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Global Profiling of Sirtuin Deacylase Substrates Using Chemical Proteomic Strategy and Validation by Fluorescent Labeling

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Abstract

Protein fatty-acylation is an important post-translational modification (PTM) and has been associated with many fundamental biological processes. Sirtuins, the nicotinamide adenine dinucleotide (NAD) dependent class of histone deacetylases have been reported to possess lysine defatty-acylase activity. Comprehensive substrate profiling of sirtuins will help to establish the function of both protein lysine fatty acylation and its regulation by sirtuins. Here, we describe a chemical proteomic strategy to globally profile sirtuin defatty-acylation substrates and a fluorescent labeling method to validate sirtuin substrates.

Keywords

protein fatty acylation; sirtuin substrates; proteomic profiling; SILAC; fluorescent labeling

1. Introduction

Most of our current understanding of protein lipidation comes from studies of N-terminal glycine myristoylation (N-myristoylation), cysteine palmitoylation (s-palmitoylation), cysteine prenylation, and modifications by GPI anchors [1]. However, recently it has been reported that NAD^+-dependent sirtuins (SIRT) 1, 2, 3, 6 and 7, as well as the zinc-dependent histone deacetylase 8 (HDAC8) possess lysine defatty-acylase activity, suggesting that protein lysine fatty acylation is an understudied, but important PTM [2-5]. To date, the only mammalian proteins that have been identified to have lysine fatty acylation are tumor necrosis factor alpha (TNF-α), interleukin-1 alpha, lens integral membrane protein aquaporin-0, Ras Related Protein 2 (R-Ras2), K-Ras4a and H-Ras, and several members of the Rac family of small GTPases [3, 6-11]. Among these, TNF-α, R-Ras2, and K-Ras4a are all sirtuin defatty-acylation substrates [3, 6, 7]. Profiling the defatty-acylation substrates of sirtuins will not only help to elucidate the physiological function of sirtuins, but will also help to understand the role of protein lysine fatty acylation.

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Chemical proteomic technology has allowed the global profiling of fatty-acylated proteins, especially N-myristoylated and S-palmitoylated proteins, in cells and even animals [12, 13]. Fatty acid analogs functionalized with either an alkyne or azide group, such as Alk14 (Fig. 1A), can be metabolically incorporated into fatty-acylated proteins [14]. Utilizing bio-orthogonal click chemistry, a biotin tag can be introduced to Alk14-labeled proteins for selective enrichment of fatty-acylated proteins on streptavidin beads. Treatment of the immobilized proteins with hydroxylamine, which cleaves fatty acids linked to cysteine, further enriches proteins that are fatty acylated at lysine residues, that can then be identified through proteomic studies. To specifically identify sirtuin defatty-acylase substrates, we have used a quantitative proteomics technique, SILAC (stable isotope labeling with amino acids in cell culture), to identify fatty-acylated proteins in sirtuin wild type (WT) and knock down or knock out (KD/KO) cells (Figure 1A) [7]. If a protein is a substrate for the defatty-acylase activity of the sirtuin, then it should have more Alk14 labeling in the KD or KO cells. The use of SILAC mitigates sample preparation variation, such as click chemistry reaction efficiency, producing more reliable quantitative results. SILAC proteomics using alkyne fatty acid analogues can also be applied to cells treated with small molecules that inhibit defatty-acylase activity of sirtuins as an alternative approach to identifying substrates.

To validate candidate substrates identified using the proteomics method described above, we compare the fatty acylation level of a specific protein in sirtuin WT and KD/KO cells by in-gel fluorescence. The protein of interest is expressed or overexpressed in WT and sirtuin KD/KO cells treated with the Alk14 probe and then isolated by immunoprecipitation. An azide-tagged fluorophore can be attached by click chemistry and the sirtuin-dependent fatty acylation can be detected through in-gel fluorescence (Fig. 1B). Confirmation that the labeled protein is fatty acylated at lysine is assessed by hydroxylamine resistance of the in-gel fluorescence.

2. Materials

Prepare reagents using analytical grade reagents and ultrapure water from a MilliQ water purification system.

2.1 Cell culture and alkyne probe treatment

1. SILAC Heavy Labeling Medium: DMEM for SILAC (without L-lysine and L-arginine) (Thermo Fisher), 10% dialyzed fetal bovine serum (FBS), 100 μg/ml [$^{13}C_6$, $^{15}N_2$]-L-lysine and 100 μg/ml [$^{13}C_6$, $^{15}N_4$]-L-arginine (Sigma). *See* Note 1
2. SILAC Light Labeling Medium: DMEM, 10% dialyzed FBS, 100 μg/ml L-lysine and 100 μg/ml L-arginine.
3. 50 mM Alkyne probe in DMF or DMSO: Alk12 or Alk14 synthesized as previously reported [15], or purchased (Caymen Chemical). *See* Note 2

[1]. SILAC labeling medium is deficient in L-lysine and L-arginine, enabling supplementation with the corresponding amino acids with substituted stable isotopic nuclei (^{13}C, ^{15}N) to generate “heavy” medium. “Light” medium is supplemented is supplemented with natural amino acids. DMEM for SILAC is used in this protocol, but a variety of tissue culture media are available commercially.

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4. DMEM Culture medium: DMEM, 10% FBS
5. Trypsin-EDTA (0,05%)
6. Phosphate-buffered saline (PBS)
7. 1% Nonidet P-40 (NP40) lysis buffer: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40, protease inhibitor cocktail (Sigma Aldrich) (add just before use).
8. SDS Solubilization Buffer: 2% SDS and 50 μM EDTA in PBS
9. Brij Buffer: 1% (w/v) Brij97, 150 mM NaCl, 50 mM triethanolamine (pH 7.4).

2.2 Click Chemistry

1. 10 mM TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) (Sigma), in DMF or DMSO.
2. 5 mM Azide-PEG3-biotin conjugate (Sigma) in DMF or DMSO.
3. 40 mM $CuSO_4$ in H_2O.
4. 40 mM TCEP (tris(2-carboxyyethyl)phosphine) in H_2O.
5. 2 mM Tetramethylrhodamine (TAMRA) azide (Lumiprobe) in DMF or DMSO.
6. High capacity streptavidin agarose (Thermo Fisher).

2.3 Trypsin digestion and peptide purification

1. 0.2% SDS in PBS
2. 20 mM Tris-HCl, 500 mM KCL, pH 7.4
3. 20 mM Tris-HCl, pH 7.4
4. 6M urea, 9.5 mM TCEP in PBS
5. 400 mM iodoacetamide in water
6. 2M urea in PBS
7. 2M urea in PBS, 1 mM $CaCl_2$
8. Trypsin (Promega)
9. 10% trifluoracetic acid (TFA)
10. 90% methanol, 0.1% TFA
11. 0.1% TFA
12. 80% acetonitrile, 0.1% TFA
13. Sep-Pak Vac C18 cartridge (Waters).

[2]. To detect lysine fatty acylation Alk12 and Alk14 are typically used as these are the best mimics of lysine myristoylation and palmitoylation.

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2.4. Immunoprecipitation and fluorescent labeling

1. NP-40 immunoprecipitation wash buffer: 25mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.2% NP-40.
2. 6X Protein loading dye: 60mM Tris pH 6.8, 0.12 % SDS (w/v), 47% glycerol, 0.6 M DTT and 0.0006% bromophenol blue (w/v)
3. Coomassie blue stain: 10% acetic acid, 40% methanol, 50% H_2O, 0.25% Blue R250 Dye (Sigma Aldrich) (w/v)
4. Destaining solution: (v/v) 10% acetic acid, 40% methanol, 50% H_2O.

3. Methods

3.1 Cell culture and alkyne probe treatment for proteomic profiling

1. Culture sirtuin WT and KD/KO cells in SILAC Light Labeling Medium (medium with L-lysine and L-arginine) and SILAC Heavy Labeling Medium (medium with [$^{13}C_6$, $^{15}N_2$]-L-lysine and [$^{13}C_6$, $^{15}N_4$]-L-arginine) medium, respectively, for six generations (*see* Note 1, 3). To generate the heavy or light cell lines, culture cells in 6-well dishes, seeding 10×10^4 cells per well. Split the cells at 80% confluency.
2. After passing the cell lines for six generations, culture the cells in 10-cm dishes. Depending on the cell line being used, the experiment may require 5–10 dishes. When cell confluency reaches 80%, treat the cells with fatty acid alkyne probe (Alk12 or Alk14) at a final concentration of 50 μM for 6 h (*see* Note 4).
3. Harvest the cells by scraping and centrifuge for 5 min at 500 × *g* at 4˚C.
4. Wash the cells twice with 15 mL of ice-cold PBS. Centrifuge for 3 min at 500 × *g* at 4˚C. Remove the supernatant and retain the cell pellet.

3.2 Click chemistry in total cell lysates

1. Prepare ice-cold methanol, ice-cold chloroform, and ice-cold water before starting the reaction.
2. Lyse the cells with 1% NP-40 lysis buffer by adding 5 mL of lysis buffer supplemented with protease inhibitor cocktail and place on a nutating rocker for 30 min (*see* Notes 5, 6). After 30 min, remove insoluble material by centrifuging

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[3]. To increase confidence in the candidates identified in the proteomics analysis, perform the reverse SILAC experiment, incubating WT cells with SILAC Heavy Labeling Medium and KD/KO cells with SILAC Light Labeling Medium.

[4]. Alkyne probes at concentrations from 20 to 50 μM show no toxicity to cells; higher concentration might cause cell death. Normally 2–6 h treatment works well for most cells, longer incubation times may lead to metabolism of alkyne probes.

[5]. Always add a protease inhibitor cocktail into lysis buffer before lysing the cells (Ratio 1:40–1:100).

[6]. The choice of cell lysis buffer is based on experimental conditions and requirements. A buffer with 4% SDS (4% SDS, 50mM trieathanolamine pH 7.4, 150 mM NaCl) maximally solubilizes proteins but will also lyse the nuclei, so universal cell nuclease (Thermo) should be included in the buffer. The 1% NP-40 lysis buffer is gentler than 4% SDS, but may not solubilize all the hydrophobic proteins (e.g. integral membrane proteins). Vorinostat, or SAHA (suberoylanilide hydroxamic acid), a pan HDAC inhibitor and nicotinamide, a pan Sirtuin inhibitor, can be added to the lysis buffer to inhibit zinc-dependent histone deacetylase and sirtuins. This is to prevent deacylation reactions from happening after lysing the cells. Do not add EDTA to the lysis buffer, as it can chelate to copper making it incompatible with click chemistry.

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at 17, 000 × *g* at 4˚C for 30 min. Quantify the protein concentration of the whole cell lysate (*see* Note 7).

3. Mix equal amounts of heavy and light samples. Typically, 5–7.5 mg of each whole cell lysate is needed.

4. To set up the click chemistry reaction, add 10–15mg of the mixed lysate per tube and make sure the protein concentration is ~2 mg/mL. Add additional 1% NP-40 lysis buffer supplemented with protease inhibitor cocktail to bring the total reaction volume to 4.6 mL. Add 150 μL of 5 mM azide-biotin, followed by 100 μL of 10 mM TBTA, 100 μL of 40 mM TCEP and 100 μL of 40 mM $CuSO_4$. The final concentrations in the reaction should be: 200 μM TBTA, 0.8 mM $CuSO_4$, 0.8 mM TCEP, and 150 μM Azide-biotin. (*see* Note 8,9).

5. Incubate for 30 min at room temperature.

6. Precipitate the proteins with ice-cold methanol-chloroform-water (v/v ratio of sample:methanol:chloroform:water is 1:4:1:3) solution. For a 5-mL click chemistry reaction, add 20 mL of methanol, 5 mL of chloroform, and 15 mL of water.

7. Vortex the mixture, and then centrifuge at 4˚C, 17,000 × *g* for 15 min or 4,500 × *g* for 30 min.

8. Remove and discard the upper aqueous phase, leaving the protein layer and lower organic phase.

9. Add 20 mL of ice-cold methanol to the sample and mix gently, causing the protein pellet to sink to the bottom of the tube. Centrifuge at 4˚C, 17,000 × *g* for 10 min, or 4,500 × *g* for 20 min.

10. Remove the liquid by pipetting, and be careful not to disturb the pellet. Wash the protein pellet by adding 20 mL of ice-cold methanol and inverting the tube. Centrifuge at 4˚C for 10 min at 17,000 × *g* or at 4,500 × *g* for 20 min.

11. Remove the methanol by pipetting. Allow the remaining methanol to evaporate by letting the pellet air dry for about 10 min at room temperature. Do not let the protein pellet dry completely. If the protein pellet is allowed to dry completely, it will be difficult to solubilize the precipitated protein.

12. Add SDS Solubilization Buffer and vortex to solubilize the protein pellet (*see* Note 10).

13. Centrifuge for 5 min at 14,000 × *g* at room temperature, and collect the supernatant. Dilute the sample to a volume of 10 mL with PBS buffer to ensure that the final SDS concentration is 0.2%.

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7. If 4% SDS buffer is used as lysis buffer, the protein concentration should be determined using the BCA assay (Thermo). if 1% NP40 lysis buffer is used, protein concentration can be determined by either BCA assay or Bradford assay.
8. Click chemistry works best when the pH of the buffer is 8.
9. The protein concentration for the click chemistry reaction should not exceed 2 mg of lysate per 1 mL of reaction. Exceeding this concentration will hinder the efficiency of the reaction.
10. EDTA is added to chelate any residual copper, allowing the protein to be solubilized more easily.

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14. Wash 50 μL of high-capacity streptavidin agarose with 1 mL of PBS (or Brij buffer) three times, and then add all of the streptavidin beads to your sample. Place on a nutating rocker for 90 min at room temperature.

3.3 On-bead trypsin digestion

1. Centrifuge the beads for 2 min at 1,000 × *g*, room temperature. Remove the supernatant and wash the beads three times with 1 mL of 0.2% SDS in PBS, followed by three washes with 1 mL PBS, three washes with 1 mL of 20 mM Tris-HCl, 500 mM KCl, pH 7.4, and finally three washes with 1 mL of 20 mM Tris-HCl, pH 7.4.
2. Add 400 μL of a PBS solution containing 6 M urea and 9.5 mM TCEP to the beads. Incubate for 20 min at 37°C with gentle rotation.
3. Add 20 μL of a fresh solution of 400 mM iodoacetamide (dissolved in water) to the suspension of beads. Incubate for 20 min at 37°C with gentle rotation.
4. Remove the supernatant, and wash the beads with 1 mL of 2M urea in PBS, and then incubate the beads with 2 μg of trypsin in 200 μL of 2M urea in PBS with 1 mM $CaCl_2$ at 37 °C overnight with gentle rotation. (*see* Note 11) Prior to adding the trypsin to the sample, it should be activated for 15 min at 30˚C in the buffer provided by Promega.

3.4 Purify digested peptides using Sep-Pak Vac C18 cartridge

1. Following trypsin digestion, pellet the beads by centrifugation at 1000 × *g*, room temperature. Transfer the supernatant to a 1.5-mL tube. Wash the beads twice with 300 μL of water. Combine the washes with the supernatant. Dilute the solution to 1 mL by adding water, and adjust the pH to ~2 by adding 15 μL of 10% trifluoroacetic acid (TFA) (*see* Note 12).
2. Condition the Sep-Pak Vac C18 cartridge by passing 1 mL of 90% methanol/ 0.1% TFA through the cartridge three times. Make sure not to let the cartridge dry out.
3. Equilibrate the cartridge by passing 1 mL of 0.1% TFA through the cartridge three times.
4. Load the sample from step 1 slowly, at a rate of 1 drop/sec. To minimize loss, pass the sample through the cartridge three times. Wash the loaded cartridge with 1 mL of 0.1% TFA, three times.

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11. To ensure complete peptide digestion, use proper working concentrations of urea, TCEP, and iodoacetamide to denature, reduce, and alkylate proteins. The concentration of urea in the trypsin digestion solution should be less than 2M. In general, 2–4 μg of trypsin should be sufficient for the recommended whole cell lysate input. The sample should be incubated with trypsin for at least 8 hours to ensure complete digestion. $CaCl_2$ is added to the solution to maximize the activity of trypsin.

12. When using a Sep-Pak Vac C18 cartridge to purify digested peptides, make sure the pH of the digested peptide solution is 2–3 before loading the sample on the C18 cartridge. The sample should be loaded slowly, at a rate of approximately 1 drop/second. Solutions should be made fresh, and avoid skin contact to prevent the sample from being contaminated by keratin from skin.

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5. Elute the sample by passing 1 mL of 80% acetonitrile/0.1% TFA through the cartridge once. Dry the cartridge with nitrogen to ensure all peptides have been eluted.

6. Lyophilize the eluted sample, and analyze nano-LC-MS/MS.

3.5 Cell culture and alkyne probe treatment for fluorescence detection

1. Culture Sirtuin WT and KD/KO cells in DMEM Culture Medium. (*see* Note 13).

2. Add alkyne probe to the cells with a final concentration of 50 μM when the confluency reaches 80% and incubate with cells for 6 h (*see* Note 3).

3. Harvest the cells (either by scraping or by treating with trypsin-EDTA) and centrifuge for 5 min at 500 × *g*, 4˚C.

4. Wash the cells using ice-cold PBS. Centrifuge for 3 min at 500 × *g*, 4˚C. Discard the supernatant, saving the cell pellet.

3.6 Fluorescent labeling of target proteins

1. Lyse cells with 1% NP40 lysis buffer supplemented with phosphatase inhibitor cocktail, by adding the appropriate amount of buffer (300μL per 10-cm dish of HEK-239T cells) and placing on a nutating rocker for 30 min at 4˚C. After 30 min, clear the lysate by centrifugation at 17,000 × *g*, 4˚C for 20 min. After lysing the samples, quantify the protein concentration (*see* Note 14).

2. Dispense at least 500 μg-2000 μg of whole cell lysate for each sample into 1.5 mL of microcentrifuge tubes. Bring the volume to 400–1000 μL with 1% NP40 lysis buffer as above (*see* Note 15).

3. Carry out standard immunoprecipitation (IP) procedure for the protein of interest with agarose beads conjugated to the appropriate antibody. Wash the agarose beads at least three times with IP washing buffer.

4. Add 10–50 μL of washing buffer or PBS buffer to the pelleted agarose beads (*see* Note 8).

5. Add click chemistry reagents into the tubes such that the final concentrations in the reaction are: 200 μM TAMRA-azide, 600 μM TBTA, 2 mM CuSO4 and 2 mM TCEP.

6. Incubate for 30 min at room temperature.

7. Add 6X protein loading dye (final 2X) and heat the sample for 10 min at 95 ˚C to denature proteins.

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13. If the use of sirtuin inhibitors is desired, WT cells can be used and we recommend trying various concentrations and treatment times with the inhibitor. When the alkyne probe is added, it is recommended to add fresh media supplemented with the inhibitor and the alkyne probe.

14. Always add protease inhibitor cocktail into lysis buffer before lysing the cells (Ratio 1:40–1:100). Choose a lysis buffer compatible with the immunoprecipitation procedure to avoid denaturation of the antibody. Avoid EDTA in the lysis buffer, which is not compatible with click chemistry.

15. The protein amount used in this step depends on the abundance of target proteins, lower abundant target proteins need more total proteins (same principle as immunoprecipitation).

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8. Centrifuge 2 min at 2,000 × *g*.
9. Transfer the supernatant to another 1.5 mL microcentrifuge tube.
10. Divide the sample in half. To detect non-cysteine fatty acylation, add hydroxylamine (pH 7.4, final concentration 400 μM) to the sample and heat for 5 min at 95 ˚C. To detect all fatty acylation, including cysteine fatty acylation add water (final concentration 400 μM) and heat for 5 min at 95˚C.
11. Load protein samples onto SDS-PAGE gel and resolve by electrophoresis.
12. To reduce background signal, destain the gel by shaking for at least 2 h in destaining solution (*see* Note 16).
13. After destaining, change the destaining solution to water. Scan the gel using a fluorescence gel scanner, such as a Typhoon 9400 variable mode imager (GE Healthcare Life Science, Piscataway, NJ). The excitation and emission settings are determined by the fluorescent dye selected. For TAMRA-N_3 the excitation and emission wavelengths are 532/580 respectively.
14. Stain the gel with Coomassie blue staining solution to detect protein loading. Expected results for a SIRT6 target, the small GTPase R-Ras2 are presented in Fig. 2.

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16. Destaining for a longer time can significantly decrease background. If time allows, destain overnight at 4˚C.

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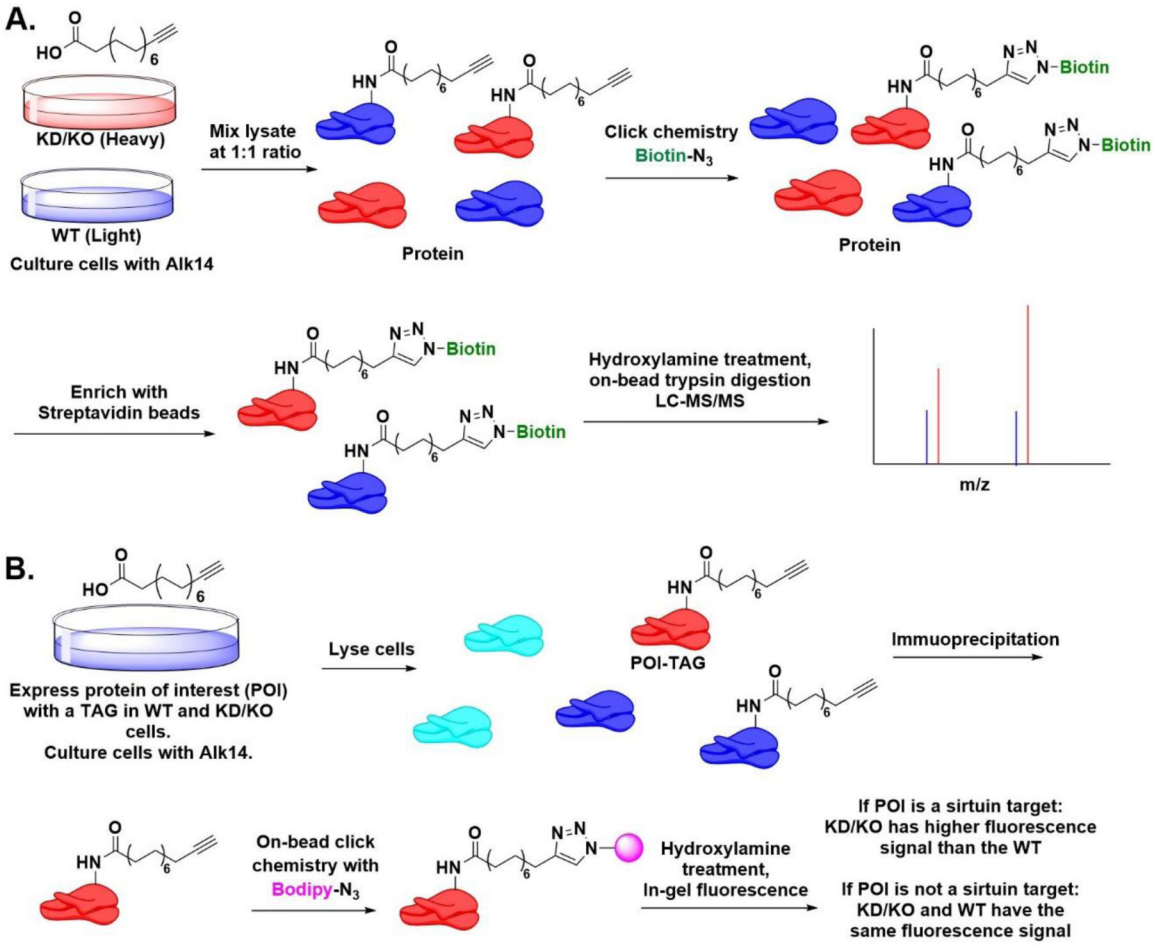

Fig. 1.

Workflows for (**A**) profiling of sirtuin deacylation substrates and (**B**) fluorescent labeling of fatty-acylated proteins.

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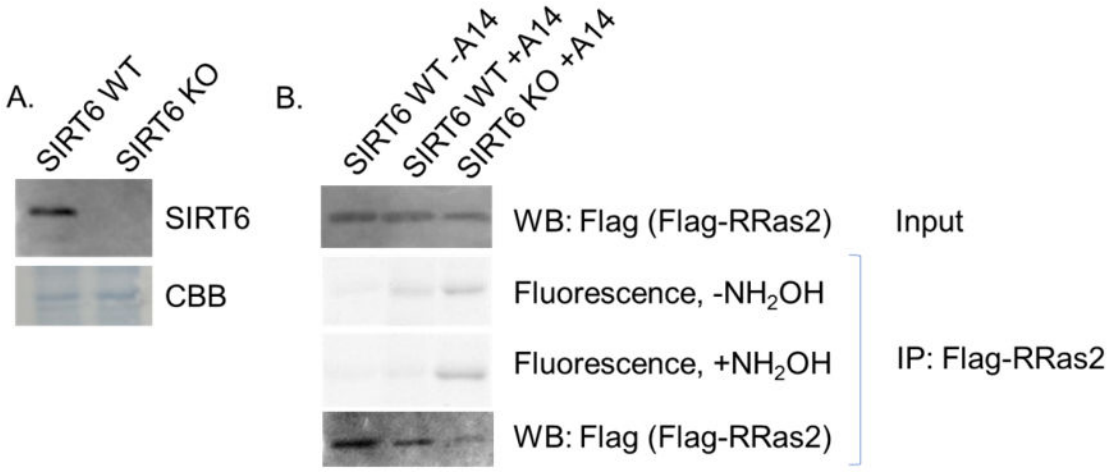

Fig. 2.

Typical results for the alkyne-tagged fatty acid labeling of sirtuin targets. (**A**) Western blot to confirm the status of SIRT6 in SIRT6 WT and KO mouse embryonic fibroblast cells (MEFs). (**B**) Fluorescent labeling of RRas-2 in SIRT6 WT and KO MEFs.