Chapter 15

Identification of Sirtuin4 (SIRT4) Protein Interactions: Uncovering Candidate Acyl-Modified Mitochondrial Substrates and Enzymatic Regulators

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Abstract

Recent studies have highlighted the three mitochondrial human sirtuins (SIRT3, SIRT4, and SIRT5) as critical regulators of a wide range of cellular metabolic pathways. A key factor to understanding their impact on metabolism has been the discovery that, in addition to their ability to deacetylate substrates, mitochondrial sirtuins can have other prominent enzymatic activities. SIRT4, one of the least characterized mitochondrial sirtuins, was shown to be the first known cellular lipoamidase, removing lipoyl modifications from lysine residues of substrates. Specifically, SIRT4 was found to delipoylate and modulate the activity of the pyruvate dehydrogenase complex (PDH), a protein complex critical for the production of acetyl-CoA. Furthermore, SIRT4 is well known to have ADP-ribosyltransferase activity and to regulate the activity of the glutamate dehydrogenase complex (GDH). Adding to its impressive range of enzymatic activities are its ability to deacetylate malonyl-CoA decarboxylase (MCD) to regulate lipid catabolism, and its newly recognized ability to remove biotinyl groups from substrates that remain to be defined. Given the wide range of enzymatic activities and the still limited knowledge of its substrates, further studies are needed to characterize its protein interactions and its impact on metabolic pathways. Here, we present several proven protocols for identifying SIRT4 protein interaction networks within the mitochondria. Specifically, we describe methods for generating human cell lines expressing SIRT4, purifying mitochondria from crude organelles, and effectively capturing SIRT4 with its interactions and substrates.

Key words Sirtuin 4, Lipoic acid, Lipoyl, Lipoamide, PDH, Protein–protein interactions

1 Introduction

Sirtuins (SIRTs) comprise a family of seven mammalian nicotinamide adenine dinucleotide (NAD$^+$)-dependent enzymes that regulate genome expression, stress response, and aging [1, 2]. Defining the functional diversity of SIRTs is of immense interest, given their roles in the regulation of critical cellular homeostasis, and involvement with conditions such as cancer, and cardiovascular and neurodegenerative diseases [3–8]. Significant progress towards characterizing individual SIRTs has begun to elucidate

their specificity in the context of cellular localization, protein interactions, enzymatic function, and substrates, shedding light on these important molecular effectors.

SIRTs display diverse subcellular localizations and enzymatic functions. For example, SIRT1, SIRT6, and SIRT7 are nuclear, SIRT3–5 are predominantly mitochondrial, and SIRT2 is cytoplasmic [9, 10]. Although SIRT3–5 are housed within mitochondria, they have different deacyl catalytic functions and impact diverse metabolic pathways. SIRT3 exerts robust deacetylation activity for numerous mitochondrial substrates [11, 12] and regulates energy homeostasis [13]. SIRT4 deacetylates malonyl-CoA decarboxylase (MCD) to regulate lipid catabolism [14], and has been shown to delipoylate pyruvate dehydrogenase (PDH) to inhibit acetyl-CoA production [15]. SIRT4 has also been shown to perform ADP-ribosylation on glutamate dehydrogenase (GLUD1) [9]. SIRT5 desuccinylates, demalonylates, and deglutarylates protein substrates such as carbamoyl phosphate synthase 1 (CPS1) to regulate the urea cycle [16–18]. Thus, mitochondrial SIRTs are emerging as important enzymes, intrinsic to pivotal metabolic reactions that dictate mitochondrial regulation and homeostasis.

The conventional approach to discover new sirtuin enzymatic activities has been to screen various acyl-modified histone peptides with recombinant SIRT proteins in *in vitro* reactions [19]. While this approach can be used to directly compare the catalytic efficiency of SIRTs to various substrates, it is somewhat limited by the number of candidates tested in the screen. Moreover, it is often difficult to translate the *in vitro* findings *in vivo*, depending on the subcellular locations of the enzyme and potential substrate in cells. Furthermore, identification of the biological protein substrates in the cell remains challenging, particularly if there are several possible proteins that contain the acyl modification, or if there are as yet unknown substrates. An alternative strategy to this systematic screening approach is provided by proteomic profiling. The interacting SIRT protein partners are identified by mass spectrometry, and statistical bioinformatic analysis are employed to discover enriched functional categories.

We recently utilized this proteomics-based strategy to discover SIRT4 as the first known cellular lipoamidase (or delipoylase), enzymatically removing lipoyl modifications from lysine residues of substrate proteins [15]. Immunoaffinity purification (IP) of SIRT4 from mitochondrial fractions revealed its interactions with lipoyl-modified dehydrogenases and biotin-dependent carboxylases. The value of defining SIRT4 protein interactions within mitochondria in order to discover novel substrates was demonstrated by a combination of follow-up experiments. In vitro steady-state enzyme kinetic assays confirmed the catalytic efficiency of SIRT4 for lipoyl- and biotin-modified peptides. Additionally, experiments performed in human cells (i.e., fibroblasts) and in livers from SIRT4 knockout mice demonstrated that SIRT4 modulates the lipoyl-lysine levels of

dihydrolipoyl acetyltransferase (DLAT), the E2 component of PDH, and the overall PDH activity. The impact of SIRT4 on other dehydrogenase complexes and on biotin-dependent carboxylases remains to be investigated. Furthermore, the contribution of the SIRT4 lipoamidase activity to various conditions of health and disease remains to be determined. Therefore, future studies of SIRT4 interactions with substrates and functional protein complexes in different cells and tissues promises to significantly expand the knowledge regarding its impact on cellular metabolism in health and disease.

In this chapter, we describe protocols that have proven effective for the identification of SIRT4 protein interactions. Firstly, retroviral transduction is used to generate stable cell lines overexpressing SIRT4-EGFP. This approach is commonly implemented when high-affinity, high-specificity antibodies required for the IP of the protein of interest are not available. While studying the interactions of the endogenous protein is always preferred, a tag can offer higher affinity (and sometimes specificity) of isolation. In addition to the studies of SIRT4 and other human sirtuins [20, 21], we have previously used this approach to discover and validate novel functional protein interactions for the eleven human histone deacetylases [22], as well as of other relevant families of proteins, such as the PYHIN proteins involved in transcriptional regulation and immune response [23]. In the second section, we describe a protocol for cellular fractionation and mitochondrial isolation. This is used to deplete the high abundance cellular contaminants from the IP, whilst enriching for trace SIRT4 interactions within the mitochondria. The third section contains methods for characterization of SIRT4 protein interactions. We have included protocols that describe conjugation of antibodies to magnetic beads, and the lysis conditions for achieving effective SIRT4 isolations. We also provide protocols for gel-based protein separation and tryptic digestion into peptides for mass spectrometry interrogation. The chapter concludes with instructions for data analysis, and determination of the specificity of the observed protein interactions based on the SAINT algorithm [24]. This integrated pipeline can be applied to any mitochondrial protein of interest to discover novel potential substrates, interacting protein partners, and expand the knowledge of its molecular function.

2   Materials

2.1   Generation of Fibroblasts Stably Expressing SIRT4-EGFP

1. Cell lines: MRC5 and Phoenix (ATCC). Cells are maintained at 37 °C with 5 % CO_2.

2. Culture medium: DMEM containing 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin–streptomycin solution.

3. Opti-MEM reduced serum medium.

4. Lipofectamine 2000.

5. 1.5 mL Eppendorf tubes (Sterile).

6. 0.45 μm syringe filters.

7. 10 mL syringes.

8. Tissue culture plates: 6, 10, and 15 cm.

9. Geneticin (G418).

10. Selection medium: Culture medium containing 400 μg / mL G418.

2.2 Conjugation of Anti-GFP Antibody to Magnetic Beads

1. Dynabeads M-270 Epoxy (Life Technologies).

2. Affinity purified antibodies against an epitope tag or, if available, the protein of interest (e.g., anti-GFP antibodies described below for the isolation of GFP-tagged SIRT4). Store at –80 °C.

3. 0.1 M sodium phosphate buffer, pH 7.4 (4 °C, filter sterilized). Prepare as 19 mM NaH_2PO_4, 81 mM Na_2HPO_4. Adjust pH to 7.4, if necessary.

4. 3 M ammonium sulfate (filter sterilized). Prepare in 0.1 M sodium phosphate buffer, pH 7.4.

5. 100 mM glycine–HCl, pH 2.5 (4 °C, filter sterilized). Prepare in water and adjust to pH 2.5 with HCl.

6. 10 mM Tris–HCl, pH 8.8 (4 °C, filter sterilized). Prepare in water and adjust to pH 8.8 with HCl.

7. 100 mM triethylamine: Prepare fresh in water. CAUTION: Triethylamine is toxic and extremely flammable, and must be handled in a chemical hood and disposed appropriately.

8. DPBS, pH 7.4 (Dulbecco's phosphate-buffered saline (1×), liquid).

9. DPBS containing 0.5 % Triton X-100. Prepare fresh in DPBS.

10. DPBS containing 0.02 % sodium azide. Prepare fresh in DPBS. CAUTION: Sodium azide is a toxic solid compound and must be handled in a chemical hood and disposed appropriately.

11. Rotator (at 30 °C).

12. Magnetic separation tube rack.

13. Tube shaker, e.g., TOMY micro tube mixer.

14. Safe-Lock tubes, 2 mL round bottom.

15. Ultrapure water (e.g., from a Milli-Q Integral water purification system).

2.3 Isolation of Mitochondria from Cultured Human Cells

1. Polycarbonate membrane filters, 14 μm (STERILTECH Corporation).

2. Swinney filter holders (Maine Manufacturing).

3. 5 mL syringes.

4. Trypsin–EDTA (0.5 %), no phenol red.

5. Dulbecco's phosphate-buffered saline (DPBS).

6. Homogenization buffer: 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES–NaOH, pH 7.4.

7. Dilution buffer: 0.25 M sucrose, 6 mM EDTA, 120 mM HEPES–NaOH, pH 7.4.

8. OptiPrep™ Density Gradient Medium.

9. 50 % OptiPrep solution: Prepare fresh just before use by mixing 4 mL OptiPrep stock with 0.8 mL dilution buffer.

10. OptiPrep gradient solutions (Prepare fresh and store on ice) (Table 1).

11. 15 mL conical tubes.

12. Benchtop centrifuge.

13. Ultracentrifuge (capable of $100,000 \times g$).

14. Ultracentrifuge rotor and respective buckets.

15. Thin-wall polypropylene 4 mL ultracentrifuge tubes.

16. Safe-Lock microcentrifuge tubes, 1.5 mL.

2.4 Characterization of SIRT4 Interacting Proteins by Immunoaffinity Isolation

1. Mitochondrial-enriched fraction. It is preferred to isolate mitochondria fresh and to use it immediately.

2. 10× IP buffer: 0.2 M HEPES–KOH, pH 7.4, 1.1 M KOAc, 20 mM $MgCl_2$, 1% Tween 20, 10 µM $ZnCl_2$, 10 µM $CaCl_2$. Filter-sterilize, and store at 4 °C for up to 6 months.

3. 10 % Triton X-100.

4. 4 M NaCl.

5. Protease inhibitor cocktail.

6. Lysis buffer: 20 mM HEPES–KOH, pH 7.4, 0.11 M KOAc, 2 mM $MgCl_2$, 0.1% Tween 20, 1 µM $ZnCl_2$, 1 µM $CaCl_2$ 0.6% Triton X-100, 200 mM NaCl, protease inhibitors. The final composition and amount of the lysis buffer has been optimized for

Table 1 Optiprep density mixtures

OptiPrep solutions (%)	50 % OptiPrep solution (mL)	Homogenization buffer (mL)
10	0.4	1.6
15	0.6	1.4
20	0.8	1.3
25	1.0	1.0
30	1.2	0.8

analyzing SIRT4 interactions within mitochondria isolated from five 15 cm culture dishes. Prepare 2 mL of buffer <u>per IP sample</u> by combining 1.58 mL of Milli-Q grade water, 0.2 mL of 10× IP buffer, 0.12 mL of 10% Triton X-100, and 0.1 mL of 4 M NaCl. Prepare fresh prior to each experiment and store on ice. Add 1/100 (v/v) protease inhibitor cocktail immediately before use.

7. Wash buffer: Same composition as lysis buffer, except without protease inhibitors. Prepare 10 mL of buffer <u>per IP sample</u>.

8. DPBS, pH 7.4 (Dulbecco's phosphate-buffered saline (1×), liquid).

9. Magnetic beads conjugated with antibodies. Stored at 4 °C and used for IP within 2 weeks after conjugation.

10. Polytron for tissue homogenization (e.g., PT 10–35 Polytron from Kinematica).

11. Centrifuge and rotor, capable of $8000 \times g$ at 4 °C.

12. Safe-Lock microcentrifuge tubes, 2 mL round bottom.

13. Magnetic separation rack.

14. 4× LDS sample buffer.

15. 10× reducing agent: Bond-breaker TCEP solution, neutral pH (Pierce).

16. 10× alkylating agent: 0.5 M chloroacetamide in water. Aliquot and store at ≤–20 °C.

17. Heat block at 70 °C.

2.5 SDS-PAGE and In-Gel Digestion of SIRT4 Interacting Proteins

1. Primary eluate from immunoaffinity purification.

2. 4–12 % Bis–Tris pre-cast SDS-PAGE gel, 10 well.

3. SDS-PAGE electrophoresis system.

4. 20× MOPS SDS running buffer.

5. 1× running buffer: Dilute 20× MOPS SDS Running Buffer to 1×.

6. Molecular weight standards.

7. 4× LDS sample buffer.

8. Coomassie blue stain.

9. Ultrapure dH_2O (e.g., from a Milli-Q purification system).

10. Rocking platform.

11. Sheet protector and scanner.

12. Solution basins.

13. Safe-Lock microcentrifuge tubes, 2 mL round bottom.

14. Axygen 96-well plates with sealing mat.

15. Multichannel pipet.

16. Pipet tips, 200 μL (Low retention tips are highly recommended).

17. Ceramic plate, forceps, razor blade, Windex.

18. Mickle Gel Slicer or equivalent tool that cuts gel lanes into slices.

19. Lens paper.

20. Water, minimum HPLC grade.

21. Acetonitrile (ACN), HPLC grade.

22. Formic acid stock (FA), LC-MS grade, 99+%.

23. 1% FA solution: Mix 1.0 mL of FA stock in 99.0 mL ultrapure water. Store at RT.

24. 0.1 M ammonium bicarbonate (ABC): Dissolve 0.80 g of ammonium bicarbonate solid in 0.1 L of HPLC grade water. Store at RT and use within 1 month, or sterile filter for long term storage.

25. Destain solution: Mix 2 mL of ACN with 2 mL of 0.1 M ammonium bicarbonate. Prepare fresh before use in a solution basin.

26. Rehydration solution: Mix 2.5 mL of ultrapure water with 2.5 mL of 0.1 M ammonium bicarbonate. Prepare fresh before use in solution basin.

27. 0.5 µg/µl trypsin stock, sequencing grade. Store at –80 °C, limit to <5 freeze–thaw cycles.

28. 12.5 ng/µl trypsin solution: Mix 8.5 µL of trypsin stock with 331.5 µL of rehydration solution. Prepare fresh immediately before use.

29. Vortex device with an adaptor for 96-well plates.

30. Glass autosampler vials, MS Certified with 200 µL fused inserts and pre-slit caps.

31. 50% ACN–0.5% FA solution. Mix 0.5 mL of ACN and 0.5 mL of FA solution (1%) in microfuge tube.

32. Vacuum concentrator.

2.6 Peptide Desalting Using StageTips

1. Microcentrifuge.

2. Pipet tips, 200 µL (Low retention tips are highly recommended).

3. 16 G needle (Hamilton).

4. Syringe plunger, 100 µL (Hamilton).

5. Empore SDB-RPS disks (3 M).

6. 10% trifluoroacetic acid (TFA) in MS grade water.

7. 0.2% TFA in MS grade water.

8. Elution buffer: 5% ammonium hydroxide and 80% acetonitrile in water.

9. FA solution: 1% formic acid and 4% acetonitrile in water.

10. Autosampler vials.

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2.7 Nanoliquid Chromatography Tandem Mass Spectrometry Analysis	1. Nanoflow HPLC system. 2. Mobile phase A (MPA): 0.1 % FA/99.9 % water. Store in amber bottle for up to 6 months. 3. Mobile phase B (MPB): 0.1 % FA/97 % ACN/2.9 % water. Store in amber bottle for up to 6 months. 4. Analytical column, e.g., Acclaim PepMap RSLC 75 μm ID × 25 cm. 5. Mass spectrometer. 6. Nanospray ESI source. 7. SilicaTip Emitter, Tubing (OD × ID) 360 μm × 20 μm; Tip (ID) 10 μm.
2.8 Mass Spectrometry Data Analysis and Interpretation	1. Multi-core/multi-CPU 64-bit PC workstation with at least 12 GB of RAM and 2 TB of storage. 2. Software for generating peaklists and scoring PSMs, with support for precursor ion quantification e.g., Proteome Discoverer 1.4, Mascot 2.3, Scaffold 4. 3. SAINT (http://www.crapome.org/). 4. Spreadsheet software.

3   Methods

3.1 Generation of Fibroblasts Stably Expressing SIRT4-EGFP

Highly specific antibodies with strong SIRT4 affinity can be used to directly study endogenous SIRT4 interactions. However, when these are not available, an alternative strategy can be to express a tagged version of the protein, such as SIRT4-EGFP. Different vectors can be used to express the tagged protein; however, it is preferable to select one that leads only to moderate levels of overexpression. For the protocol below, the retroviral vector pLXSN is used to express SIRT4-EGFP together with a neomycin-resistance marker in human fibroblasts (specifically, MRC5 cells). This approach was proven effective for discovering a novel enzymatic activity of SIRT4, as well as its substrates within the mitochondria [15]. The process involves transfection of Phoenix cells to generate retroviral particles in the cellular supernatant, followed by transduction of MRC5 cells (Fig. 1a). The protocol below describes reagent amounts required to generate one MRC5 cell line. For construction of additional cell lines, adjust reagents accordingly. For instance, MRC5 cells expressing EGFP should be generated in parallel to use as a control for nonspecific protein associations to magnetic beads or the tag.
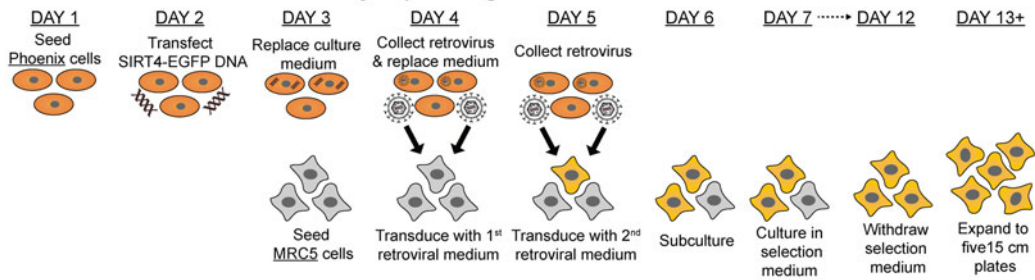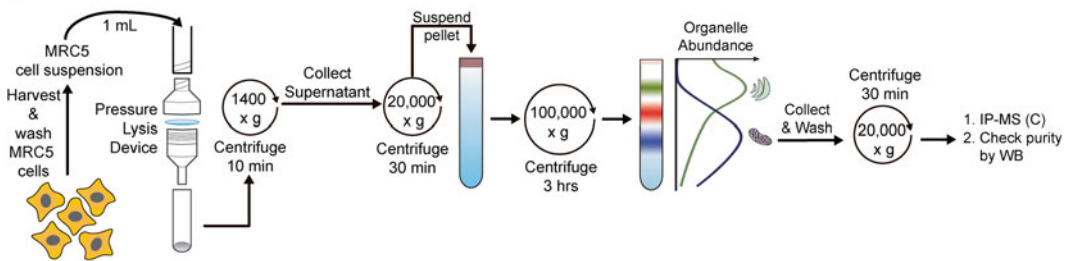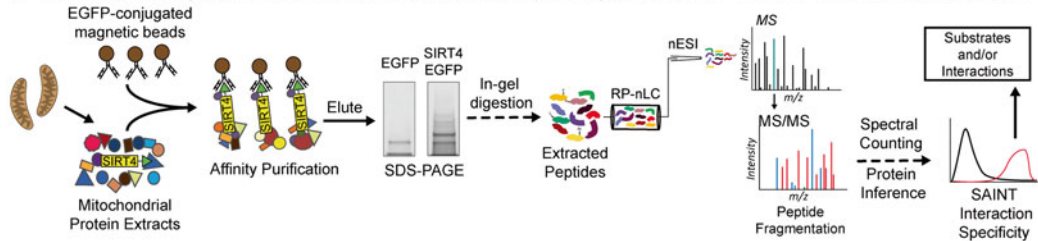
Fig. 1 Experimental approach for studying interactions and substrates of SIRT4. (**a**) Timeline for the generation of MRC5 fibroblasts (*bottom row*) stably expressing SIRT4-EGFP (*orange cells*) by transduction of retrovirus generated from Phoenix cells (*top row*). Two rounds of transduction are performed, followed by G418 selection of EGFP-tagged SIRT4 expressing cell. MRC5 cells that survive the selection are expanded to 5×15 cm plates in normal growth medium. (**b**) Scheme for isolation of mitochondria from EGFP-tagged SIRT4 expressing MRC5 cells. A crude organelle fraction is first obtained by $3\times$ passage of MRC5 cells through a pressure lysis apparatus containing two polycarbonate filter disks. Organelles are then resolved by ultracentrifugation through a self-prepared OptiPrep density gradient. Western blot analysis should be used to confirm mitochondria are enriched in fractions 3 and 4, and also to check purity. (**c**) Graphic workflow for proteomic analysis of SIRT4-containing complexes isolated from purified mitochondria. Using optimized lysis buffer, SIRT4-EGFP and associated proteins are extracted from mitochondria and mixed with EGFP-conjugated magnetic beads. Affinity purified proteins are then eluted from the beads, resolved by SDS-PAGE, and digested in-gel with trypsin. Tryptic peptide mixtures are then analyzed by reverse-phase nanoliquid chromatography (RP-nLC) coupled to nanoelectrospray (nESI) tandem mass spectrometry. The total number of spectra collected and assigned to each protein (spectral counts) between control and SIRT4 isolations are used to determine specific interactions, which are subsequently analyzed by bioinformatics

3.1.1 Day 1

1. For each unique MRC5 cell line that will be transduced, seed one 6 cm plate with 1.7×10^6 Phoenix cells in 5 mL culture medium. Place in a humidified incubator set at 5 % CO_2 and 37 °C (unless otherwise stated, all subsequent overnight cell culture incubations are performed under these conditions).

3.1.2 Day 2

1. In the afternoon, ensure Phoenix cells are 85–90 % confluent.
2. Label two 1.5 mL microfuge tubes, A and B.
3. In Tube A, add 500 μL Opti-MEM and 7 μL Lipofectamine.
4. In Tube B, add 500 μL Opti-MEM and 2 μg DNA (e.g., SIRT4-EGFP or EGFP).
5. Incubate for 5 min at room temperature (RT).
6. Gently add contents of Tube B to Tube A, and incubate for 20 min at RT.
7. Replace culture medium of Phoenix cells seeded on Day 1 with 4 mL of fresh medium (without penicillin–streptomycin).
8. Add Lipofectamine–DNA mixture dropwise to Phoenix cells, swirl gently to mix, and incubate overnight.

3.1.3 Day 3

1. In the morning, aspirate culture medium from Phoenix cells and replace with 5 mL of fresh medium. Incubate overnight. This supernatant will be used for first retroviral transduction on Day 4 (**steps 1–6**).
2. In the afternoon, seed 1.5×10^6 MRC5 cells in a 10 cm plate in culture medium. Incubate overnight.

3.1.4 Day 4

1. Gently collect first retrovirus-containing supernatant from Phoenix cells in 15 mL conical tube (from Day 3, **step 1**).
2. Add 5 mL fresh culture medium to Phoenix cells and incubate overnight. This supernatant will be used for second retroviral transduction on Day 5.
3. Centrifuge Phoenix cell supernatant (first retrovirus collection) at $500 \times g$ for 5 min at RT to pellet lifted cells.
4. Pass supernatant through 0.45 μm syringe filter and collect in a 15 mL conical tube.
5. Add 5 mL fresh culture medium to the filtered Phoenix cell supernatant containing retrovirus (*see* **Note 1**).
6. Remove culture medium from MRC5 cells (seeded on Day 3, **step 2**) and replace with retrovirus-containing medium. Incubate overnight.

3.1.5 Day 5

1. Gently collect second retrovirus-containing supernatant from Phoenix cells in 15 mL conical tube (from Day 4, **step 2**).

2. Perform a second round of retroviral transduction, following the same procedure as described above (Day 4, **steps 3–6**).

3.1.6 Day 6

1. Passage MRC5 cells, and re-seed to have 60–70 % confluence in 10 mL culture medium. Incubate overnight.

3.1.7 Day 7

1. Aspirate MRC5 cell culture medium and replace with 10 mL selection medium (*see* **Note 2**).

2. Culture in selection medium for 3–6 days. During this time, monitor the amount of cell detachment/lysis and change medium as needed (*see* **Note 3**). Expression of SIRT4-EGFP can be evaluated by direct fluorescence microscopy and/or western blotting.

3. Withdraw selection medium when cell lysis has stopped (6 days or less). Continue to passage cells in culture medium (no selection) for 2–3 passages (*see* **Note 4**) and expand into five 15 cm plates.

4. At ~95 % confluence, cells should be harvested for mitochondria isolation (*see* Subheading 3.3). It is recommended to perform mitochondria isolation from freshly harvested cells and then proceed immediately to immunoaffinity purification of SIRT4-EGFP (*see* Subheading 3.4). Therefore, preparation of the EGFP antibody-conjugated magnetic beads (*see* Subheading 3.2) should be started 2 days prior to the collection of cells for mitochondria isolation.

3.2 Conjugation of Antibodies to Magnetic Bead for SIRT4 Immunoaffinity Purifications

This protocol has been optimized for the conjugation of M-270 Epoxy Dynabeads with affinity purified antibodies [25]. The use of other magnetic beads (e.g., M-450 or MyOne Dynabeads) is possible, but the amount of antibody used for conjugation should be adjusted based on the binding capacity of the bead. Commercially available antibodies can be used with the M-270 Epoxy Dynabeads. However, if the antibody is stored in buffer containing free amines (e.g., Tris), the amount of antibody covalently conjugated to the surface epoxy groups will be limited; it is best to avoid such buffers. It is recommended to begin this section of the protocol in the afternoon and resume (**step 7**) in the morning of the following day.

1. For each immunoisolation to be performed, weigh out 7 mg of magnetic Dynabeads in a round-bottom tube (*see* **Note 5**).

2. Add 1 mL sodium phosphate buffer (pH 7.4) to the beads. Mix by vortexing for 30 s, followed by 15 min on a tube shaker (vigorous setting).

3. Place the tube on a magnetic rack. Once the beads have attached to the magnet and the supernatant has a clear appearance (~30 s), aspirate the wash buffer.

4. Remove the tube from the rack. Add 1 mL sodium phosphate buffer (pH 7.4) and mix by vortexing for 30 s. Aspirate the wash buffer as above.

5. Remove the tube from the rack. In the following order, add the necessary amount of (1) antibodies, (2) sodium phosphate buffer, and (3) ammonium sulfate solution.

   (a) The optimal total volume for bead conjugation is a 20 μL reaction volume per mg of M-270 epoxy beads.

   (b) The amount of antibody that should be used during conjugation is 4–5 μg Ab per mg M-270 epoxy beads.

   (c) The 3 M ammonium sulfate solution is added last to give a final concentration of 1 M.

   (d) For example, for 14 mg of washed beads, add 42 μg of antibody. Second, add 0.1 M sodium phosphate buffer such that its volume equals 280 μL, minus the volume of antibody and the volume of 3 M ammonium sulfate. Finally, add 93 μL, of 3 M ammonium sulfate.

6. Wrap the tube with parafilm and incubate the bead suspension overnight on a rotator at 30 °C (*see* **Note 6**).

7. The following morning, place the tube against the magnetic rack.

8. OPTIONAL: Retain the supernatant to assess the efficiency of bead conjugation by SDS-PAGE.

9. Wash the beads sequentially with the following buffers (1 mL each): sodium phosphate buffer; 100 mM glycine–HCl, pH 2.5 (FAST WASH); 10 mM Tris–HCl, pH 8.8; 100 mM triethylamine (FAST WASH); D-PBS (4 WASHES); DPBS containing 0.5 % Triton X-100 (15 min wash with gentle agitation); DPBS (*see* **Note 7**).

10. Resuspend washed beads in 12.5 μL DPBS + 0.02 % NaN$_3$ per mg of beads. Measure the final volume of the bead slurry to determine the bead concentration (mg of beads/μL DPBS).

11. Beads can be used immediately or stored for up to 2 weeks at 4 °C. After 1 month of storage, their efficiency for isolation decreases by approximately 40 %.

3.3 Isolation of Mitochondria from Cultured Cells

Given that SIRT4 is known to be primarily mitochondrial [10], subcellular enrichment of mitochondria prior to immunoaffinity purification is advantageous. This approach will deplete highly abundant cytoplasmic and nuclear proteins that are unlikely to be physiological substrates of SIRT4, improving the detection of trace and/or transient protein interactions that may be in lower abundance (Fig. 1b).

1. Precool ultracentrifuge rotor and buckets at 4 °C.

2. Prepare five pressure filtration apparatuses. Unscrew and open swinney filter holder, and place two polycarbonate membrane filters inside (shiny side facing towards syringe attachment side of swinney). Screw swinney closed extremely tight, to ensure sample does not leak out during cell lysis (*see* **Note 8**). Attach syringe to swinney.

3. Remove culture medium, and rinse cells with 10 mL DPBS.

4. Lift cells with 3 mL/plate Trypsin–EDTA solution. Briefly incubate at 37 °C, if necessary.

5. Add 12 mL culture medium to inactivate trypsin, and centrifuge at $500 \times g$ for 5 min to pellet cells.

6. Aspirate and discard medium, and wash cells with 10 mL DPBS. Centrifuge at $500 \times g$ for 5 min to pellet cells.

7. Resuspend cells in 5 mL homogenization buffer (*see* **Note 9**).

8. Transfer 1 mL to the each syringe of each pressure filtration apparatus, and gently push syringe plunger down to force cell suspension through filtration apparatus, collecting filtrate in a clean 15 mL tube in ice.

9. Unscrew syringe from swinney, and separate plunger from syringe. Reconnect syringe to swinney.

10. Repeat **steps 8–9** twice, using the filtrate from the first lysis (*see* **Note 10**).

11. Pool filtrates from the same cell lines and centrifuge at $1400 \times g$ for 10 min at 4 °C to pellet nuclei.

12. Carefully aspirate the supernatant, and transfer to new 15 mL tube. Centrifuge at $20,000 \times g$ for 30 min at 4 °C to pellet crude organelles.

13. During centrifugation, prepare two 10–30% discontinuous OptiPrep gradients (*see* Subheading 2.3) in thin wall ultracentrifuge tubes by top-layering 0.7 mL of each OptiPrep solution, starting with the densest solution (30%) at the bottom of the tube (*see* **Note 11**). Store gradients on ice.

14. After centrifugation, aspirate and discard the supernatant.

15. Gently resuspend the crude organelle pellet in 0.7 mL homogenization buffer by pipetting until the pellet has been dispersed to a homogenous suspension.

16. To one OptiPrep gradient, carefully overlay the 0.7 mL crude organelle suspension on top of the 10% OptiPrep solution. To the other OptiPrep gradient, layer 0.7 mL of Homogenization Buffer to serve as the balance during ultracentrifugation.

17. Transfer tubes to opposing ultracentrifuge buckets.

18. Attach all six buckets to their respective position on the SW60 rotor and perform ultracentrifugation at $100,000 \times g$ for 3 h at 4 °C.

19. Carefully aspirate six 0.7 mL fractions from the top of the gradient. Collect Mitochondria should be primarily enriched in fraction 4, and to a lesser extent, in fraction 3 (*see* **Note 12**).

20. Wash mitochondria by adding 0.8 mL DPBS, and mixing gently.

21. Centrifuge at $20,000 \times g$ for 30 min at 4 °C to pellet mitochondria.

22. Assess purity by western blotting (*see* **Note 13**).

23. Ideally, as mentioned above, the immunoaffinity isolation of SIRT4-EGFP (*see* Subheading 3.4) should be performed on freshly isolated mitochondria. This will produce the most consistent isolation of SIRT4-EGFP and its interacting proteins, particularly for less stable interactions. However, if experimental limitations require collection of mitochondria on different days, freshly isolated mitochondria should be immediately flash frozen in liquid nitrogen and stored at –80 °C until ready to proceed with the immunoaffinity isolation section.

3.4 Immunoaffinity Isolation of SIRT4 Interacting Proteins

SIRT4 enzymatic activity towards various acyl-modified peptides has been traditionally screened by systematically testing candidate peptides using in vitro enzymatic assays, e.g., as in Ref. [19]. This approach can be laborious and is often prohibited by the number of candidate substrates in the screen. Proteomics can be employed for a less biased approach for identifying potential substrates, which can then be validated by in vitro enzyme kinetic assays, as in [15]. Using optimized mitochondrial lysis conditions and stringency, SIRT4 and its interactions are isolated and subjected to mass spectrometry-based identification (Fig. 1c). Following immunoaffinity isolation, there are several time points when the protocol can be paused and continued at a later time (as indicated below).

1. Prepare lysis buffer (containing protease inhibitors) and wash buffer.

2. Add 1 mL of lysis buffer to purified mitochondria (isolated from five 15 cm plates, Subheading 3.3) and vortex 3×20 s, cooling in between on ice. Mix by rotation for 10 min at 4 °C.

3. Centrifuge the lysate at $5000 \times g$ for 10 min at 4 °C.

4. During centrifugation step, place tube of antibody-conjugated magnetic beads against a magnetic rack for 30–60 s. Discard the storage buffer and wash 3×1 mL with wash buffer by gently pipetting to resuspend the beads. Do not vortex.

5. Resuspend the beads in 100 μL of wash buffer and place on ice.

6. Carefully transfer the clarified mitochondrial lysates (supernatant) into a clean round-bottom microfuge tube and store on ice. Retain (1) the insoluble cell/tissue pellet and (2) 40 μL of the clarified lysates as the input fraction for isolation efficiency analysis by western blot.

7. Mix the antibody-conjugated beads to a homogenous suspension by gently pipetting. Pipette a 7 mg equivalent of beads into each sample of clarified mitochondrial lysates.

8. Rotate the lysate–bead suspension on a rotator at 4 °C for 1 h (*see* **Note 14**).

9. During the incubation, prepare 1× NuPAGE sample buffer and three clean round-bottom microfuge tubes per IP sample. Ensure that wash buffer and DPBS are cooled on ice.

10. After incubation, place samples on magnetic rack and allow the beads to adhere to the tube wall (~20 s). Transfer the flow-through (unbound) fraction to a clean tube and retain for isolation efficiency analysis by western blot.

11. Gently resuspend the beads in 1 mL of wash buffer and transfer the bead suspension to a clean round-bottom tube.

12. Separate the beads from the buffer wash buffer using the magnetic rack as above. Aspirate the wash buffer. *Perform this step between all subsequent wash steps.*

13. Wash the beads 3 × 1 mL with wash buffer. On the third wash, transfer the bead slurry to a second clean round-bottom tube.

14. Wash the beads 2 × 1 mL with wash buffer.

15. Wash the beads with 1 mL DPBS and transfer slurry to a third clean round-bottom tube.

16. Repeat DPBS wash and ensure buffer is completely removed.

17. To elute the protein complexes from the beads, add 40 μL of 1× sample buffer to beads.

18. Incubate for 10 min at 70 °C, then 10 min at RT with agitation (*see* **Note 15**).

19. Isolate beads on the magnetic rack and transfer the primary eluate to a clean microcentrifuge tube.

20. For assessment of elution efficiency, resuspend the beads in 1× sample buffer and repeat **steps 18** and **19**, except incubate the beads for 5 min at 95 °C.

21. Isolate beads on the magnetic rack and transfer the secondary eluate to a clean microcentrifuge tube.

22. Add 5 μL of 10× reducing agent and 5 μL of 10× alkylating agent to primary and secondary eluates. Heat at 70 °C for 10 min. Retain 10% aliquots of primary and secondary eluates for western analysis of isolation efficiency.

23. At this point, samples can either be stored at ≤ -20 °C, or the remaining 90 % of the primary eluate can be processed immediately by SDS-PAGE and in-gel digestion to identify the co-isolated proteins by mass spectrometry (*see* Subheading 3.5).

24. To assess the efficiency of immunoisolation, analyze equal percentages (e.g., 5 %) of the following samples by western blotting: cell pellet and input (**step 6**), flow-through (**step 10**), primary and secondary eluates (**step 22**).

3.5 SDS-PAGE and In-Gel Digestion of SIRT4 Interacting Proteins

The proteins co-isolated with SIRT4 are next identified by mass spectrometry. Samples can be prepared for mass spectrometry analysis by digesting the proteins with an enzyme using either an in-gel or in-solution protocol. An example of an in-solution digestion protocol was described in detail in [26]. Here, we provide an in-gel digestion protocol that was successfully implemented when studying SIRT4 interactions [15]. This protocol is performed over the course of 3 days. To minimize keratin and other environmental contaminants, it is recommended to wear a lab coat and hair protection, to avoid close contact and limit environmental exposure of the pre-cast gel during sample loading and protein staining. While many SDS-PAGE systems can be employed for proteomic analysis, a system that has pre-cast gels available is highly recommended to further reduce environmental contaminants. Here we use the NuPAGE system as we have found that using the MOPS running buffer in combination with a 4–12 % Bis–Tris pre-cast acrylamide gels excels at resolving larger molecular weight proteins, which usually generate more peptide complexity (*see* **Note 16**).

3.5.1 Day 1

1. If necessary, thaw <u>primary</u> eluate sample(s) at 70 °C.

2. Set up the electrophoresis system.

3. Load 30 µL of primary eluates, leave 1–2 empty lanes between samples (*see* **Note 17**).

4. Load 20 µL of 1× sample buffer into empty wells to ensure even running of the samples.

5. Electrophorese briefly (2–3 min) at 100 V, then turn off system and load remaining sample volume (~15 µL).

6. Continue electrophoresis at 150 V until the dye front has migrated approximately 1/3 of the way down the gel (*see* **Note 18**).

7. Open gel cassette to expose gel and discard the wells. Working with wet gloves, transfer gel (by the thick ridge at the bottom) into a plastic tray containing ultrapure water. Remove bottom ridge.

8. Wash the gel 3×5 min with ultrapure H_2O while rocking.

9. Cover the gel completely in Coomassie blue stain. Incubate for 1–3 h until the protein bands become clearly visible.

10. Dispose of the staining solution. Rinse briefly with ultrapure H$_2$O. Add fresh ultrapure H$_2$O and destain overnight at 4 °C with gentle rocking (*see* **Note 19**).

3.5.2 Day 2

Working solution volumes are calculated for 16 total gel fractions and based on 90 μl of solution per well, except where noted (*see* **Note 20**). For higher throughput, a 96-well plate with sealing mat and a multichannel pipette is recommended. A different set of tips should be used for each set of gel pieces from the sample lane, but each set can be used for all steps (Day 2 and 3).

1. Briefly wash the destained gel several times with ultrapure water to remove any excess staining reagent.

2. Place the gel in a sheet protector. Keep the gel wet with ultrapure water. Place on a bed scanner to digitize the gel image. Print out the image to use as a reference during grouping of the gel slices (**step 9**).

3. Wash the ceramic plate, forceps, blade of the gel slicer, and a razor blade with Windex, then with ultrapure water.

4. Transfer the gel to the ceramic plate and cut out the vertical stained region of one sample lane. Trim the lane to remove the lower portion just at the dye front and the top 2 mm near the well.

5. Place a small piece of lens paper on the cutting stage of the Mickle Gel Slicer, wet the lens paper with ultrapure water and flatten it out.

6. Place the excised gel lane on the lens paper and add a drop of ultrapure water.

7. Use the Mickle Gel Slicer (or equivalent) to cut the gel lane into 1 mm slices (~20–30 total slices).

8. Lift the lens paper and flip the cut gel lane onto the ceramic plate. Carefully remove the lens paper. Keep cut gel slices slightly wet for ease of sample handling.

9. Starting from the bottom of the gel, divide/group gel pieces such the amount of protein per group (estimated by the Coomassie blue stain intensity) is normalized. For example, for darkly stained bands/band clusters, group only 1–2 gel pieces, while lightly stained regions (usually upper MW region) can be grouped into 3–4 gel slices (*see* **Note 21**)

10. Once the grouping has been defined, use a razor blade to cut each group gel slices into three segments (*see* **Note 22**).

11. Transfer gel pieces using the forceps or the flat side of razor blade into one well of 96-well plate.

12. Repeat **steps 4–12** for additional sample lanes.

13. Add 90 µL of destain solution to each well, seal plate, and agitate on a vortexer (medium setting) at 4 °C for 10 min. Aspirate and discard. For all aspiration steps, make sure the gel pieces are not stuck to pipet tips when discarding waste solution as sample loss could occur.

14. Repeat the previous step with fresh destain solution. While destaining, prepare and aliquot rehydration solution and ACN (5 mL each) in separate solution basins.

15. Aspirate the destain solution, add 90 µL ACN to the gel pieces, seal plate with mat, and invert manually several times. Let it stand at RT until the gel pieces are white (1–2 min).

16. Remove ACN and add 90 µL of rehydration solution, seal the plate, and manually invert several times. Incubate at 4 °C until the gel pieces have swelled and are translucent (<5 min).

17. Aspirate rehydration solution and perform another round of dehydration–rehydration.

18. Aspirate rehydration solution and perform a final dehydration with ACN. Aspirate the ACN and let residual evaporate.

19. While ACN is evaporating, prepare the trypsin solution. Add 20 µL of trypsin solution to the dried gel pieces and incubate at RT until the gel pieces swell and become translucent (~5 min).

20. Add ~30 µL of rehydration solution to the gel pieces (use enough volume to fully cover the gel pieces). Seal the plate and incubate overnight at 37 °C.

3.5.3   Day 3

1. In the morning, add 30 µL 1 % FA to each sample and incubate for 4 h at RT.

2. **CRITICAL STEP**: Transfer the extracted peptides to separate microfuge tubes. Ensure no gel pieces are transferred to the microfuge tubes. Keep extracted peptides at 4 °C.

3. Add 30 µL of 50 % ACN–0.5 % FA solution to the gel pieces. Incubate for 2 h at RT.

4. Combine the second extraction with each respective first extraction.

5. To further normalize the amount of peptides per fraction, pool peptides from neighboring gel regions that were minimally stained (*see* **Note 23**).

6. Concentrate the samples (to ~ 25 µL) by vacuum centrifugation to remove ACN.

7. Proceed to peptide desalting procedure (Subheading 3.6).

3.6 Peptide Desalting Using StageTips

1. Prepare one StageTip for each sample by using a 16 G needle to cut and deposit a single Empore SDB-RPS disk into the bottom of a 200 μL pipette tip using the syringe plunger (*see* **Note 24**).

2. Using 10 % TFA stock, acidify samples to final concentration of 1 % TFA.

3. Apply the sample to the top of the StageTip (*see* **Note 25**) and centrifuge at $1000 \times g$ until all solution has passed through the StageTip (*see* **Note 26**).

4. Wash disk with 100 μL of 0.2 % TFA.

5. Apply 50 μL of Elution buffer to the StageTip and manually collect the eluate in an autosampler vial.

6. Concentrate samples by vacuum centrifugation to near-dryness.

7. Add FA solution to achieve a final volume of 9 μL. Vortex briefly to mix.

8. Proceed immediately to nLC-MS/MS analysis (Subheading 3.7) or store at −80 °C for future analysis.

3.7 Nanoliquid Chromatography-Tandem Mass Spectrometry Analysis

Many HPLC and MS system configurations are suitable for analyzing tryptic peptide digests obtained from in-gel digestion. Here, we present an approach that is tailored for the acquisition of label-free spectral counting data, since we have effectively implemented this label-free approach [24] for determining the specificity of interactions for SIRT4 and for other proteins [15, 22, 23]. Towards this goal, the instrumentation and associated method should be optimized for the acquisition rate of MS/MS spectra, while also balancing the sensitivity and the depth of analysis, improving the detection of low abundance interactions. An LC system capable of low flow rates (<0.5 μL/min) and high pressure support (>400 bar) is highly preferable, as these capabilities allow the use of analytical columns with inner diameters ≤75 μm and lengths ≥ 25 cm. Additionally, a high sensitivity detector for MS/MS acquisition is encouraged. For example, in our study of SIRT4 interactions [15], we used a Dionex Ultimate 3000 nanoliquid chromatography system directly coupled to an LTQ Orbitrap Velos mass spectrometer.

1. Ensure that the system is properly calibrated according to the manufacturer's specifications.

2. Using MS instrument software, create an appropriate acquisition method (*see* **Note 27**).

3. Using the LC instrument software, create a reverse-phase LC method. Program the method to separate peptides over 90 min using a linear gradient of 4– 40 % mobile phase B.

4. Create a shorter length (e.g., 30 min) gradient method to use for analysis of a standard sample that serves as a quality control for instrument performance before, during, and after the analysis of the SIRT4 samples. A typical quality control sample would be a tryptic digest of a single protein (e.g., albumin) or whole cell lysates (e.g., HeLa).

5. Perform duplicate (at a minimum) injections of the standard sample to ensure that the system is performing at an acceptable level prior to injecting experimental samples.

6. For experimental samples, inject 4 μL of each fraction using the appropriate LC-MS/MS method designed above.

7. After an experimental set of injections has been complete, inject the standard peptide mixture to confirm that instrument performance has been maintained throughout the analysis.

3.8 Data Analysis and Interpretation

3.8.1 Peptide Identification and Protein Assignment

1. Extract all MS/MS spectra from raw mass spectrometry data, removing MS/MS spectra that do not contain at least ten peaks.

2. Create instrument and experiment-specific database search methods.

   (a) Define static peptide modification for cysteine carbamidomethylation.

   (b) Define variable modification for methionine oxidation (*see* **Note 28**).

3. Submit spectra to an appropriate workflow to obtain peptide spectrum matches and protein group assignments (*see* **Note 29**).

4. Select peptide and protein scoring filters to achieve a desired false discovery rate (e.g., ≤1 %).

5. Export data tables for interaction specificity analysis using the SAINT algorithm. The tables should contain, at minimum, protein group descriptions with respective accession numbers and total spectrum counts.

3.8.2 SAINT Interaction Specificity Analysis Using Label-free Spectral Counting

1. Register for a free user account at the website, www.crapome. org (*see* **Note 30**).

2. Select "Workflow 3: Analyze Your Data".

3. OPTIONAL: Select additional negative controls from the CRAPOME database (*see* **Note 31**).

4. Using the exported data tables (*see* above), generate a compatible SAINT matrix input file, as specified in the workflow step 2, Upload Data.

5. Upload SAINT matrix file and proceed to step 3, Data Analysis.

6. Under the "Analysis Options", enable "Probability Score", choose the "SAINT" model, and increase the "n-iter" option to 10,000 (*see* **Note 32**). Run Analysis.

7. After the analysis has completed, save and open the output file, which reports the individual and average SAINT scores (AvgP) for each identified protein. SAINT scores range from 0 (lowest probability of specific interaction) to 1 (highest probability of specific interaction).

8. Evaluate the performance of SAINT to distinguish between specific interactions and nonspecific background. If many interactions are known for a particular protein of interest, the sensitivity and specificity of the analysis can be estimated by constructing ROC plots. If no prior interaction knowledge is available, then construct a histogram for the distribution of SAINT scores. Use these analyses to select a SAINT score cut-off that eliminates the majority of nonspecific interactions (false positives), while retaining the highest scoring interactions (*see* **Note 33**).

3.9 Conclusion

The protocol detailed above provides a method for isolating SIRT4 and identifying its protein interactions in the mitochondria of human fibroblasts (Fig. 1a). The approach to generate cell lines stably expressing SIRT4-EGFP using retroviral transduction is also amenable for other cell types. By performing an upstream biochemical organelle fractionation (Fig. 1b) to obtain enriched mitochondria, a major source of potential nonspecific interactions from abundant cytoplasmic and nuclear proteins during immunoaffinity isolation is reduced (Fig. 1c). This overall approach was proven effective for the identification of SIRT4 interactions and has led to the discovery of SIRT4 enzymatic delipoylase activity [15]. In addition to the identification of candidate SIRT4 substrates, the resulting interaction datasets can contain novel information about proteins that may regulate the diverse enzymatic activities of SIRT4, including its ability to remove lipoyl, biotinyl, and acetyl modifications. Furthermore, such studies can reveal protein complexes that may facilitate the targeting of SIRT4 to its substrates. New hypotheses can be generated by analyzing candidate interactions using bioinformatics and functional pathway enrichment analyses. Different bioinformatics platforms can be used for this purpose and, while the description of these computational approaches is outside the scope of this chapter, we point the reader to data workflows used in several protein interaction studies [15, 22, 23, 26, 27]. As the knowledge regarding the substrates and functions of SIRT4 still remains limited, we hope that this protocol will aid future studies to better understand its contribution to diverse metabolic pathways in health and disease states.

4   Notes

1. For some cell types, polybrene (4–8 µg/mL) can be used to enhance gene transfer efficiency, however, this is usually toxic to MRC5 cells.

2. Prior to selection, a G418 killing curve should be optimized to determine appropriate concentration of G418 required to kill non-transduced cells.

3. Un-transduced cells will begin to lift and undergo cell lysis, leading to the medium becoming cloudy with cellular debris. Replace medium daily, and continue to select in G418-containing medium until cells stop lifting, and begin to expand (usually within 6 days). As an alternative, or in addition to G418, FACS may also be used to select for transfected cells which will express EGFP.

4. MRC5 cells cultured in G418 will have reduced proliferation and can acquire enlarged morphology. Two to three passages in regular medium are usually required for them to recover and stabilize.

5. Unless otherwise indicated, all steps should be performed at room temperature. Round-bottom tubes are the preferred tube shape, which minimizes bead trapping during the conjugation. The required amount of beads is dependent on both the experimental objective and the abundance of the protein to be immunoaffinity purified. As an approximate guide, 1–2 mg beads are appropriate for small-scale optimization experiments, 5–7 mg beads are usually sufficient for single immunoaffinity purifications, and 10–20 mg beads may be required for proteins of high abundance. During the washing steps, proceed immediately from one wash step to the next and do not allow the beads to sit without a washing solution between each step.

6. For efficient conjugation, tubes should rotate end-over-end (not a lateral rocking). Also, ensure the beads remain wet during this rotation. A minimum conjugation volume of 200 µl is recommended, which equates to a minimum of 10 mg beads used for conjugation. This is achieved in the protocol by performing at least two affinity enrichments (7 mg beads each for EGFP IP and SIRT4-EGFP IP).

7. Bead washes with the acidic (glycine) and basic (trimethylamine) solutions should be performed quickly (labeled as "FAST"), so as not to denature the antibody. To perform a FAST wash, the beads should only be pipetted in these solutions enough to disperse the pellet, which can usually be achieved by adding the solution directly over the pellet, followed by one additional rinse (if necessary). The beads should be immediately placed on the magnet, then when the solution

turns clear, the wash buffer should be aspirated and the subsequent neutralization buffer added to the tube.

8. For each plate of cells, prepare one pressure filter apparatus containing two filters. Usually each device will efficiently lyse 1 mL of cell suspension after 3 rounds. After use, the filters are disposed; however, the swinneys can be reused following short treatment with bleach, and extensive washing with water.

9. 1 mL of homogenization buffer per plate of cells is recommended.

10. The efficiency of each round of lysis can be observed by viewing a few microliters of the cell lysis suspension under the microscope to determine percentage of cell lysis (cell free nuclei). Continue until 70–90% of cells have been lysed, which is expected to take approximately three rounds with MRC5 cells.

11. Samples in this protocol have been top-loaded; we have found that alternatives, such as bottom-loading, are not as reproducible. When preparing the gradient, carefully layer the fractions on top of each other to minimize the mixing of the solutions. This can be done by angling the pipette tip towards the wall of the tube, and dispensing solutions gently as the pipette moves upwards out of the tube.

12. To estimate the density of specific fractions, fractions can be collected from the empty OptiPrep gradient that was used as a balance. These fractions can then be diluted 10,000-fold with water, and the absorbance can be measured at 244 nm.

13. Location of mitochondria on gradient can be evaluated by western blotting across all 6 fractions for mitochondrial markers COXIV, SIRT4, or EGFP (for tagged SIRT4). Mitochondria should be enriched in fractions 3 and 4. These fractions can be pooled and washed together to increase yield of mitochondria. Purity estimations can be performed simultaneously, by probing fractions with markers for other organelles such as endosome (EEA1), endoplasmic reticulum (calreticulin), Golgi (GM130), and lysosome (LAMP1).

14. Longer incubation times tend to promote the accumulation of nonspecific binders and the loss of weak interacting partners [25].

15. We have found that when using high affinity antibodies (e.g., anti-GFP) stringent heat and detergent denaturing conditions are sometimes required for efficient elution of the target proteins from the beads.

16. If improved resolution of proteins in the lower molecular weight region is desired, use the 20× MES running buffer instead of the 20× MOPS running buffer.

17. Since the total volume of primary eluates is ~45 μL, each sample is loaded in two separate additions (30 and 15 μL).

18. If it is known that the sample complexity or protein load is high, then resolving proteins for entire gel length may be beneficial.

19. If necessary, the stained gel can be stored in ultrapure water at 4 °C, wrapped tightly, up to several weeks until proceeding to in-gel digestion, though some sample loss may occur.

20. The total number of gel slice fractions per lane will vary slightly depending on the number of distinct bands and the resolving distance. Working solution volumes should be adjusted accordingly.

21. When using a 96-well plate, do not place more than 4×1 mm gel slices per well.

22. Do not mince gel slices as this increases the likelihood of transferring them during extraction.

23. For the study of SIRT4 interactions, after pooling of neighboring gel fractions, four fractions were subjected to LC-MS/MS analysis. For samples with more complexity or greater amount of co-isolated proteins, increasing the number of fractions may be beneficial. Alternatively, if an appropriate HPLC system is available, longer reverse phase separation gradients can be used.

24. Ensure that the disk makes contact with the walls of the pipette tip and is located a few mm above the tapered end of the tip. Each disk can bind up to ~20 µg of peptides. Though rarely necessary for in-gel digestion, if greater capacity is required, an additional disk can be layered in the same StageTip to increase binding capacity. The number of washes should be increased, equal to the total number of disks.

25. In contrast to C_{18} Empore disks, SDB-RPS Empore disks do not require activation with organic solvents.

26. Binding and washing of peptides over StageTips can be performed manually by applying pressure with a small plastic syringe or by centrifugation of the StageTip in a collection tube with an adapter. Independent of method, it is important that the peptide binding is done slowly (~25 µL / min). For the centrifugation method, usually a speed of ~$1000 \times g$ is appropriate for sample binding and $2000 \times g$ for washing.

27. Several considerations are required when designing an LC-MS/MS method, many of which are instrument-specific. However, in general the MS acquisition cycle should be designed based on the performance characteristics of the LC system. It is critical that the MS cycle time, determined largely by the number of full and tandem MS scans, permits acquisition of multiple full scans over the average LC elution peak.

For example, given LC peak widths of 15–30 s, an optimal time for a single acquisition cycle would be in the range of 2–4 s for data-dependent methods.

28. Other variable modifications may be included in the primary database search, such as phosphorylation, acetylation, or deamidation. However, as addition of modifications increases both search time and space, it is recommended to include only those modifications that are a frequent occurrence, e.g., > 5 % relative to the total number of identified peptides.

29. When selecting an analysis workflow, ensure that it incorporates the ability to control for false positive sequence matches, e.g., by performing database searching against reversed protein sequences to estimate false discovery rates. If available, it is highly recommended to use a software platform that also controls false identification rates at the protein level.

30. An alternative to the online SAINT algorithm is to download the latest version of the SAINT source files (www.source-forge.com) and compile it for your appropriate operating system. This strategy allows the SAINT algorithm to be run locally in the command-line, but requires additional computational knowledge. For a more detailed description of the underlying SAINT algorithm and its associated parameters *see* Ref. 28.

31. To compute meaningful SAINT specificity scores at least two biological replicates of the experimental and control isolations are required. Ideally, control isolations are "user" controls performed in parallel to the experimental samples; however, user controls can be replaced and/or supplemented with negative control data from the CRAPOME database [29] to provide additional stringency. These datasets are easily added when using the online SAINT workflow #3.

32. Several user-defined options are available when running SAINT. A thorough discussion of their recommended usage can be found in [28].

33. For examples of ROC curves and histogram distributions that illustrate the distribution of SAINT scoring *see* Refs. 22 and 23.

Acknowledgements

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