Chapter 2

Detection of Sumo Modification of Endogenous Histone Deacetylase 2 (HDAC2) in Mammalian Cells

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Abstract

Small ubiquitin-related modifier (SUMO) is an ubiquitin-like protein that is covalently attached to a variety of target proteins and has a significant role in their regulation. HDAC2 is an important epigenetic regulator, promoting the deacetylation of histones and non-histone proteins. HDAC2 has been shown to be modified by SUMO1 at lysine 462. Here we describe how to detect SUMO modification of endogenous HDAC2 in mammalian cells by immunoblotting. Although in this chapter we use this method to detect HDAC2 modification in mammalian cells, this protocol can be used for any cell type or for any protein of interest.

Key words HDAC2, SUMO, UBC9, Immunoblotting, Posttranslational modification, PTM

1 Introduction

Histone deacetylase 2 (HDAC2) belongs the human class I HDACs that regulates gene expression by deacetylation of histones and non-histone proteins. Among class I HDACs (HDAC1 and HDAC2, HDAC3 and HDAC8), HDAC1 and HDAC2 are believed to regulate most of the changes observed in histone acetylation. HDAC2, with high homology to yeast RPD3, was identified in a yeast two-hybrid screen as a corepressor that binds the YY1 transcription factor in Edward Seto's laboratory [1]. Transcriptional repression by YY1 was shown to be mediated by tethering HDAC2 to DNA as a corepressor. Mammalian HDAC2 was then cloned and characterized by the same group [2].

HDACs activity is regulated by their binding to corepressor complex partners and depends on their posttranslational modifications (PTMs) (reviewed in Ref. [3]). In particular, HDAC2 has been shown to be phosphorylated by CK2 [4], acetylated by CBP in response to cigarette smoke treatment [5], ubiquitinated for proteasomal degradation [6, 7], nitrosylated [8, 9] and carbonylated [10]. Sumoylation is a PTM that involves the covalent attachment of

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SUMO (small-ubiquitin-like modifier) to lysine residues of proteins. Four SUMO proteins (SUMO1–4) have been identified in humans (reviewed in Ref. [11]). Covalent conjugation of SUMO proteins to their substrates requires an enzymatic cascade, comprising the sequential action of three enzymes: a modifier activating enzyme (E1), the E2 conjugating enzyme (UBC9), and a member of the ligases (E3s). Many proteins modified by SUMO contain an acceptor lysine residue within the consensus motif ψKxE (where ψ is an aliphatic branched amino acid) [12]. The covalent attachment of SUMO to target proteins regulates many functional properties of target proteins, such as protein localization, binding, and transactivation functions of transcription factors [13, 14]. Recently Brandl et al. [15] showed that HDAC2 is covalently modified by SUMO1 at lysine 462 and this modification promotes deacetylation of p53 at lysine 320. p53 acetylation blocks its recruitment into promoter-associated complexes and its ability to regulate the expression of genes involved in cell cycle control and apoptosis. Thus, HDAC2 sumoylation inhibits p53 functions and attenuates DNA damage-induced apoptosis. Moreover, HDAC2 has been shown to have a sumoylation-promoting activity in a deacetylase-independent manner. Indeed, HDAC2 seems to promote sumoylation of the eukaryotic translation initiation factor 4E (elF4E), stimulating the formation of the eukaryotic initiation factor 4F (elF4F) complex and the induction of protein synthesis of a subset of elF4E-responding genes [16].

This chapter describes the detection of HDAC2 sumoylation by immunoblotting. SUMO is typically conjugated only to a small fraction of the target protein, yet having a global and significant effect, but, as a result, the detection of the modification can be difficult. Here we show how to increase the amount of endogenous modified HDAC2 by overexpressing SUMO and the E2 conjugating enzyme UBC9 in mammalian cells. We describe how to prepare lysates from mammalian cells using denaturing lysis buffer designed to block the action of desumoylating enzymes and all the best settings to improve the detection of this modification, as previously shown for HDAC1 [17]. This method is the quicker way to detect HDAC2 sumoylation, and can be used to check changes in this modification upon different stimuli (e.g., upon treatment with the proteasomal inhibitor MG132 or deacetylase inhibitors). Although only conjugation of SUMO1 to HDAC2 has been previously described [15], this protocol shows that also conjugation of SUMO2 can be detected. This chapter focuses on the detection of sumoylated HDAC2 in mammalian cells; however, this protocol can be adapted to any cell type to detect SUMO modification for any intracellular protein.

2 Materials

2.1 Cell Culture

1. HeLa, MCF7, and U2OS cell lines (American Type Culture Collection).

2. 100 mm treated tissue culture dishes.
3. Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics, 2 mM L-glutamine, and 10% fetal bovine serum (FBS).
4. Trypsin–EDTA.
5. Sterile phosphate buffered saline (PBS).

2.2 Transfection

1. DMEM with L-glutamine, without FBS and antibiotics.
2. Fugene® 6 transfection reagent (Promega).
3. Plasmid DNA: pcDNA3/HA-N vector containing human SUMO1 (described in Ref. [18]), pcDNA3/HA-N vector containing human SUMO2 (described in Ref. [19]), pcDNA3 vector containing human UBC9 (described in Ref. [20]), and pcDNA3 empty vector.

2.3 Cell Lysis

1. SDS lysis buffer:
   (a) Solution I (5% SDS, 0.15 M Tris–HCl pH 6.8, 30% glycerol), stored at room temperature.
   (b) Solution II (25 mM Tris–HCl pH 8.3, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS), stored at 4 °C.
2. Protease inhibitors: 100 μg/mL phenyl-methyl-sulfonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL aprotinin (*see* **Note 1**).
3. 5 mM NEM (*see* **Note 2**).
4. Five times loading buffer (312 mM Tris–HCl pH 6.8, 10% SDS, 40% glycerol, 20% β-mercaptoethanol, bromophenol blue).
5. Protein quantitation assay: Bradford or Lowry.

2.4 Immunoblotting

1. Resolving gel buffer: 1.5 M Tris–HCl pH 8.8.
2. Stacking gel buffer: 0.5 M Tris–HCl pH 6.8.
3. 30% acrylamide–bis solution.
4. Ammonium persulfate (APS): 10% solution in water (*see* **Note 3**).
5. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED).
6. Resolving portion of polyacrylamide gel: acrylamide–bis solution, resolving solution, 0.1% APS, and TEMED.
7. Stacking portion of polyacrylamide gel: acrylamide–bis solution, stacking solution, 0.1% APS, and TEMED.
8. SDS-PAGE running buffer: 2 M glycine, 0.25 M Tris–HCl pH 8.3, 0.02 M SDS.
9. Benchmark prestained molecular weight standards.
10. Immobilon® Polyvinylidene difluoride (PVDF) membrane (Millipore).
11. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol.

12. Tris buffered saline (TBS; 1×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4, containing 0.1% Tween 20 (TBST).
13. Blocking solution: 5% low-fat milk in TBST.
14. Enhanced chemiluminescence (ECL) solutions.
15. Antibodies: anti-HDAC2 rabbit polyclonal (Abcam, ab7029), anti-HA mouse monoclonal (Covance), anti-UBC9 rabbit polyclonal (Santa Cruz, sc-10759).
16. Blot Stripping solution: 62.5 mM Tris–HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS.

3 Methods

3.1 Transfection of Human Cancer Cell Lines HeLa, MCF7, and U2OS (See Note 4)

1. Plate cells the day before transfection in 100 mm tissue culture dishes in order to obtain a 60% of confluence the day of transfection.
2. For each transfection sample prepare one tube of transfection reagent: add 24 μl of Fugene® 6 in 600 μl of serum and antibiotic-free DMEM, and incubate at room temperature for 5 min. Add 5–10 μg of each DNA to the solution, and incubate for 20 min at room temperature to promote the formation of membrane delimited vesicles containing DNA.
3. Once waiting for the complex to form, remove old media from cells, wash once with PBS 1×, and add 5 mL of serum and antibiotic-free DMEM.
4. Add the mixed DNA–Fugene® 6 solution dropwise on the cells.
5. Incubate at 37 °C in a 5% CO_2 incubator for 6 h.
6. Replace with fresh complete media.
7. Incubate at 37 °C in a 5% CO_2 incubator for 24 h.

3.2 Cell Lysis

1. Collect cells by trypsinization and wash them in cold PBS containing SUMO and ubiquitin protease inhibitor: 5 mM *N*-ethylmaleimide (NEM) (*see* **Note 5**).
2. Lyse cells under denaturing condition using an SDS containing lysis buffer composed of one volume of buffer I and three volumes of buffer II and containing protease inhibitors and NEM.
3. Incubate lysate on ice for 15 min.
4. Sonicate each sample for 20 s (*see* **Note 6**).
5. Centrifuge in microcentrifuge for 10 min at 10,000 × *g* at 4°C and transfer supernatants to clean tubes.
6. Use a protein quantitation assay to determine the protein concentration of each sample (*see* **Note 7**).
7. Dilute sample using equivalent amount of proteins in 5× loading buffer (*see* **Note 8**).

3.3 Immunoblotting

1. Prepare a 8% polyacrylamide gel composed by a resolving portion and a stacking portion (*see* **Notes 9** and **10**).
2. Heat samples at 95 °C for 5 min.
3. Load sample on the gel together with a prestained molecular weight standard (*see* **Note 11**).
4. Run gel in running buffer at 100 mV until samples has entered the resolving gel, and then continue at 180 mM until the dye front has come out from the gel.
5. Transfer protein from the gel to a PVDF membrane at 250 mA for 1 h in transfer buffer (*see* **Note 12**).
6. Incubate membrane in blocking solution for 1 h at RT.
7. Incubate with primary antibody diluted in 5% milk/TBST overnight at 4 °C.
8. Wash membrane three times with TBST.
9. Incubate with secondary antibody diluted in 5% milk/TBST for 1 h at RT.
10. Wash membrane three times with TBST.
11. Develop the membrane using ECL according to manufacturer's instructions (*see* **Note 13**). The results of the immunoblotting analysis are shown in Figs. 1 and 2 (*see* **Note 14**).

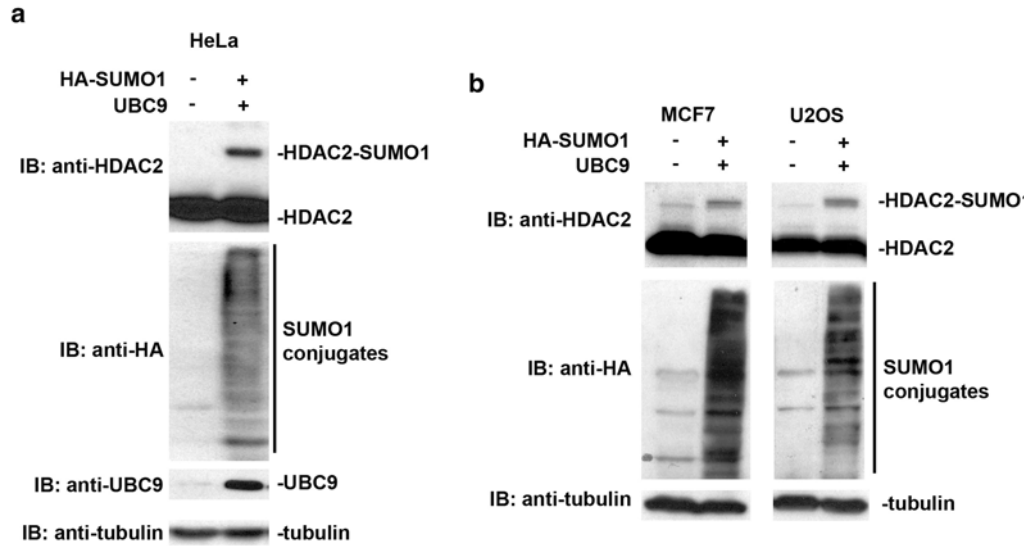

Fig. 1 Western blot analysis of cell lysates from HeLa (**a**), U2OS and MCF7 cells (**b**) co-transfected with HA-SUMO1 and UBC9 or empty vector. Cells were lysed in SDS lysis buffer and lysates were resolved on denaturing polyacrylamide gel. The expression level of HDAC2 and sumoylated-HDAC2 proteins was determined by immunoblotting (IB) using an anti-HDAC2 antibody. Tubulin was used as loading control and overexpression of HA-SUMO1 and UBC9 was checked by immunoblotting (IB) with anti-HA and anti-UBC9 antibodies respectively

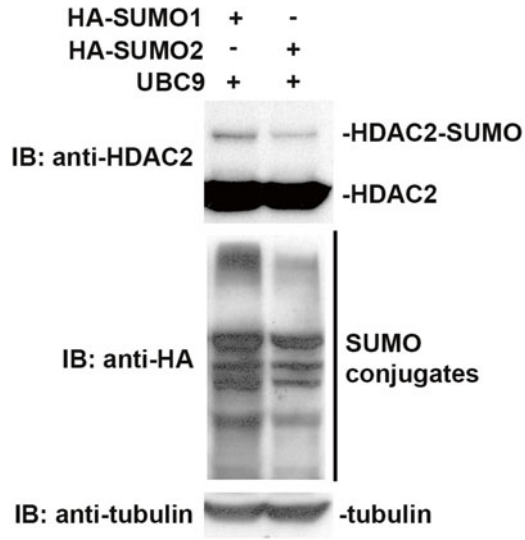

Fig. 2 Western blot analysis of cell lysates from HeLa cells co-transfected with HA-SUMO1 or HA-SUMO2 and UBC9. Cells were lysed in SDS lysis buffer and lysates were resolved on denaturing polyacrylamide gel. The expression level of HDAC2 and sumoylated-HDAC2 proteins was determined by immunoblotting (IB) using an anti-HDAC2 antibody. Tubulin was used as loading control and overexpression of HA-SUMO1 and HA-SUMO2 was checked by immunoblotting with anti-HA antibody

4 Notes

1. The inhibitors are freshly added to the buffers. A protease inhibitor cocktail for mammalian cell extract commercially available can be used instead.
2. 0.5 M NEM is prepared in absolute ethanol, stored at −20 °C and freshly added to the buffers.
3. APS can be dissolved in water and aliquots can be stored at −20 °C.
4. HeLa cells can be efficiently transfected using also calcium phosphate transfection procedure. MCF7 and U2OS can be also transfected using Lipofectamine® 2000 (Life Technologies), but we noticed an increased cell death using this reagent compared to Fugene® 6.
5. NEM is a SUMO and ubiquitin protease inhibitor used in the buffers to avoid deconjugation of sumoylated proteins.
6. Due to the high concentration of SDS in the lysis buffer, lysates appear very viscous.
7. Cell lysates can be also processed for anti-HDAC2 Immunoprecipitation. Samples have to be diluted at least 1:4 in a non-denaturing lysis buffer to dilute the amount of SDS before incubating them with the antibody.

8. β-mercaptoethanol can be replaced by dithiothreitol (DTT), used at a final concentration of 0.04 M in loading buffer 1×.
9. HDAC2 molecular weight is around 55 kDa, the addition of one SUMO protein covalently bound to HDAC2 increases the size of the protein in a range of 15–17 kDa. HDAC2 seems to have only one lysine modified by SUMO, but in the case of a protein with multiple target sites, multiples of this size increase are expected.
10. UBC9's molecular weight is around 18 kDa, and thus, a 15% polyacrylamide gel is necessary to detect it.
11. A consistent amount of proteins in necessary to detect SUMO-modified HDAC2; at least 80 μg of proteins need to be loaded on the gel for each sample.
12. PVDF membranes are preferred compared to nitrocellulose membranes. PVDF has to be activated by soaking it in pure methanol. After 1 min or less of incubation, methanol is removed and the membrane is washed once in TBST. Do not let the membrane dry. It is preferred not to stain the membrane with Ponceau after transfer.
13. Detection can be done by preferred method.
14. After the incubation and detection with the first antibody (anti-HDAC2), the membrane can be stripped using a blot stripping solution and probed with other antibodies (e.g., anti-HA and anti-tubulin, as shown in Figs. 1 and 2).

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