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Detection of the ubiquitinome in cells undergoing oncogene-induced senescence

Hengrui Zhu¹, Linh Le^{1,3}, Hsin-Yao Tang², David W. Speicher², and Rugang Zhang^{1,*}

¹Gene Expression and Regulation Program, The Wistar Institute, Philadelphia, PA 19104, U.S.A

²Molecular and Cellular Oncology Program and Proteomics Core, The Wistar Institute, Philadelphia, PA 19104, U.S.A

³Cell and Molecular Biology Graduate Group, Perelman School of Medicine of the University of Pennsylvania, Philadelphia PA 19104

Summary

Senescent cells exhibit dramatic changes in protein post-translational modifications. Here, we describe a method, stable isotope labeling with amino acids in cell culture (SILAC) coupled to liquid chromatography tandem mass spectrometry (LC-MS/MS), to identify changes in the ubiquitinome in cells that have undergone oncogene-induced senescence.

Keywords

Senescence; Post-translational modification; Ubiquitin; Ubiquitin-proteasome pathway; Ubiquitinome; Stable isotope labeling with amino acids in cell culture (SILAC); Liquid chromatography tandem mass spectrometry (LC-MS/MS)

1. Introduction

Cellular senescence is a state of stable cell growth arrest and is implicated in many biological processes, including embryogenesis (1), aging, tissue repair and tumor suppression (2, 3, 4). Cellular senescence can be triggered by many different stimuli, such as DNA damage, telomere shortening (5, 6), loss of tumor suppressor genes (7), and activation of oncogenes, termed oncogene-induced senescence (OIS). Activation of oncogenes such as RAS in primary mammalian cells induces cellular senescence and thus prevents primary cells from abnormal proliferation and transformation (8, 9). During OIS, senescent cells undergo profound changes in cellular morphology, chromatin structure, and global gene expression.

Post-transcriptional modifications (PTM) also play an important role in regulating gene expression and protein function during cellular senescence (10). Ubiquitination is a common post-translational modification critical for protein recognition by the ubiquitin-proteasome pathway, which can affect cellular protein levels and function by regulating protein

*Corresponding author: Rugang Zhang, Ph.D., Gene Expression and Regulation Program, The Wistar Institute, Room 312, 3601 Spruce Street, Philadelphia, PA 19104, rzhang@wistar.org.

degradation. The initial step of the pathway involves the covalent addition of one (monoubiquitination) or a chain of several ubiquitin molecules (polyubiquitination) to a substrate protein (11, 12). Ubiquitination is carried out by 3 types of enzymes – E1, E2, and E3 ligases – which consecutively activate, transfer and covalently link ubiquitin to lysine residues in a substrate protein (13, 14, 15). Recent studies have shown that the E3 ligase SPOP is upregulated in OIS (16, 17). To systematically identify changes to the ubiquitinome during oncogene-induced senescence in primary human fibroblasts, here we described a stable isotope labeling with amino acids in cell culture (SILAC) combined with liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

2. Materials

2.1. Cell culture for expression of oncogenic RAS

1. pBABE-puro and pBABE-puro-H-RAS^{G12V} constructs (Addgene)
2. 2.5 M CaCl₂
3. 2X BBS: 50 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95 (*see Note 1*)
4. Sterile-filtered Milli-Q water
5. Laemmli sample buffer [50 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue, pH 6.8]
6. Equipment and reagents for SDS-polyacrylamide gel electrophoresis (PAGE)
7. Bradford reagent (Bio-Rad) and 1 mg/mL bovine serum albumin (BSA, Pierce) as standard
8. 0.45 µm filter
9. Phoenix cells (a kind gift from Dr. Gary Nolan at The Stanford University) growing in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Clontech), 1% (w/v) penicillin-streptomycin, and 1% (w/v) L-glutamine in a humidified 37°C, 5% (v/v) CO₂ incubator (*see Note 2*).
10. IMR90 cells (ATCC) growing in DMEM supplemented with 20% (v/v) FBS, 1% (w/v) L-glutamine, 1% (v/v) Non-Essential Amino Acids Solution (Cellgro), 2% (v/v) Essential Amino Acids (Cellgro), 1% (v/v) Vitamins (Cellgro), and 1% (w/v) Penicillin-Streptomycin in a humidified 37°C, 5% (v/v) CO₂ incubator
11. 0.25% (w/v) Trypsin + 1 mM EDTA
12. Dulbecco's phosphate-buffered saline (DPBS), pH 7.3

¹The pH value of the 2X BBS solution is critical for efficient plasmid transfection using this method.

²Passage Phoenix cells every 2–3 days, never let cells reach confluence. Phoenix cells should be cultured in 300 µg/mL hygromycin (Roche) and 1 µg/mL diphtheria toxin (Sigma) for 1 week every month to maintain transfection efficiency. For more information, please visit Gary Nolan's website: (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html).

13. 1 mg/mL puromycin in PBS, pH 7.3 (Clontech)
14. 8 mg/mL (w/v) Polybrene (Sigma) in ddH₂O
15. 10 cm cell culture dishes

2.2 SILAC labeling

- 1 SILACTM Protein ID & Quantitation Media Kit, with Lysine and D-MEM-Flex (Invitrogen #MS10030)
- 2 [¹³C₆]-L-arginine, or [¹³C₆,¹⁵N₄]-L-arginine (Thermo Scientific #88433, #89990)
- 2 MG132 (Sigma #C2211) (*see* Note 3).
- 3 Cell lysis buffer: 8 M urea, 50 mM Tris-Cl (pH8), 1 mM EDTA, 1 mM Na₃VO₄, 2.5 mM sodium pyrophosphate
- 4 BCA protein assay kit (Thermo Scientific #PI-23227)
- 5 1 M DTT, pH 8.0
- 6 120 mM Iodoacetamide, pH 8.0
- 7 130 mM cysteine, pH 8.0
- 8 Sequencing Grade Modified Trypsin (Promega)
- 9 50 mM Tris-Cl, pH 8.0
- 10 Sep-Pak C18 (Waters Corporation, Milford, MA, USA)
- 11 PTMScan® Ubiquitin Remnant Motif (K-ε-GG) Kit (Cell Signaling Technology)

2.3 Sep-Pak® C18 Purification of Lysate Peptides

- 1 SepPak Vac 1 cc (100 mg) C18 cartridges (Waters #WAT023590)
- 2 20% (v/v) trifluoroacetic acid (TFA): add 10 mL TFA to 40 mL water to a total volume of 50 mL (*see* Note 4).
- 2 Solvent A (0.1% TFA): add 5 mL of 20% TFA to 995 mL water.
- 3 Solvent B (0.1% TFA, 40% acetonitrile): add 400 mL of acetonitrile (MeCN) and 5 mL of 20% TFA to 500 mL of water, adjust final volume to 1 L with water.

2.4 LC-MS/MS

- 1 Q Exactive mass spectrometer (Thermo Scientific)

³Dissolve MG132 in DMSO, store it at -20°C to keep its activity.

⁴Prepare solutions with RODI or equivalent grade water. Organic solvents (trifluoroacetic acid, acetonitrile) should be of the highest grade. All percentage specifications for solutions are vol/vol.

- 2 BEH C18 nanocapillary analytical column (75 mm i.d. × 25 cm, 1.7 μm particle size; Waters)
- 3 Symmetry C18 trap column (180 μm i.d. × 2 cm; 5 μm; Waters)
- 3 0.1% formic acid in water
- 4 0.1% formic acid in acetonitrile
- 5 MaxQuant version 1.3.0.5 or later software

3. Methods

3.1 Culturing IMR90 cells

Human primary fibroblasts IMR90 (between population doubling #25–30) are cultured in heavy $^{13}\text{C}_6$ -lysine and $^{13}\text{C}_6$ -arginine or control light $^{12}\text{C}_6$ -lysine and $^{12}\text{C}_6$ -arginine SILAC medium for 5 passages prior to induction of senescence and maintained in the respectively labeled medium during the entire experimental period.

3.2 Induction of cellular senescence in IMR90 cells with oncogenic RAS

The retrovirus encoding the constitutively activated H-Ras^{G12V} oncogene is generated by transfecting the plasmid DNA into a packaging cell line Phoenix cells cultured in a 37°C, 5% (v/v) CO₂ incubator. The plasmid DNA for transfection is made using a Qiagen Plasmid Maxi kit according to the manufacturer's instructions. The tissue culture supernatant from Phoenix cells transfected with H-Ras^{G12V} or control are collected and applied to IMR90 cells to infect the cells to induce senescence. The infected cells are cultured in heavy $^{13}\text{C}_6$ -lysine and $^{13}\text{C}_6$ -arginine or control light $^{12}\text{C}_6$ -lysine and $^{12}\text{C}_6$ -arginine SILAC medium with 1 μg/ml puromycin for 3 days to eliminate non-infected cells.

1. Day 0: 5×10^6 Phoenix cells are plated into a 10-cm dish in 6 mL medium 24 h prior to transfection.
2. Day 1: 4 h prior to transfection, change the medium to 9 mL of pre-warmed fresh media [DMEM supplemented with 10% FBS, 1% (w/v) penicillin-streptomycin, and 1% (w/v) L-glutamine].
3. Dilute the required amount of 2.5 M CaCl₂ to 250 mM in sterile ddH₂O and aliquot 0.5 mL per transfection to separate, sterile 15 mL polystyrene tubes.
4. Add 30 μg of the plasmid DNA of the intended viral construct to each tube (see Note 5).
5. Add 0.5 mL of 2X BBS by dripping slowly from a 1 mL pipette vertically down the center of the tube (1–2 drops per second). Do not mix. Incubate the mixture at room temperature for 15 min. At this time, the precipitate should be barely visible to the naked eye.

⁵Here we used calcium phosphate method for transfection. Lipofectamine 2000 method can also be used for transfection to make virus, typically 10 μg of plasmid with 2 fold of lipofectamine 2000 (20 μL) is sufficient for transfecting 10 cm dish Phoenix cells.

6. After the incubation, mix the precipitate by blowing air bubbles through the solution with a 1 mL pipette. Add the mixture drop-wise into the prepared Phoenix cells (*see* Note 6).
7. Rock the plates back and forth very gently to make the calcium phosphate precipitate distribute evenly in the medium and then culture the cells in a humidified 37°C incubator with 5% (v/v) CO₂.
8. Day 2: Change the medium to 6 mL fresh, pre-warmed growth medium approximately 24 h later.
9. Day 2: On the same day as **Step 8**, prepare IMR90 primary human embryonic fibroblast cells for infection. 1.5×10⁶ IMR90 cells are plated into a 10 cm dish for infection on the next day (*see* Note 7).
10. Day 3, 1st virus collection: 24 hours after **step 8**, collect the supernatant containing virus from the transfected Phoenix cells and put through a 0.45 µm filter. Add 6 mL of fresh, pre-warmed growth media to the Phoenix cells.
11. Day 3, 1st infection: Infect IMR90 cells from **step 9** with virus collected from **step 10**. Aspirate the IMR90 media from the dish, add 3 mL fresh media and 3 mL of virus-containing supernatant harvested from the Phoenix cells.
12. Add 6 µL 8 mg/mL (w/v) polybrene to make a final concentration of 8 µg/mL, and swirl the dishes to mix. Put the cell dish back into a 5% (v/v) CO₂-containing incubator.
13. Day 4, 2nd infection: Culture the infected cells for 24 h. Repeat the infection (**Steps 10–12**) to increase infection efficiency.
14. Day 5: Remove the medium from the IMR90 cells 24 h post-infection and replace with 10 mL of fresh, pre-warmed IMR90 growth media (different labelling) containing 1 µg/mL puromycin (*see* Note 8).
15. Day 9: Infected IMR90 cells are harvested for preparation for LS-MS/MS. At the same time, cells infected with control or H-Ras^{G12V} virus are assayed for senescence-associated β-galactosidase (SA-β-gal) activity and BrdU incorporation to validate cellular senescence status.

3.3 Harvest senescent cells for LC-MS/MS

Carry out all procedures on ice unless otherwise specified.

1. Treatment of MG132: On day 6 post infection, to prevent the degradation of ubiquitinated proteins, infected cells are treated with 5 µM MG132 and cultured in a 37°C, 5% (v/v) CO₂ incubator for 5 hours prior to harvest.

⁶Be very gentle when adding the mixture or fresh media to Phoenix cells as the cells do not adhere to the dishes very well.

⁷A confluent 10 cm dish of IMR90 cells should be sufficient to be split 1:4 in regular growth media for infection on next day.

Generally, cells should be 70–80% confluent when infection

⁸Typically, 3 days after selection in 1 µg/mL puromycin, all of the non-infected cells should be dead. At this time, there should be no surviving cells left in the control mock infected dish.

2. Discard culture medium, wash cells once with PBS, pH 7.3 to remove all culture media.
3. Trypsinize cells with 1 mL 0.25% (w/v) Trypsin + 1 mM EDTA, incubate at 37°C for 5 min.
4. Stop trypsin reaction by add 5 mL complete culture medium (same as culture medium), resuspend cells by pipetting up and down, transfer to a 15 mL polystyrene tubes.
5. Spin cells down at 300 g at 4°C for 5 min to collect cell pellet.
6. Wash with PBS for three times to remove the medium and spin at 300 g at 4°C for 5 min to collect cells.
7. Resuspend cells in freshly prepared cell lysis buffer (8 M urea, 50 mM Tris-Cl (pH8.0), 1 mM EDTA, 1 mM Na₃VO₄, 2.5 mM sodium pyrophosphate), transfer samples to 1.5 mL microcentrifuge tube.
8. Sonicate the lysate with 2 bursts of 30 s prime and 1 min between 2 bursts on ice.
9. Spin samples at 12,000 g at 4°C for 15 min, transfer supernatant to a new 1.5 mL microcentrifuge tube.
10. Quantification: quantify protein concentration of each sample with BCA protein assay kit (Thermo Scientific, #23227).
11. Prepare test sample: mix equal amount (2.7 mg) of lysates from IMR90 cells infected with oncogenic RAS cultured in heavy SILAC labeled medium and control virus cultured in light SILAC labeled medium.
12. Prepare control sample: to set a control for evaluation of the proteomic analysis, equal amount (2.7 mg) of lysates from IMR90 cells infected with control virus cultured in either heavy SILAC labeled or light SILAC labeled medium are mixed.
13. Prepare non-enriched sample: to set a global non-enriched combined lysate proteome analysis, take 18 µg of protein sample from **step 11** and save for future use.
14. Reduction: all samples are reduced by addition of 1 M DTT (pH8.0) to 5 mM final concentration and incubation at 37°C for 45 min.
15. Alkylation: all samples are alkylated by addition of 120 mM Iodoacetamide (pH8.0) to 10 mM final concentration, and incubation at 37°C for 30 min in the dark.
16. All samples are then quenched by adding 130 mM cysteine (pH8.0) to a final 10 mM concentration, and incubated at 37°C for 30 min.
17. Trypsin digestion: Dilute samples to 4 M urea with 50 mM Tris (pH8.0) and digest with modified trypsin (enzyme:protein=1:100) for 4 h at 37°C.

18. Further dilute urea concentration to 2 M with 50 mM Tris (pH8.0) and add another aliquot of modified trypsin (enzyme:protein=1:100), incubate overnight at 37°C.
19. Stop trypsin digestion by acidifying with TFA as described below.

3.4 Sep-Pak® C18 Purification of Lysate Peptides

Sep-Pak® C18 purification utilizes reversed-phase (hydrophobic) solid-phase extraction. Peptides and lipids bind to the chromatographic material. Large molecules such as DNA, RNA, and most protein, as well as hydrophilic molecules such as many small metabolites are separated from peptides using this technique. Peptides are eluted from the column with 40% acetonitrile (MeCN) and separated from lipids and proteins, which elute at approximately 60% MeCN and above. Purification is performed at room temperature.

1. Acidification of Digested Cell Lysate: Add 1/20 volume of 20% TFA to the digest for a final concentration of 1% TFA, incubate 15 min on ice to allow precipitate to form. Check the pH of the sample on a pH strip (the pH should be under 3) (*see* Note 9).
2. Centrifuge the acidified peptide solution for 15 min at 12,000 g at room temperature to remove the precipitate.
3. Gently transfer peptide-containing supernatant into a new 50 mL conical tube.
4. Place Sep-Pak column in a 15 mL polypropylene tube and pre-wet the column with 2 mL 100% MeCN. Discard flow-through (*see* Note 10).
5. Wash the column with 3 mL of Solvent A (0.1% TFA) for three times. Discard flow-through.
6. Load acidified and cleared digest from step 2 and allow it to flow through the column.
7. Wash the column four times with 3 mL of Solvent A (0.1% TFA), followed by wash with 2 ml of 5% MeCN, 0.1% TFA.
8. Elute peptides with a sequential wash of 2 × 0.9 mL of Solvent B (0.1% TFA, 40% acetonitrile) and collect the elute to a new 2 ml tube.
9. Freeze the elute on dry ice-ethanol bath and lyophilize frozen peptide solution, save the non-enriched sample for future LC-MS/MS use (*see* Note 11).

⁹Before loading the peptides from the protein digest on the column, the digest must be acidified with TFA for efficient peptide binding. The acidification step helps remove fatty acids from the digested peptide mixture.

¹⁰From this step, application of all solutions should be performed by gravity flow.

¹¹The digested peptides are stable at -80°C for several months (seal the closed tube with parafilm for storage). The PTMScan® procedure can be interrupted before or after lyophilization. Once the lyophilized peptide is dissolved in IAP buffer, continue to the end of the procedure.

3.5 Immunoaffinity Purification (IAP) with Ubiquitin Remnant Motif (K-e-GG) Antibody Bead Conjugate

1. Centrifuge the lyophilized peptide and add 1.4 mL 1x IAP (plus detergent) buffer. Dissolve lyophilized samples by sonication in a water bath sonicator (*see* Note 12).
2. Centrifuge dissolved peptide for 5 min at 10,000 g at 4°C to remove insoluble pellet. Keep the samples on ice.
3. Transfer the supernatant from **step 2** into the microfuge tube containing motif antibody beads. Pipet sample directly on top of the beads at the bottom of the tube to ensure immediate mixing. Avoid creating bubbles upon pipetting.
4. Seal the microfuge tube with parafilm and incubate on a rotator at 4°C for 2 hr.
5. Centrifuge at 2,500 g for 1 min and transfer the supernatant to a labeled Eppendorf tube to save for future use. (*see* Note 13).
6. Add 1 mL of IAP buffer to the beads, mix by inverting tube 5 times, centrifuge at 2,500 g for 1 min, and gently remove supernatant without disrupting beads (*see* Note 14).
7. Repeat step 6 once for a total of TWO IAP buffer washes.
8. Wash the bead with 1 mL chilled Burdick and Jackson water, mix by inverting tube 5 times, centrifuge at 2,500 g for 1 min, and remove supernatant with a P-1000 micropipettor (*see* Note 15).
9. Repeat step 8 two times for a total of THREE water washes. During the last water wash, the tube may need to be shaken while inverting in order to ensure efficient mixing (*see* Note 16).
10. Add 55 µl of 0.15% TFA to the beads, tap the bottom of the tube several times (do not vortex), and let stand at room temperature for 10 min, mixing gently every 2–3 min.
11. Centrifuge 30 sec at 2,000 g in a microcentrifuge and transfer supernatant to a new 1.7 mL Eppendorf tube.
12. Repeat step **10–11**, combine both eluents in the same 1.7 mL tube. Briefly centrifuge the eluent to remove any remaining beads.

¹²After dissolving the peptide, check the pH of the peptide solution with a pH indicator paper. The pH should be close to neutral, or no lower than 6.0. In the rare case that the pH is more acidic (due to insufficient removal of TFA from the peptide under suboptimal conditions of lyophilization), titrate the peptide solution with 1 M Tris base solution that has not been adjusted for pH. 5–10 µl is usually sufficient to neutralize the solution.

¹³In order to recover the beads quantitatively, do not spin the beads at lower g-forces than what is specified in this procedure. Avoid substantially higher g-forces as well, since this may cause the bead matrix to collapse. All centrifugation steps should be performed at the recommended speeds throughout the protocol.

¹⁴All wash steps should be performed at 0–4°C. In all wash steps, the supernatant should be removed reasonably well. Avoid removing the last few microliters since this may cause inadvertent carry-over of the beads.

¹⁵All steps from this point forward should be performed with solutions prepared with Burdick and Jackson or other HPLC grade water.

¹⁶After the last wash step, remove supernatant with a P-1000 micropipettor as before, then centrifuge for 5 sec to remove fluid from the tube walls, and carefully remove all remaining supernatant with a gel loading tip attached to a P-200 micropipettor.

13. Transfer eluent to a new 0.3 mL polypropylene autosampler vial, lyophilize the sample and redissolve in 25 μ l of 0.1% TFA with 3% MeCN.
14. Dissolve saved non-enriched sample [section 3.4, step 9] in 25 μ l of 0.1% TFA with 3% MeCN.

3.6 LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis is performed on a Q Exactive mass spectrometer (Thermo Scientific) coupled with a Nano-ACQUITY UPLC system (Waters).

1. Place sample vials in the cooled autosampler and setup Xcalibur instrument sequence control to automatically pick up samples and inject onto a UPLC Symmetry trap column (180 mm i.d. $\times$ 2 cm packed with 5 mm C18 resin; Waters), follow by separation on a BEH C18 nanocapillary analytical column (75 mm i.d. $\times$ 25 cm, 1.7 mm particle size; Waters).
2. Use a reversed phase gradient formed by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile).
3. An example of the gradient used is: 5–28% B over 42 min, 28–50% B over 25.5 min, 50–80% B over 5 min, and constant 80% B over 7.5 min.
4. Run a quick blank before analyzing the next sample to minimize carryover.
5. Set the mass spectrometer to repetitively scan m/z from 400 to 2000 for eluted peptides. Collect the full MS scan at 70,000 resolution follow by data dependent MS/MS scans at 17,500 resolutions on the 20 most abundant ions exceeding a minimum threshold of 8,300.

3.7 MS data analysis

1. Analyze MS data with appropriate software such as MaxQuant version 1.3.0.5 or later (18).
2. Set first search peptide tolerance at 20 ppm, main search peptide tolerance at 4.5 ppm, and MS/MS match tolerance at 20 ppm.
3. Search MS/MS data against the human protein database using full trypsin specificity with up to 2 missed cleavages, static carboxamidomethylation of Cys, and variable oxidation of Met, protein N-terminal acetylation and diglycine addition to Lys.
4. Generate consensus identification lists with false discovery rates of 1% at protein, peptide and site levels.

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