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Molecular, Cellular, and Physiological Characterization of Sirtuin 7 (SIRT7)

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Abstract

Sirtuin 7 (SIRT7), a histone 3 lysine 18 (H3K18) deacetylase, functions at chromatin to suppress endoplasmic reticulum (ER) stress and mitochondrial protein folding stress (PFS^{mt}), and prevent the development of fatty liver disease and hematopoietic stem cell aging. In this chapter, we provide a methodology to characterize the molecular, cellular, and physiological functions of SIRT7.

Keywords

SIRT7; Myc; NRF1; Mitochondrial UPR (UPR^{mt}); Mitochondrial protein folding stress; ER UPR (UPR^{er}); ER stress; Fatty liver; Hematopoietic stem cell; Aging

1 Introduction

Silent information regulator 2 (Sir2) proteins, or sirtuins, are a class of highly conserved proteins found in organisms ranging from bacteria to humans that possess nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase activity, including deacetylase activity [1–3]. There are seven mammalian sirtuins, SIRT1–7, that localize to various cellular compartments [4–15]. SIRT7 is a chromatin binding protein that deacetylates H3K18 at specific gene promoters to repress transcription [4]. A major class of SIRT7 target genes is ribosomal proteins and mitochondrial ribosomal proteins [4]. SIRT7 does not have a known DNA binding domain and is recruited to chromatin through its interaction with transcription factors, such as Myc, NRF1, and ELK4 [4, 10, 15]. SIRT7 expression is induced upon various stress conditions, such as ER stress and PFS^{mt}, and represses the expression of ribosomal proteins and mitochondrial ribosomal proteins to alleviate stresses [10, 15]. Mouse genetics studies reveal that SIRT7 prevents the development of fatty liver disease and hematopoietic stem cell aging [10, 15].

Here, we describe the methods we utilized to characterize the molecular, cellular, and physiological role of SIRT7. We describe a co- immunoprecipitation (co-IP) method to identify SIRT7-interacting transcription factors. We present a method to assess the effect of SIRT7 on PFS^{mt} using an aggregation prone mutant mitochondrial protein ornithine

transcarbamylase (ΔOTC) as a marker. Finally, we provide a guide to study SIRT7 physiology with a focus on hepatic lipid metabolism and hematopoietic stem cell aging.

2 Materials

2.1 Molecular and Cellular Characterization of SIRT7

2.1.1 Endogenous Co-immunoprecipitation

1. High salt lysis buffer: 10 % glycerol (w/v), 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 % NP-40, 20 mM $MgCl_2$. Freshly add protease inhibitor cocktail before use.
2. Co-IP buffer: 10 % glycerol (w/v), 0.6 % Triton™ X-100 in PBS. Freshly add protease inhibitor cocktail before use.
3. Laemmli sample buffer (5×): 10 % SDS (w/v), 10 mM dithiothreitol, 20 % glycerol (w/v), 0.2 M Tris–HCl (pH 6.8), 0.05 % bromophenol blue.

2.2 Physiological Characterization of SIRT7

1. Homogenization buffer: 1:2 ratio of 100 % MeOH and 100 % chloroform.
2. Reconstitution buffer: 1 % Triton™ X-100 in 100 % EtOH.

2.2.1 Physiological Function of SIRT7 in the Liver

Triglyceride (TG) Quantification in the Livers of Wild Type (WT) and SIRT7$^{-/-}$ Mice

Histological Analyses of the Livers of WT and SIRT7$^{-/-}$ Mice

1. Paraformaldehyde: 4 % solution in water.
2. Oil Red O Solution: Add 0.7 g of Oil Red-O powder into 100 mL of isopropanol. Stir overnight in a glass bottle. Filter the mixture through two layers of Whatman papers. Store at 4 °C. Before staining, prepare a fresh Oil Red-O working solution (WS) by adding 6.0 mL of stock to 4.0 mL of DD water. Then filter the mixture through two layers of Whatman papers (*see* Note 1).
3. Mayer’s hematoxylin.
4. Blocking buffer: 5 % FBS, 0.3 % Triton™ X-100 in PBS.
5. Antibody dilution buffer: 2.5 % FBS, 0.3 % Triton™ X-100 in PBS.
6. Rat anti-mouse F4/80 antibody: diluted 1:200 in antibody dilution buffer.
7. Donkey anti-rat IgG antibody: diluted 1:500 in antibody dilution buffer.
8. DAPI: 10 mg/mL in H_2O.
9. Prolong Gold antifade reagent.

1. It is important to remove any precipitates.

2.2.2 Functional Assessment of SIRT7 in Hematopoietic Stem Cell (HSC) Aging

Infection of HSCs with Control or SIRT7 Overexpression Lentivirus

1. HEK-293T growing media: 10 % fetal bovine serum in high-glucose Dulbecco's modified eagle medium
2. Stem cell stimulation media: 9 mL of StemSpan SFEM, 1 mL of stem cell fetal bovine serum, 100 μL Pen-Strep, cytokine mix: 100 μL SCF, 20 μL TPO, 25 μL Flt3, 10 μL IL3, 10 μL IL6 (all stocks at 10 μg/mL, all cytokines murine).
3. Syringe fi lter: 0.45 μm, nitrocellulose.
4. Polybrene: 10 mg/mL, used in 1:1000 dilution.

3 Methods

3.1 Molecular and Cellular Characterization of SIRT7

3.1.1 Endogenous Co-immunoprecipitation

1. The day before starting with the experiment, seed 1×10^7 HEK- 293T cells in 15 cm dish.
2. Aspirate media off of the cells and wash cells with ice-cold PBS.
3. Lyse cells by adding 700 μL of high salt lysis buffer directly to the plate.
4. Incubate the cells in the plate on a shaker in the cold room (4 °C) for 30 min.
5. Use cell scraper to dislodge all cells and transfer to 1.5 mL tubes. Centrifuge the cell lysate for 10 min at 16,100 × *g* at 4 °C.
6. Transfer supernatant to new 1.5 mL tube.
7. Measure protein concentration by BCA assay.
8. Dilute 1 mg of protein to 1 mL total volume with co-IP buffer (*see* Note 2).
9. Pre-clear lysates by adding 15 μL of washed protein A beads.
10. Place samples in a rotator in the cold room for 30 min at 4 °C.
11. Spin down at 1000 × g for 5 min at 4 °C.
12. Transfer supernatant to new tubes. Reserve a 50 μL aliquot in a separate new 1.5 mL tube for input controls and set aside.
13. For the rest of the supernatant add either 1 μg of IgG control antibody or 1 μg of SIRT7 antibody.
14. Place the samples in a rotator overnight at 4 °C.
15. Wash 20 μL worth of protein A/G bead slurry with 1 mL Co-IP buffer.

2. You would need one IgG control IP and one SIRT7 IP.

16. Spin down for 30 s at 1000 × *g* at 4 °C.
17. Aspirate supernatant, and add 950 μL of protein + antibody that had been incubating the night before to the washed protein A/G bead slurry.
18. Place the samples in the rotator for 3 h at 4 °C.
19. Spin down for 30 s at 1000 × *g* at 4 °C.
20. Aspirate off supernatant. Add 1 mL Co-IP buffer.
21. Spin down for 30 s at 1000 × *g* at 4 °C.
22. Repeat **steps 20** and **21** twice more.
23. Using 25 G needle, pierce a hole on the lid of 1.5 mL tube and a second "half" hole at the bottom, only going half the way through the tube bottom for the elution of the protein off the beads. Place the tube with holes on top of a new 1.5 mL tube.
24. Spin down for 30 s at 1000 × *g* at 4 °C. Repeat this step until the beads are dry (*see* Note 3).
25. Add 40 μL of 100 mM glycine (pH 3.0) to the beads. Incubate for 10 min at room temperature, flicking intermittently to mix.
26. Place the tube on top of a new 1.5 mL tube. Spin down for 30 s at 1000 × *g* at 4 °C (*see* Note 4).
27. Add 10 μL 5× Laemmli sample buffer (for a final concentration of 1×) to the Input and IP eluents.
28. Boil samples at 95 °C in a heat block for 10 min.
29. Continue with Western blotting and probe for SIRT7 interacting transcription factors.

3.1.2 *Overexpression of ΔOTC in SIRT7 Knockdown Cells*

1. The day before starting with the experiment, seed 5×10^5 of control or SIRT7 knockdown (KD) HEK-293T cells in a 6 well dish.
2. Transfect OTC or ΔOTC constructs into control or SIRT7 KD cells. Mix 2 μg of DNA with 250 μL of Opti-MEM and 6 μL of Lipofectamine 2000 in a sterile 1.5 mL tube.
3. Incubate for 20 min at room temperature.
4. Add dropwise to cells.
5. Collect cells at 48 h post transfection.
6. Continue with Western blotting and probe for OTC.

[3]The beads will turn white once they are dry. If the beads remain transparent and wet after spinning down, you would need to make your "half" hole bigger and spin down again.
[4]Make sure eluents are clear of beads, but bead pellet in the eluted tube is dry.

3.2 Physiological Characterization of SIRT7

3.2.1 Physiological Function of SIRT7 in Liver

TG Quantification in the Livers of WT and SIRT7$^{-/-}$ Mice

1. Cut 50–100 mg of liver samples on dry ice and weigh.
2. Homogenize samples in 1 mL of homogenization buffer using a motorized pestle in a 1.5 mL tube (*see* Note 5).
3. Rotate samples in fume hood at RT for 2 h.
4. Spin down homogenates at 15,000 × *g* for 5 min in a tabletop centrifuge.
5. Transfer supernatant to glass GC/MS tubes.
6. Evaporate under N_2 gas for ~5–20 min and ~10–40 μL of viscous fat layer will remain.
7. Reconstitute with 200–500 μL of reconstitution buffer.
8. Perform enzymatic TG measurement assay according to manufacturers' instructions.

Histological Analyses of the Livers of WT and SIRT7$^{-/-}$ Mice

Oil Red O Staining

1. Embed liver tissues in Optimal Cutting Temperature compound (OCT) and make 5 μm sections using the Cryostat.
2. Fix for 40 min in 4 % paraformaldehyde (*see* Note 6).
3. Wash with PBS.
4. Immerse into Oil Red-O working solution (WS) for 30 min.
5. Wash briefly in 60 % isopropanol.
6. Stain with hematoxylin for 1 min.
7. Wash with H_2O.
8. Observe under fluorescent microscope with magnifications of ×20.

Immunohistochemistry for F4/80

1. Embed liver tissues in OCT and make 16 μm sections using the Cryostat.
2. Fix for 40 min in 4 % paraformaldehyde.
3. Wash with 1× PBS.
4. Permeabilize in 1 % Triton™ X-100 in PBS for 15 min.
5. Rinse once in PBS for 1 min.

5. Prepare a mastermix of 1:2 MeOH–chloroform freshly before cutting samples.
6. The sections can be stored in −20 °C freezer for later use.

6. Block for 1 h in blocking buffer
7. Incubate sections with rat anti-mouse F4/80 antibody diluted 1:200 in antibody dilution buffer overnight at 4 °C.
8. Rinse three times in PBS, 5 min each wash.
9. Incubate sections with secondary antibody diluted 1:500 in antibody dilution buffer for 1 h at room temperature (*see* Note 7).
10. Rinse three times in PBS, 5 min each wash.
11. Incubate sections with DAPI diluted 1:500 in PBS for 5 min at room temperature.
12. Rinse once in PBS for 5 min.
13. Add coverslip with anti-fade reagent, avoiding air bubbles.
14. Observe under microscope.

3.2.2 Functional Assessment of SIRT7 in Hematopoietic Stem Cell Aging

Infection of Enriched HSCs with Control and SIRT7 Overexpression Lentivirus: *Day 1*: Transfect ~90 % confluent HEK-293T cells in 10 cm dish

1. Add 1 mL serum free Opti-MEM to 1.5 mL tube.
2. Add 6 μg each of: pFUGW/pFUGW-SIRT7; pMDLg-pRRE; pRSV-Rev; pMD2.G (Addgene).
3. Mix and add 60 μL of Lipofectamine 2000 to the tube and vortex briefly.
4. Incubate at room temperature for 20 min.
5. Add the mixture dropwise into 10 cm dish.
6. Mix by gentle swirling of the plate.
7. Change media the next day (~18 h after transfection).

Day 3: Infection

1. Plate 50,000 of sorted Lineage− c-Kit+ Sca-1+ (LSK) cells from aged mice (18–24 months old) in a round bottom 96-well plate and incubate in stem cell stimulating media for 3 h.
2. Filter viral media from 10 cm dish through 0.45 μm syringe filter into centrifuge bottles.
3. Add 10 mL of HEK-293T growing media back to viral producing 293T plates for a second round of viral infection to be carried out the next day.
4. Equilibrate weights of bottles with sterile PBS and cap.

[7]·Protect from light whenever handling the secondary antibody, as well as your samples after you have added the secondary antibody.

5. Spin bottles in Sorval Centrifuge, using SS34 rotor, at 20,000 × *g* for 90 min at 4 °C.
6. Gently pour off supernatant and use aspirator at the mouth of the bottle to remove any remaining supernatant (*see* Note 8).
7. Resuspend invisible pellet with 200 μl of Stem cell stimulating media.
8. Add polybrene to the resuspended media (final concentration of polybrene in the media is 10 μg/mL).
9. Transfer media containing sorted LSK cells from 96 well round- bottom dish to 96 well flat-bottom dish (*see* Note 9).
10. Add resuspended virus to each well.
11. Spin plate at 270 × *g* for 90 min at room temperature.
12. Return to 37 °C CO_2 incubator and incubate for 24 h.

Day 4: Second round of infection

1. Repeat **steps 2–12** on *Day 3*.
2. Give the cells a few hours to rest. Transduced LKS can be used for transplantation on this day.

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8. Since the viral pellet is invisible, care should be taken while aspirating, such as tilting the centrifuge tubes and aspirating near the neck of the tubes.
9. Enriched HSCs are usually plated in round-bottom plates, however, we have found more consistent transduction when it is carried out in flat-bottom plates.

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