Chapter 20

Systematic Localization and Identification of SUMOylation Substrates in Knock-In Mice Expressing Affinity-Tagged SUMO1

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Abstract

Protein SUMOylation is a posttranslational protein modification that is emerging as a key regulatory process in neurobiology. To date, however, SUMOylation in vivo has only been studied cursorily. Knock-in mice expressing His_6-HA-SUMO1 from the *Sumo1* locus allow for the highly specific localization and identification of endogenous SUMO1 substrates under physiological and pathophysiological conditions. By making use of the HA-tag and using wild-type mice for highly stringent negative control samples, SUMO1 targets can be specifically localized in and purified from cultured mouse nerve cells and mouse tissues.

Key words SUMOylation, Knock-in mice, Affinity purification, Immunoprecipitation, HA-tag, In vivo

1 Introduction

SUMOylation is a reversible, highly dynamic posttranslational protein modification [1]. The consequences of SUMOylation depend on the target protein, and include alterations of protein localization, enzymatic activity, solubility, stability, or interactions [2–4]. In view of this broad functional relevance of SUMOylation, immense efforts have focused over the past two decades on the biochemical enrichment of SUMO targets using anti-SUMO antibodies [5], heterologous expression of tagged SUMOs [6, 7], Ubc9 fusion-dependent SUMOylation [8], or SUMO-interaction motif domains [9, 10]. Combined with mass spectrometric identification of candidate proteins, these studies have provided a huge resource of information on SUMO substrates—often including the identification of relevant modified lysine residues—and established protein SUMOylation as a crucial posttranslational protein modification that operates in every eukaryotic cell to regulate its growth, proliferation, differentiation, and function [7, 11–13].

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Unfortunately, however, the analysis of endogenous SUMOylation in complex tissues and organisms, such as mouse brain, liver, or heart, has remained challenging. This is a substantial concern, not least because growing evidence indicates an important role of SUMOylation in human diseases that can partly be modeled in genetically modified mice, particularly in neurodegenerative disorders. Consequently, several mutant mouse models have been developed to study SUMOylation in vivo [14–21]. However, most methods described so far focus on enriching SUMOylated protein species for further proteomic analysis, and only few methods are available to combine the specific localization of endogenously SUMOylated protein species with their enrichment. As a consequence, the exact subcellular distribution of endogenous SUMO targets in cells—especially in neurons—is highly debated.

To allow for the precise analysis of the localization of endogenous SUMO1 targets and their stringent enrichment, we generated His_6-HA-SUMO1 knock-in (KI) mice that express His_6-HA-SUMO1 from the endogenous *Sumo1* locus, so that overexpression artifacts can be largely excluded [15]. Additionally, these KI mice facilitate the localization and enrichment of SUMO1 substrates because anti-HA antibodies usually have higher epitope affinities than anti-SUMO1 antibodies and thus provide better signal-to-noise ratios. Further, cells or tissues from wild-type (WT) mice provide highly stringent negative controls when compared to KI material, which boosts the confidence in corresponding results. Finally, the His_6-HA-SUMO1 line can be crossed into any disease model, thus providing the opportunity to study SUMO1 conjugation in a plethora of disease-relevant processes. These are clear advantages of the His_6-HA-SUMO1 KI mice over other tools that make them a very useful model system for the analysis of SUMOylation.

Our own research focus is on SUMOylation in neurons for which we used the His_6-HA-SUMO1 KI model and WT controls. We thus describe step-by-step methods to (1) enrich SUMO1 substrates from His_6-HA-SUMO1 KI mouse brain for subsequent proteomic analysis, based on an anti-HA immunopurification protocol, and to (2) study SUMO1 localization in mouse neurons and brain sections. These methods are generally applicable and can be easily adapted to other cell types and tissues. The KI mice can be obtained from us freely, based on an MTA.

2 Materials

2.1 Immunoprecipitation

Radioimmunoprecipitation assay (RIPA) buffer, 150 mM NaCl, 20 mM Tris–HCl pH 7.4 (at 4 °C), 1% (w/v) Triton X-100, 0.5% (w/v) Na-deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS). RIPA should be made fresh and cooled at 4 °C. Protease inhibitors (see below) are added shortly before lysis.

Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, 50 mM Tris–HCl pH 6.8, 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 10 mM dithiothreitol (DTT, added freshly).

Glycine elution buffer, 0.1 M glycine-HCl pH 2.

Bead storage buffer, 20 mM Tris–HCl pH 7.5 (at 4 °C), 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.09% (w/v) NaN_3.

Protease inhibitors: Aprotinin dissolved in water as a 500 µg/ml stock solution and used at a final concentration of 0.5 µg/ml, leupeptine dissolved in water as a 1 mg/ml stock solution and used at a final concentration of 1 µg/ml, phenylmethylsulfonyl (PMSF) dissolved in isopropanol as a 17.4 mg/ml stock solution and used at a final concentration of 17.4 µg/ml, *N*-ethylmaleimide (NEM) dissolved in DMSO as a 1 M stock solution and used at a final concentration of 20 mM.

Ultrasonic homogenizer (e.g., Bandelin Sonopuls HD2200, tapered tip KE76).

100% (w/v) methanol.

100% (w/v) chloroform.

HA peptide (custom made).

Chromatography columns (10 ml reservoir).

Peristaltic pump.

Anti-HA beads.

50 ml Falcon tubes.

1.5 ml Eppendorf tubes.

Porcelain mortar and pestle.

Liquid N_2.

Precast gels (e.g., Invitrogen 4–12% BisTris).

Eppendorf Thermomixer.

Benchtop centrifuge (e.g., Eppendorf 5416, fixed-angle rotor).

Ultracentrifuge (e.g., Beckmann Coulter Optima L-70, rotor 50.2Ti).

Shaker.

2.2 Immunostaining

1× PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

Blocking and antibody buffer, 1× PBS, 5% (v/v) goat serum, 0.3% (w/v) Triton X-100.

Slides (ThermoScientific, SuperFrost Plus).

Mounting medium containing DAPI.

Cover slips.

Anti-HA antibody.

Secondary goat anti-mouse Alexa-Fluo 555 antibody.

3 Methods

3.1 Immunoprecipitation

The following procedure has been optimized for mouse brain but can also be used in order to enrich SUMOylated protein species from other mouse tissues such as heart or liver. Detergent extraction conditions can be altered depending on the tissue and target proteins to be recovered.

3.1.1 Preparation of Brain Lysate and Chromatography Column

1. Kill mice by cervical dislocation.
2. On ice, quickly remove brains from His_6-HA-SUMO1 KI and WT mice and remove brainstem.
3. Flash-freeze brains in liquid N_2.
4. Grind each brain to fine powder using a precooled porcelain pestle and a precooled porcelain mortar filled with liquid N_2.
5. Transfer frozen powder to a 50 ml Falcon tube.
6. Once all liquid N_2 has evaporated, add 10 ml of fresh, ice-cold RIPA buffer supplemented with protease inhibitors.
7. Triturate samples by pipetting up and down until complete dissolution of the powder.
8. Sonicate samples on ice for 15 s, 8-pulsed cycles, 75 % of power.
9. Ultracentrifuge samples (100,000 × *g*, 1 h, 4 °C). Carefully remove supernatants for further use.
10. During ultracentrifugation, sediment 0.5 ml of anti-HA beads into a plastic chromatography column and wash with 10 ml of RIPA buffer to equilibrate the beads.

3.1.2 Immunoaffinity Binding and Washing of the Column

1. Put the supernatant obtained after ultracentrifugation into a 50 ml Falcon tube and add fresh NEM to a final concentration of 20 mM. Take a small aliquot and keep on ice for later analysis (Input sample, INP).
2. For efficient depletion of SUMOylated protein species from the lysates, pump samples over the column for 12 h at a flow rate of 1 ml/min in the cold room (*see* **Note 1**).
3. After 12 h, take an aliquot of the lysate and keep on ice for later analysis (flow-through sample, FT).
4. Drain the column of lysate until the meniscus of the lysate almost reaches the column bed (do not let column run dry). Then wash the beads with 40 ml of RIPA containing fresh protease inhibitors and NEM. Once all the washing buffer has passed through the column, start with the elution.

3.1.3 Elution

1. Take 3 mg of lyophilized HA-peptide from −20 °C and let it warm up to room temperature for 30 min.
2. Add 600 µl of water to make a stock solution of 5 mg/ml. Mix well until peptides are completely dissolved.
3. Add 5.4 ml of RIPA to make elution buffer at a peptide concentration of 0.5 mg/ml. Keep at room temperature.
4. Carefully resuspend the washed beads in the column with RIPA buffer and transfer them to a fresh 2 ml Eppendorf tube. Pellet the beads by centrifuging gently (1000 × *g*, 3 min, room temperature). Carefully remove with a 1 ml pipet all buffer on top of beads.
5. Add 1.5 ml of elution buffer to each bead aliquot and shake (1400 rpm) in the Thermomixer at 30 °C.
6. Pellet the beads by centrifugation (1000 × *g*, 3 min, room temperature), transfer the first eluate to a fresh tube, and keep on ice.
7. Add another 1.5 ml of elution buffer to the beads and repeat elution as described above. A syringe with a 24 G needle can be used to remove all the eluate without taking beads.
8. Pool both eluates and centrifuge at maximum speed (23,100 × *g*, 5 min, room temperature) to remove eventual residues of beads. Again, a syringe with a 24 G needle can be used to remove all the eluate without taking beads.
9. From here onwards, eluates can be precipitated and separated on SDS-PAGE (*see* Subheading 3.1.4) or eluates can be further submitted to Ni-NTA chromatography (*see* **Note 2**).

3.1.4 Precipitation of Proteins from Eluates

All steps are performed at the bench at room temperature.

1. Prepare 300 µl aliquots of pooled eluate in 1.5 ml Eppendorf tubes.
2. Add 400 µl of methanol and vortex for 10 s.
3. Add 200 µl of chloroform and vortex for 10 s.
4. Add 400 µl of distilled water and vortex for 10 s.
5. Centrifuge (5000 × *g*, 3 min, room temperature).
6. Remove upper phase but leave interphase undisturbed as it contains proteins.
7. Add 400 µl of methanol and vortex for 10 s.
8. Centrifuge (23,100 × *g*, 5 min, room temperature).
9. Remove supernatant and leave pellet undisturbed.
10. Let the pellet dry at room temperature (~10 min).
11. Pool all pellets in a final volume of 50 µl of Laemmli SDS-PAGE sample buffer.

3.1.5 Bead Recovery

1. After elution, resuspend beads in PBS and transfer back to the column.
2. Drain the PBS and add 20 bead volumes of glycine elution buffer.
3. Immediately re-equilibrate the beads with 20 bead volumes of PBS.
4. For storage, drain equilibration buffer and add 20 bead volumes of storage buffer, close the column tightly, and keep at 4 °C.
5. Beads can be reused 3–4 times for Western blot purposes (*see* **Note 3**).

3.1.6 Analysis of Purified Proteins

1. *Western blotting*

   The efficiency of the anti-HA affinity purification is determined by Western blotting (Fig. 1). In this example, proteins containing an HA tag were efficiently bound to the matrix as revealed by a reduced anti-HA signal in the flow-through (FT) fraction as compared to the input fraction (INP). Anti-HA substrates were enriched in eluate fractions of the His_6-HA-SUMO1 sample (EL KI) but not the WT sample (EL WT). Putative SUMO1-conjugated protein candidates are validated by a Western blotting approach as well. For example, SUMOylated forms of RanGAP1 and KAP1 were enriched in eluates from KI as compared to WT (Fig. 2).

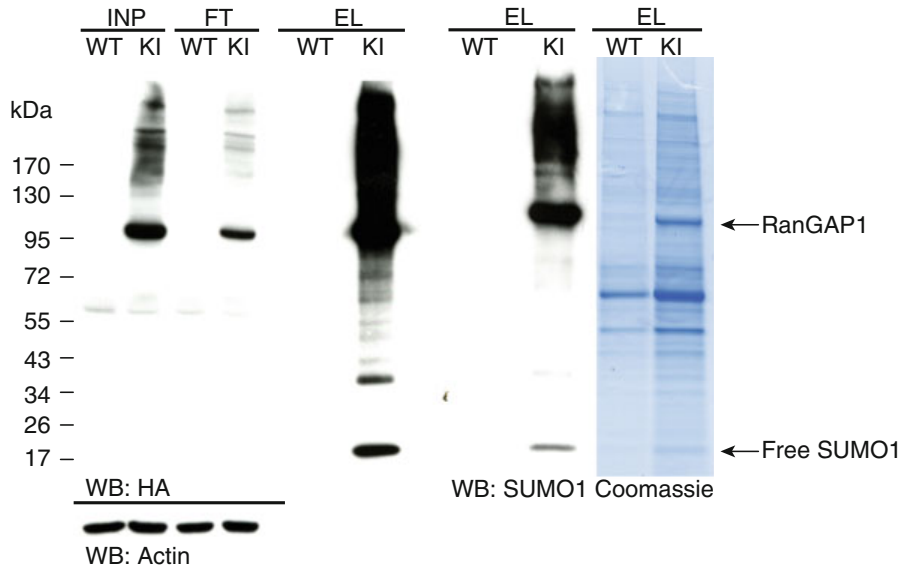

Fig. 1 Anti-HA affinity purification of HA-SUMO1 conjugates from P10 wild-type (WT) and His_6-HA-SUMO1 KI mouse brain (KI). Input (INP), flow-through (FT), and eluate fractions (EL) were analyzed by SDS-PAGE followed by either Coomassie staining (*right*) or Western blotting (*left*) using anti-HA and anti-SUMO1 antibodies

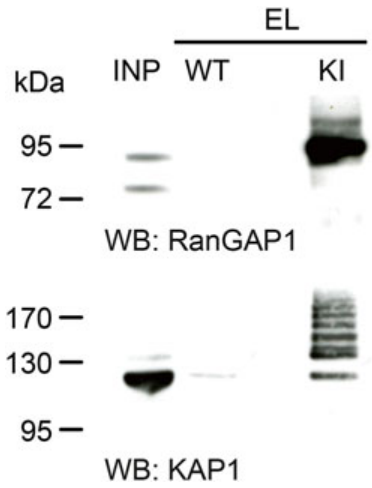

Fig. 2 Western blot analysis of the SUMO1 substrates RanGAP1 and KAP1 after anti-HA immunoaffinity purification from WT and His_6-HA-SUMO1 KI adult mice brain. Input material (from WT) and anti-HA peptide eluates of HA-immunopurified samples from WT and His_6-HA-SUMO1 KI were analyzed by Western blot using either anti-RanGAP1 (*top*) or anti-KAP1 (*bottom*) antibodies

2. *Mass Spectrometry*

   A possible follow-up procedure for proteomic analysis is described elsewhere [22]. Coomassie staining of SDS-PAGE gels loaded with HA peptide eluate fractions from WT mice reveals binding of contaminant proteins to the beads, but increased levels of protein material corresponding to enriched His_6-HA-SUMO1 targets are seen in eluate fractions from His_6-HA-SUMO1 KI mouse brain (Fig. 1).

3.2 Immunostaining

As regards the immunostaining of His_6-HA-SUMO1-conjugated proteins, we focus on the specific features of the KI mouse model. We only provide a summary of general routine techniques such as perfusion fixation of mice, neuron culture, and fixation of cultured neurons, and refer to the published literature for more details [23, 24].

3.2.1 Sample Preparation

1. *PFA fixation of mouse brain*

   His_6-HA-SUMO1 KI mice and WT littermate are first briefly anesthetized using isoflurane and then deeply anesthetized using Avertin. Mice are transcardiacally perfused with 4% cold PFA in 0.1 M PB. Brains are then post-fixed for 1 h in 4% PFA in 0.1 M PB at 4 °C and then placed in 30% sucrose in 0.1 M PB. Brains are then frozen either on dry ice or directly in the cryostat prior to cutting 30 μm thick sections (*see* **Note 4**). Sections are kept in PBS with 0.09% azide at 4 °C until further use.

2. *Primary neuron culture*

   Hippocampal or cortical neurons from His_6-HA-SUMO1 KI and WT littermates are prepared form newborn animals. Brain regions of interest (hippocampi or cortex) are carefully dissected out and digested for 45 min in a papain solution (25 units/ml) at

37 °C with gentle shaking. Papain is then inactivated by incubating the samples in stop solution containing 2.5 mg/ml bovine serum albumin, 2.5% (wt/vol) ovalbumin, and 10% (vol/vol) fetal bovine serum for 15 min at 37 °C with gentle shaking. Hippocampi or cortex pieces are then triturated in neurobasal medium complemented with B27. Neurons are then plated on poly-l-lysine-coated cover slips at a density of 13,000 cells per cm^2. After 14 days in vitro, neurons are fixed on ice for 10 min using 4% PFA in PBS with gentle shaking. Cover slips are then washed three times with PBS and kept in PBS at 4 °C until used.

3.2.2 Immuno-labeling HA

1. Incubate brain section or cover slip with neurons in 200 µl of blocking/permeabilization buffer for 1 h at room temperature with gentle shaking (*see* **Note 5**).
2. Remove blocking solution and incubate samples either overnight at 4 °C (brain sections) or for 2 h at room temperature (neurons on cover slips) with 200 µl blocking/permeabilization solution containing anti-HA primary antibody at a final dilution of 1:1000.
3. Carefully remove the primary antibody buffer and slowly add 0.5 ml of PBS. Shake gently for 10 min at room temperature.
4. Repeat the washing step described above (3) at least three times.
5. Remove washing buffer and incubate samples with 200 µl blocking/permeabilization solution containing Alexa-goat anti-mouse 555 at a final dilution of 1:2000 for 2 h (brain sections) or for 1 h (neurons on cover slips) at room temperature with gentle shaking.
6. Carefully remove the buffer with primary antibodies and add 0.5 ml of PBS. Shake gently for 10 min at room temperature.
7. Repeat the washing step described above (3) at least three times.

3.2.3 Mounting and Imaging

1. *Mounting free-floating sections*
   (a) Fill up a large glass petri dish with PBS and carefully transfer brain sections into it using a thin brush.
   (b) Submerge the glass slide below the brain section.
   (c) With a thin brush, mount and flatten the brain section on the slide.
   (d) Slowly remove the slide with the brain section from the PBS solution.
   (e) Let sample dry for a few minutes by holding the slide vertically.
   (f) Add a small drop of mounting medium to the partially dried brain section without touching.
   (g) Cover with a cover slip slowly, avoiding air bubble formation.

(h) Let dry overnight at 4 °C. Imaging can proceed on the next day.

2. *Mounting cover slips*

(a) Place a small drop of mounting medium on a slide.

(b) Carefully and slowly reverse the cover slip of stained neurons onto the drop of mounting medium, avoiding air bubble formation.

(c) Leave overnight at 4 °C to dry. Imaging can proceed on the next day.

3.2.4 *Imaging*

Image acquisition is performed as described [15]. Briefly, confocal laser-scanning microscope Leica SP2 or SP5 was used to acquire serial confocal images. Settings (gain and offset) were kept constant for a given staining and genotypes to allow for fluorescence intensity comparison. High-resolution analysis of anti-HA labeling of His_6-HA-SUMO1 brain sagittal sections (Fig. 3) or cultured neurons (Fig. 4) revealed a strong nuclear and nuclear envelope labeling of cells as compared to WT. Line scanning through cell bodies and dendrites of triple-labeled CA3 hippocampal neurons using anti-HA, anti-MAP2, and anti-Synapsin 1 antibodies showed that extra nuclear His_6-HA-SUMO1 conjugates are not localized at synapses (Fig. 3, white arrow), an observation that was further confirmed by double immunostaining of primary hippocampal neurons using anti-HA and anti-Synapsin 1 (Fig. 4, white arrow).

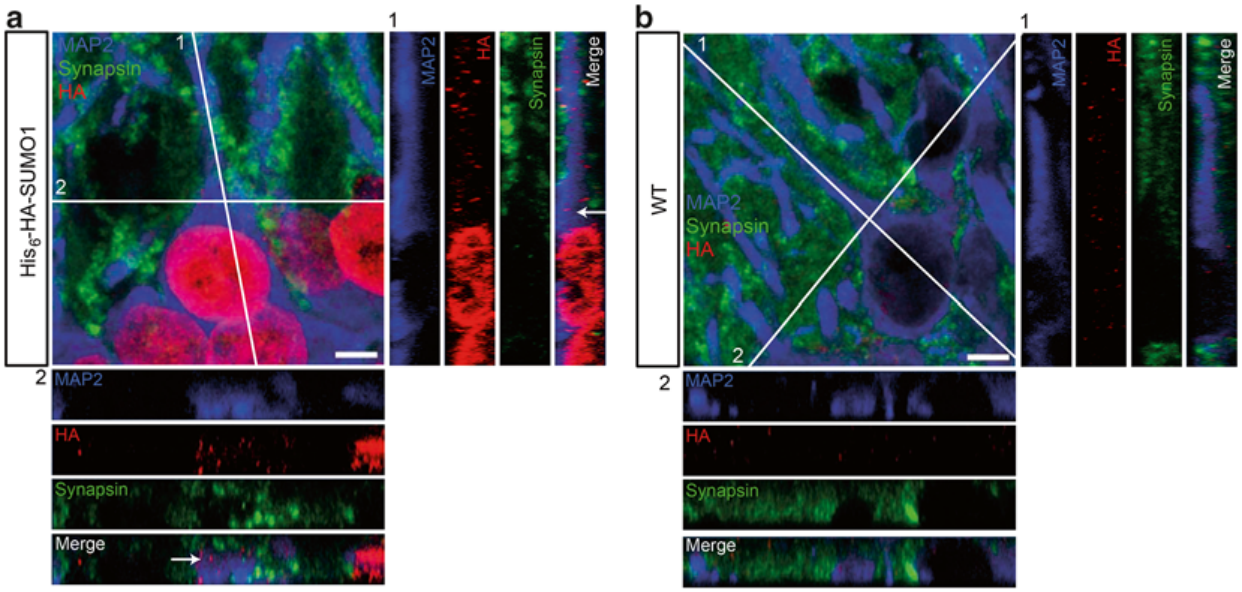

Fig. 3 Localization of His_6-HA-SUMO1 conjugates in the cytosol and the nucleus of CA3 hippocampal neurons of His_6-HA-SUMO1 mice. Sagittal brain sections from KI (**a**) and WT (**b**) mice were stained using antibodies to HA (*red*), Synapsin 1 (*green*; presynaptic terminals), and MAP2 (*blue*; neuronal dendrites). The *white line* shows the orientation of the scan used to generate the image stacks shown in side view on the *right* and *bottom*. Scale bar, 10 μm. The white arrows indicate that extra nuclear His_6 -HA-SUMO1 conjugates are not localized at synapses

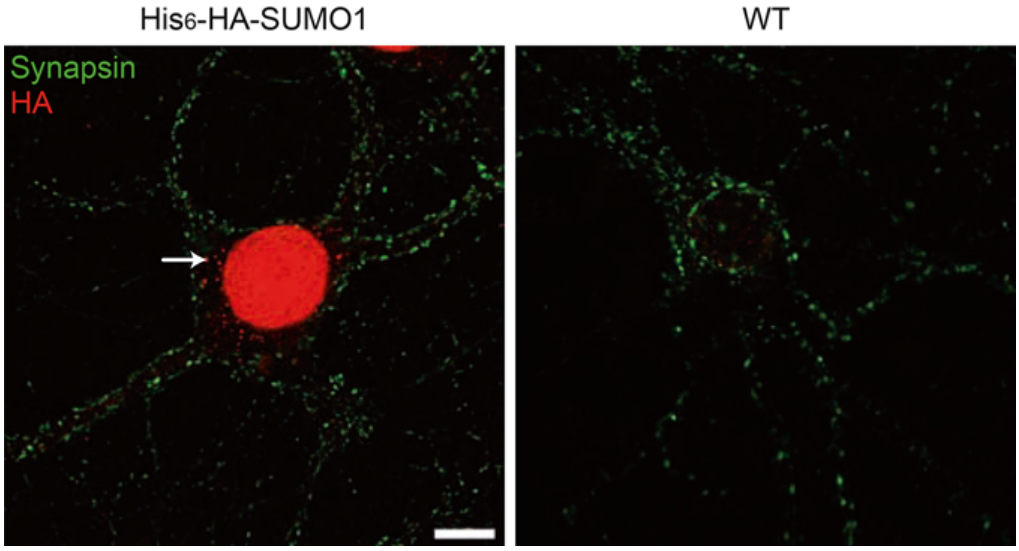

Fig. 4 Localization of His_6-HA-SUMO1 conjugates in the cytosol and nucleus of His_6-HA-SUMO1 KI neurons. Images of KI (*left*) and WT control (*right panel*) primary hippocampal neurons are shown. Neurons were stained using antibodies to HA (*red*) and Synapsin 1 (*green*; presynaptic terminals). Scale bar, 10 μm. The white arrow indicates that extra nuclear His_6 -HA-SUMO1 conjugates are not localized at synapses

4 Notes

1. For higher enrichment of His_6-HA-SUMO1 targets, it is recommended to use a chromatography-based procedure instead of a batch adsorption protocol.
2. A two-step purification (nickel-nitrilotriacetic acid (Ni-NTA) combined with anti-HA affinity purification) successfully enriches His_6-HA-SUMO1 substrates for Western blot analysis as compared to WT but does not yield enough material for routine proteomics analysis, independently of whether the Ni-NTA chromatography is performed before or after anti-HA affinity chromatography. The reason for this is the loss of proteins when changing between biological and denaturing buffers. Additionally, single Ni-NTA chromatography to enrich His_6-HA-SUMO1 substrates from His_6-HA-SUMO1 KI and WT leads to major nonspecific binding [16]. Therefore, we recommend performing the Ni-NTA chromatography as a second purification step. A detailed description of our Ni-NTA chromatography protocol from mouse brain is described elsewhere [25].
3. When a mass spectrometric comparative analysis of WT and His_6-HA-SUMO1 KI material is planned, a fresh batch of beads should be used.
4. Isopentane freezing of mouse brains did not result in proper staining of RanGAP1 at the nuclear pore complex.
5. We do not recommend using digitonine for the permeabilization of neurons, as in our hands it does not prevent the strong labeling of the nuclear envelope and leads to poor labeling of neuronal synapses with antibodies to synaptic markers.

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