 14-3-3 proteins inhibit the translocation of the forkhead transcription factor FKHRL1 and the cell cycle-regulating phosphatase CDC25C from the nucleus to the cytoplasm [19]. To study whether HDAC4 interacts with 14-3-3β in cells, we carried out co-immunoprecipitation as described [7]. The following protocol is adapted from our published study [7].

3.3.1 Co-immunoprecipitation of HDAC4 with 14-3-3

1. Seed HEK293 cells into four 6 cm dishes (~10^6 each dish).
2. When cells reach appropriate confluency (~95 %), 3 μg of Flag-HDAC4 expression plasmid is co-transfected into the cells with or without an expression plasmid for hemagglutinin (HA)-tagged human 14-3-3β (3 μg) by use of a transfection reagent such as lipofectamine 2000 and Superfect according to the manufacturer's instructions.
3. Incubate the cells in a 37 °C incubator (5 % CO_2), and replace the transfection medium with fresh complete DMEM medium 3 h later.
4. About 48 h after transfection, wash the cells twice with PBS.
5. If the cells will not be lysed immediately, remove the PBS completely and keep the dishes at −80 °C. Otherwise, add 0.5 ml buffer B containing 0.5 M KCl, scrap the cells, and collect the suspension into a 1.5 ml Eppendorf tubes.
6. Centrifuge at 16,000 × *g* and 4 °C for 5 min.
7. Incubate the supernatant with 30 μl M2 agarose beads, or with the mouse anti-HA monoclonal antibody bound to protein G beads, overnight at 4 °C.
8. Wash the beads four times with buffer B supplemented with 0.15 M KCl (*see* **Note 8**).
9. Add 30 μl buffer B and 1.5 μl FLAG peptide (4 mg/ml) to M2 beads, rotate the suspension at 4 °C for 60 min, centrifuge briefly at 5,000 × *g*, transfer 20 μl of the supernatant for mixing with 10 μl 3×SDS sample buffer, and boil for 5 min. For protein G beads, add 30 μl 1× SDS sample buffer to the beads and boil for 5 min.
10. Use boiled samples for Western blotting with anti-Flag or anti-HA antibody.

3.3.2 Mapping 14-3-3-Binding Sites on HDAC4

14-3-3 proteins bind to two types of consensus sites: R-(S/Ar)-(+/S)-pS-(L/E/A/M)-P and R-X-(Ar/S)-(+)-pS-(L/E/A/M)-P, where Ar is an aromatic amino acid, pS is phosphoserine, + is a basic amino acid, and X is any amino acid [20, 21]. There are five potential 14-3-3-binding sites in HDAC4: 242- RKTASEP-248, 464-RTQSAP-469, 516-RQPESHP-522, 629-RAQSSP-632,

and 703-RGRKATL-709 (conserved residues are underlined) [7]. Various HDAC4 mutants were used to determine which parts of HDAC4 are responsible for binding to 14-3-3 proteins. Deletion analyses showed that residues 531–1084 of HDAC4 contain one actual 14-3-3-binding site [7]. Two of the predicted motifs are within this range, namely 629-RAQSSP-632 and 703-RGRKATL-709. To test if S632 is essential, S632 was replaced with alanine. This mutant was unable to bind to 14-3-3 proteins [7], indicating that S632 but not T708 is important for 14-3-3 binding. To investigate whether S246 is important for 14-3-3 binding, S246 was replaced with alanine, and the resulting mutant was unable to bind to 14-3-3. Similarly, S467 was shown to be important for binding as well. Thus, S246, S467, and S632 of HDAC4 mediate the binding of 14-3-3 proteins (Fig. 1) [7]. Western blotting with an antibody against phosphor-S246 showed that in wild-type HDAC4, S246 is phosphorylated [9]. In agreement with this, no phosphorylation signal was detectable in the S246A mutant [9].

3.4 Determination of HDAC4 Deacetylase Activity

The following protocols are adapted from our published paper [22]. Although not described here, there are alternatives [23]. For example, another method is to perform deacetylation assays with hyperacetylated histones and subsequent immunoblotting analyses with anti-acetylated histone antibodies that are commercially available from different vendors.

3.4.1 Preparation of [^{3}H] Acetyl-Histones

1. Seed HeLa cells in a 6-well plate (0.3×10^6 cells per dish).
2. Replace culture medium containing 50 μCi of [^{3}H]acetate (2.4 Ci/mmol) per ml and 3 μM trichostatin A (TSA) when the cells reach 95 % confluency (~10^6 cells).
3. After incubation for 2–6 h, wash the cells once with ice-cold PBS.
4. Lyse HeLa cells with 500 μl buffer N and rotate the lysate at 4 °C for 1 h.
5. Centrifuge at 10,000 × *g* and 4 °C for 1 min to collect the nuclei. Wash the pellet three times with buffer N.
6. Discard the supernatant, add 0.1 ml of 0.4 N H_2SO_4, and resuspend the pellet completely by vortexing. Then add 9 volumes of acetone for precipitation of histones and incubate the mixture on ice for at least 1 h.
7. Centrifuge at 15,000 × *g* and at 4 °C for 10 min to collect histones and discard the supernatant.
8. Dissolve the precipitated histone pellet in 0.1 ml of 100 mM Tris–HCl (pH 8.0) and precipitate histones with cold acetone. Repeat **steps 6–8** for 3–4 times.
9. Air-dry the histones and dissolve in 100 μl 2 mM HCl. Determine protein concentration using Bradford methods.

10. Levels of histone acetylation are verified by using triton–acetic acid-urea gels.

    Alternatively, [^{3}H] acetyl-histones could also be prepared by in vitro labeling [22].

1. Incubate 50 μg histones with 50 pmol of [^{3}H]acetyl-coenzyme A (4.7 Ci/mmol) and 0.5 μg of Flag-PCAF (p300/CBP-associated factor) in 100 μl of Buffer A at 30 °C for 30 min.
2. Add 2 μl of 5 M NaCl, 1 ml of cold acetone, and 65 μg of BSA to precipitate histones (*see* **Note 9**).
3. Leave the tube on dry ice for 2 h.
4. Centrifuge at 15,000 × *g* for 5 min at 4 °C.
5. Wash the resulting pellet with 1 ml of cold acetone, air-dry, and dissolve in 100 μl of 2 mM HCl.

3.4.2 Determination of Deacetylase Activity of HDAC4

1. Reactions are carried out in 0.2 ml of buffer H, with purified Flag-HDAC4.
2. Allow the reaction to proceed at 37 °C for 90 min.
3. Stop the reaction by addition of 0.1 ml of 0.1 M HCl–0.16 M acetic acid.
4. To extract the released [^{3}H]acetate, add 0.9 ml of ethyl acetate.
5. After vigorous vortexing, take 0.6 ml of the upper organic phase for quantification by liquid scintillation counting.

3.5 Transcriptional Repression by HDAC4

The MEF2 family of transcription factors plays important roles in muscle, bone, heart, brain, hematopoiesis and other developmental processes [24]. In 1999, we and others identified MEF2 proteins as interaction partners of HDAC4 [16, 22, 25]. The binding site was mapped to a small region in the N-terminal part of HDAC4 (Fig. 1) [16, 22, 25]. Similarly, MEF2 interacts with HDAC5, HDAC7, and HDAC9 [14, 26]. Co-immunoprecipitation procedures are similar as those described in Subheading 3.3.1. HDAC4 interacts with MEF2 to repress transcription [22].

1. Plate 293T or NIH3T3 cells onto 6 cm dishes (1×10^6).
2. On the next day, when the cells reach 95 % confluency, start transfection.
3. The luciferase report plasmid Gal4-tk-Luc (50–200 ng) is co-transfected with a vector for expressing HDAC4 as a protein fused to the DNA-binding domain of the yeast Gal4 transcription factor (residues 1–147) or for the DNA-binding domain alone (50–200 ng) with transfection reagents. pBluescript KSII (+) s used to normalize the total amount of plasmids used in each transfection, and pCMV-β-Gal (50 ng) s cotransfected for normalization of transfection efficiency (*see* **Note 10**).

4. About 32 h after transfection, expose the transfected cells to 0.3 μM trichostatin A for 16 h.
5. Then the cells are lysed in situ with a lysis buffer and the luciferase reporter activity is determined by use of D-(−)-luciferin as the substrate.
6. The chemiluminescence from activated luciferin or Galacto-Light Plus is measured on a Luminometer plate reader.

4 Notes

1. Combine the antigen and the adjuvant using two syringes and locking connector (for instance, three-way stopcock) and emulsify until it no longer separates.
2. Injection sites must be sufficiently distant to prevent coalescence of the local inflammatory response.
3. If primary injection, use FCA. Then use FIA or other alternative adjuvant.
4. Extensive dialysis is necessary as any amine-based buffers (such as Tris and EDTA) inactivate CNBr-Sepharose and interfere antigen cross-linking.
5. Throughout the entire procedure, it is important not to allow the gel bed to run dry. Add sufficient solution or install the bottom cap on the column whenever no buffer is above the top of the gel bed.
6. **Steps 9–16** are to block nonspecific binding sites on the affinity gel.
7. All buffers to be run through a column can be degassed to avoid air bubbles.
8. For washing, it is better to use low-salt buffer in order to reduce the nonspecific background.
9. This step is to remove unincorporated [^{3}H]acetyl-coenzyme A.
10. Because the transfection efficiency varies in different wells, the expression plasmid CMV-β-Gal is co-transfected as an internal control for transfection efficiency normalization. The luciferase activities are normalized based on β-galactosidase activity.

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