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Generating Mammalian Sirtuin Tools for Protein-Interaction Analysis

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Abstract

The sirtuins are a family of NAD⁺-dependent deacylases with important effects on aging, cancer, and metabolism. Sirtuins exert their biological effects by catalyzing deacetylation and/or deacylation reactions in which Acyl groups are removed from lysine residues of specific proteins. A current challenge is to identify specific sirtuin target proteins against the high background of acetylated proteins recently identified by proteomic surveys. New evidence indicates that *bona fide* sirtuin substrate proteins form stable physical associations with their sirtuin regulator. Therefore, identification of sirtuin interacting proteins could be a useful aid in focusing the search for substrates. Described here is a method for identifying sirtuin protein interactors. Employing basic techniques of molecular cloning and immunochemistry, the method describes the generation of mammalian sirtuin protein expression plasmids and their use to overexpress and immunoprecipitate sirtuins with their interacting partners. Also described is the use of the Database for Annotation, Visualization, and Integrated Discovery for interpreting the sirtuin protein-interaction data obtained.

Keywords

Sirtuin; SIRT3; Deacetylation; Protein–protein interaction; Immunoprecipitation; DAVID

1 Introduction

Sirtuins are a family of NAD⁺-dependent deacylases that regulate protein function by removing acetyl and/or acyl modifications from lysine residues of proteins. This activity has critical regulatory roles in aging, cancer, and metabolism. Originally identified as an activity occurring uniquely with histones, reversible protein acetylation is now considered a widespread regulatory mechanism that controls the function of several nonhistone proteins. For example, recent proteomics studies reveal widespread acetylation of mitochondrial proteins with greater than 30 % of all mitochondrial proteins acetylated on at least one lysine residue [1]. Although the mechanism of mitochondrial protein acetylation remains unclear, deacetylation is well characterized and identification of sirtuin substrates is a common approach for understanding how reversible acetylation modulates mitochondrial function. Although hundreds of acetyllysine residues have been identified, in only a few cases have proteins targeted by SIRT3, the most studied mitochondrial sirtuin, been identified; acetyl-CoA synthetase (AceCS2) long-chain acyl-CoA dehydrogenase (LCAD), 3-hydroxy,3-methylglutaryl-CoA synthase (HMGCS2), and glutamate dehydrogenase (GDH) are examples (for a review, see ref. 1). These studies show that these validated SIRT3 substrates, as well as several other substrates identified to date, physically associate with SIRT3. Therefore, identification of sirtuin interacting partners represents one approach to focus the search for substrates. Described here is a method for identifying sirtuin interacting

partners and for interpreting this information. Employing basic techniques of molecular cloning and immunochemistry [2, 3], the method describes the generation of mammalian sirtuin expression plasmids and their use to overexpress and immunoprecipitate sirtuins with their interacting partners. The interacting proteins are identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to create a list of potential sirtuin targets. The list is then analyzed with bioinformatics approaches such as DAVID to map the identified interactors to an associated biological annotation such as a pathway or disease [4, 5]. Presented is a list of SIRT3 interactors identified from HEK293T cells using this method and analyzed with DAVID. We anticipate that by varying the sirtuin, the cell type used for overexpression and the cell culture conditions, unique data sets can be obtained and novel sirtuin substrates discovered. In this way, the method can be used to expand the scope of sirtuin substrate identification and provide a deeper understanding into sirtuin biology.

2 Materials

2.1 Generating Sirtuin Mammalian Expression Plasmids

1. Sirtuin cDNA (Addgene, Cambridge, MA, USA or Open Biosystems, Huntsville, AL, USA).
2. Custom-made PCR primers.
3. Taq DNA Polymerase, such as Platinum High Fidelity.
4. pcDNA3.1 plasmid (Invitrogen Life Technologies).
5. Plasmid Maxi Kit.

2.2 Transfection

1. HEK293T cells.
2. Cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) with 4,500 mg/L glucose, L-glutamine, and sodium bicarbonate, containing 10 % fetal bovine serum.
3. Lipofectamine LTX Plus.
4. Cell culture PBS without calcium, without magnesium.
5. NP-40 Lysis buffer: 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 % NP-40, 2 mM EGTA, cocktail of protease inhibitors.
6. Bicinchoninic acid (BCA) Kit for determining protein concentration.
7. Monoclonal ANTI-FLAG M2 antibody.
8. Tris-buffered saline (TBS): 150 mM NaCl, 3 mM KCl, 25 mM Tris base, pH adjusted to 8.0 with HCl.

2.3 Immunoprecipitation

1. Anti-FLAG M2 Affinity Gel.
2. TBS: 150 mM NaCl, 3 mM KCl, 25 mM Tris base, pH adjusted to 8.0 with HCl.
3. 3× FLAG Peptide.

3 Methods

3.1 Prepare Sirtuin Mammalian Expression Plasmids

1. Obtain sirtuin cDNA that encodes the entire sirtuin coding region (*see* Note 1).

2. Design primers for introducing an in-frame FLAG affinity tag (peptide sequence DYKDDDKD) at the sirtuin N- or C-terminus by PCR (*see* Note 2). Design these primers so they also introduce appropriate restriction enzyme sites at each end of the amplified PCR product for subsequent sub-cloning into the mammalian expression plasmid. This process uses primers with additional 5' restriction enzyme or FLAG sequence not complementary to the target sirtuin. The extensions are added to the new product sequence during PCR. This process is illustrated in Fig. 1 with primers used to introduce a FLAG tag at the C-terminus of human *SIRT3*.
3. Perform PCR using the sirtuin cDNA (Subheading 3.1, **step 1**) as template, the mutagenic primers (Subheading 3.1, **step 2**), and Platinum Taq DNA Polymerase High Fidelity following the cycling recommendations of the manufacturer (*see* Note 3).
4. Check the PCR fragment size on an agarose gel and sequence the entire PCR fragment to confirm that correct changes have been introduced and that unintended mutations are not present.
5. Follow standard molecular biology techniques for cloning the restriction enzyme-digested PCR product into the pcDNA3.1 mammalian expression vector (*see* Note 4).
6. Purify the sirtuin-pcDNA3.1 plasmid on a large scale with Qiagen Plasmid Maxi Kit following the manufacturer's instructions. In parallel purify empty pcDNA3.1 plasmid to use as a control. The resulting DNA is of suitable quality for transfection into cell lines (*see* Note 5).

3.2 Transfect Mammalian Cells

1. Culture HEK293T cells in cell culture medium (*see* Note 6). In preparation for transfection grow the cells to near-confluence.
2. Transfect HEK293T cells with the purified sirtuin-pcDNA3.1 plasmid using Lipofectamin LTX Plus reagent following the manufacturer's instructions. Optimize the concentrations of Lipofectamin reagent and DNA as described by the manufacturer since the most effective concentrations will vary depending on the HEK293T cell passage number and history. Perform the optimization step on a small-scale in 6-well dishes plated with near-confluent HEK293T cells.
3. Control cells receive Lipofectamin LTX Plus reagent and empty pcDNA3.1 plasmid.

¹The more commonly studied human and mouse sirtuin cDNAs can be obtained through plasmid repository services such as Addgene and Open Biosystems.

²The FLAG tag should be introduced at the C-terminus of the mitochondrial sirtuins, SIRT3-5. Like many mitochondrial proteins, SIRT3-5 are synthesized as larger polypeptides carrying N-terminal mitochondrial localization pre-sequences that are cleaved by peptidases once the precursor has reached its final destination within the mitochondria. An affinity tag positioned at the N-terminus will thus be removed at this cleavage step. The affinity tag should therefore be placed at the C-terminus. Alternatively, affinity tags can be introduced at the N-terminus of truncated versions of the mitochondrial sirtuins that lack the N-terminal pre-sequence. Nuclear and cytosolic sirtuins may have affinity tags introduced at either the N- or C-terminus.

³A high fidelity polymerase with proofreading activity, such as Platinum Taq DNA Polymerase High Fidelity should be used to avoid introduction of unwanted mutations within the sirtuin coding sequence during PCR.

⁴The vector pcDNA3.1 works well for high-level sirtuin expression in mammalian cells. However, other commercially available mammalian expression vectors work well also.

⁵DNA plasmid prepared by some methods is toxic to mammalian cells and does not transfect well. Methods using phenol at any step should be avoided.

⁶HEK293T are human cells and recommended because they grow quickly, are easy to care for, and transfect well. They can be purchased from ATCC who provides detailed instructions for maintaining the cells in culture. However, other cell lines, challenged in various ways, may be desirable depending on the specific research goals and can be substituted in this step.

4. Culture cells at 37 °C for 24 h.
5. All remaining steps are completed with the sirtuin-pcDNA3.1 and empty pcDNA3.1 plasmid samples performed in parallel.
6. Prepare HEK293T cell extract and verify expression of FLAG-sirtuin protein using the following steps below.
7. Remove culture media from 6-well plate.
8. Wash cells 2× with ice-cold cell culture PBS.
9. Mechanically scrape attached cells into 200 µL of ice-cold NP-40 lysis buffer (*see* Note 7).
10. Transfer to a chilled 1.5 mL Eppendorf tube and incubate 30 min on ice.
11. Vortex, then homogenize with glass Dounce homogenizer (five slow passes up and down).
12. Centrifuge 14,000×*g*, 10 min at 4 °C.
13. Discard pellet.
14. Quantify the protein concentration in the supernatant (cell extract) with the BCA assay.
15. Run 50 µg protein on a 10 % SDS polyacrylamide gel.
16. Before dye front runs off the gel stop electrophoresis and transfer proteins to membrane for immunoblotting with Sigma M2 FLAG antibody at 1:1,000.

3.3 Immunoprecipitate FLAG-Sirtuin with Interactors

1. Using transfection conditions from Subheading 3.2 that yield a FLAG-sirtuin protein signal by immunoblotting, scale-up the transfection reaction.
2. Calculate the amount of LF reagent and DNA to scale-up from the 6-well to a p150 dish of cells (a 15-fold increase).
3. Transfect four p150 dishes with the scaled-up amounts of LF reagent and FLAG-sirtuin or empty plasmid DNA.
4. Incubate 24 h at 37 °C.
5. Prepare cell extracts by removing culture medium from each p150 dish of cells.
6. Wash each dish 2× with 10 mL ice-cold PBS.
7. Mechanically scrape cells from each dish into 2 mL ice-cold NP-40 lysis buffer.
8. Pool scraped cells from the 4 dishes into one 15 mL conical tube and incubate on ice 30 min.
9. Vortex and then homogenize with glass Dounce homogenizer (five slow passes up and down).
10. Centrifuge 7,000×*g* for 20 min at 4 °C.
11. Discard pellet.

⁷The use of strong detergents such as SDS and sodium deoxycholate denature the FLAG antibody during the immunoprecipitation step and should be avoided. A full list of chemicals incompatible with FLAG resin is available from the manufacturer.

12. Retain supernatant (cell extract) for immunoprecipitation.
 13. Prepare FLAG resin for immunoprecipitation. For each set of 4 p150 dishes worth of cell extract, remove 160 μ L of FLAG resin from the 50 % glycerol stock bottle.
 14. Pellet the resin by centrifugation for 2 min at $2,000 \times g$, discard supernatant.
 15. Resuspend resin in 1 mL TBS.
 16. Centrifuge for 2 min at $2,000 \times g$ and discard supernatant.
 17. Repeat wash step one additional time (*see* Note 8).
 18. Set up immunoprecipitation. Add washed, TBS-equilibrated resin to pooled cell extract in 15 mL conical tube and rotate end-to-end at 4 °C overnight.
 19. Wash FLAG resin with NP-40 lysis buffer (*see* Note 9). After overnight incubation, pellet resin by centrifugation at 4 °C for 2 min at $2,000 \times g$.
 20. Discard supernatant and wash resin by rotating tube in 10 mL NP-40 lysis buffer for 10 min.
 21. Pellet resin as before and discard supernatant.
 22. Repeat washing step with lysis buffer 2 $\times$ more.
 23. Final FLAG resin wash. To the FLAG resin pellet from above, add 10 mL ice-cold TBS to 15 mL conical tube.
 24. Rotate end-to-end at 4 °C for 10 min.
 25. Remove 1 mL for elution step (*see* Note 10).
 26. Pellet resin from remaining 9 mL and discard supernatant.
 27. Add TBS to barely submerge resin pellet.
 28. Store at -20 °C until digestion with trypsin.
 29. Elute FLAG-sirtuin protein from FLAG resin to confirm success of the FLAG-sirtuin immunoprecipitation.
- Pellet the 1 mL FLAG resin reserved from above and discard supernatant.
- Resuspend in 100 μ L 3 $\times$ FLAG peptide at 300 μ g/mL.
- Incubate at room temperature for 15 min, occasionally flicking tube to mix.
- Pellet resin at $2,000 \times g$ for 2 min.
- Remove supernatant with 1 mL syringe fitted with 31 gauge needle to avoid picking up resin (*see* Note 11).
- Repeat elution step 1 $\times$ and pool.
- Run 20 μ L on 10 % SDS polyacrylamide gel.

⁸Avoid centrifugation of FLAG resin above $2,000 \times g$. Higher force may damage the resin and FLAG antibody.

⁹The detergent and salt present in the NP-40 lysis buffer make it a good buffer for washing FLAG resin.

¹⁰FLAG-sirtuin protein is eluted from a small aliquot of resin for analysis by immunoblotting. This is performed to verify that immunoprecipitation has worked.

¹¹It is necessary to avoid contamination of eluted protein with FLAG resin. The FLAG antibody attached to the resin is released during preparation of sample for immunoblotting and produces high background signal through interaction with the anti-mouse secondary antibody.

Before dye front runs off transfer protein to membrane and immunoblot with FLAG M2 antibody diluted 1:1,000; Fig. 2 shows eluted hSIRT3-FLAG protein that had been overexpressed and immunoprecipitated from HEK293T cells.

3.4 Identify Immunoprecipitated Sirtuin Interactors by Mass Spectrometry

1. Transfer the FLAG resin from above, containing the immuno precipitated FLAG-sirtuin or empty vector, to a mass spectrometry lab for trypsin digestion and identification of sirtuin interacting proteins by mass spectrometry analysis.

3.5 Filter Sirtuin Interactor Data in Preparation for Bioinformatics Analysis

1. Remove background proteins from list of interactors. Protein hits that are not significant as determined by mass spectrometry analysis in Subheading 3.4 and proteins used as controls for mass spectrometry should not be used for subsequent bioinformatics analysis (*see* Fig. 3).
2. Filter out non-mitochondrial proteins (*see* Note 12). Utilizing the Human MitoCarta dataset published by the Broad Institute [6], remove proteins that are not localized to the mitochondria.

3.6 Bioinformatics Analysis

1. Go to the DAVID website (<http://david.abcc.ncifcrf.gov>) for pathway analysis (*see* Note 13).
2. Upload the gene names of the proteins identified in Subheading 3.5 to DAVID and select “Official Gene Symbol” as an identifier. Select *Homo sapiens* as the species for both the gene list and background.
3. Examine the gene ontology terms (cellular component, biological process, and molecular function) to verify that the gene list is specific for protein products found in the mitochondria.
4. Identify diseases associated with the genes listed by accessing the OMIM (Online Mendelian Inheritance in Man) database.
5. Identify pathways that include genes from the uploaded inter-actor data. Kegg pathway analysis provides a list of pathways that contain a number of genes from your list that are statistically significant.
6. Synthesize a list of proteins of interest identified by DAVID analyzes and follow up with studies to verify interaction with the sirtuin and investigate the regulatory role of reversible deacetylation of the substrate.

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¹²Other criteria can be used to filter the interactor data for subsequent bioinformatic analysis according to your specific research goals.

¹³DAVID (database for annotation, visualization, and integrated discovery) is a free and publically available database developed at the National Institute of Allergy and Infectious Diseases, NIH. The methods included here are particularly relevant to our analysis. Please refer to references by Huang et al. for an introduction to DAVID analysis and a step-by-step procedure for using DAVID [4, 5].

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Fig. 1.

Addition of restriction enzyme and FLAG sequences to sirtuin by PCR. **(a)** General schematic of PCR reaction. The template is untagged sirtuin cDNA. Custom primers are designed that are complementary to the target sirtuin with additional 5' restriction enzyme or FLAG sequence extensions. The extensions are added to the new product sequence during PCR. FLAG-encoding, restriction enzyme (RE), and restriction enzyme flanking sequences (see Note 14) are color coded *blue*, *yellow*, and *red*, respectively. **(b)** Primer sequence for addition of EcoR1 and FLAG sequences to hSIRT3. The relevant sequence of untagged hSIRT3 template cDNA is shown (sequence encoding the first five amino acids of the protein, “MAFWG,” and the C-terminal 5 amino acids, “DGPDK,” plus the STOP codon). The forward primer contains an EcoR1 restriction site (gaattc, shown in *yellow*) and a region complementary to the antisense strand of SIRT3. The reverse primer has a region complementary to the sense strand of SIRT3 and encodes the FLAG peptide (DYKDDDDK, shown in *blue*) followed by a STOP codon. After the STOP codon is an EcoR1 site, shown in *yellow*. In *red* are restriction enzyme flanking nucleotides (see Note 14)

¹⁴Many restriction enzymes require at least three nucleotides flanking either end of their recognition sequence in order to cut efficiently.

Fig. 2.

Immunoprecipitation of hSIRT3-FLAG from HEK293T cells. Cells were transfected with hSIRT3-FLAG or with empty plasmid. Cell extracts were made, incubated with FLAG resin, and the FLAG proteins eluted with FLAG peptide for analysis by western blotting with FLAG antibody. The major band observed matches the predicted molecular weight (32 kDa) of the mitochondrial processed hSIRT3. A lesser amount of the cytosolic unprocessed form (MW 45 kDa) is also observed

Fig. 3.

Flowchart description of bioinformatic analysis. The list of sirtuin interactors is filtered to remove background, controls, and non-mitochondrial proteins. The list of genes associated with these proteins is uploaded into DAVID and analyzed by gene ontology terms, disease association, and pathway identification. Based on information gathered from multiple analyzes, a unique protein is identified as being regulated by a sirtuin and a hypothesis is generated for further investigation of the role of reversible deacetylation of the identified substrate