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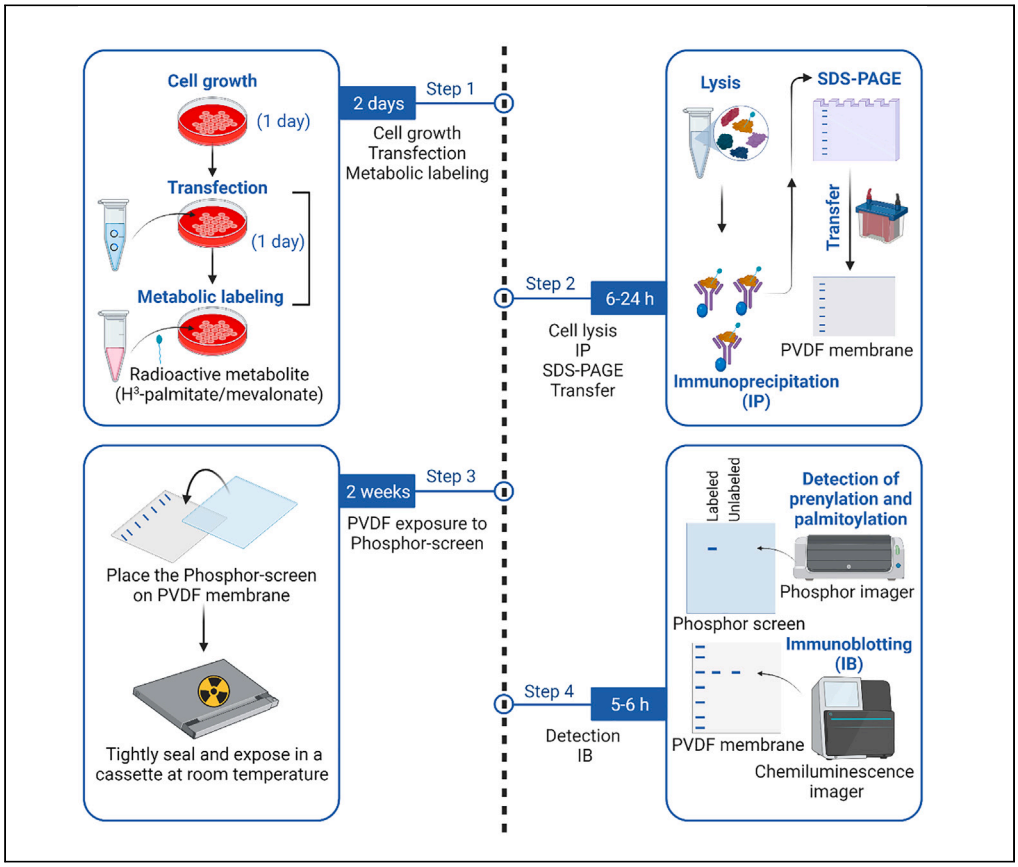
Protocol

Detection of membrane-anchoring lipid modifications of proteins in cells by radioactive metabolic labeling



Prenylation and palmitoylation are two major lipid modifications of cellular proteins that anchor proteins to cell membranes. Here, we present a protocol for detecting these modifications in cellular proteins by radioactive metabolic labeling. We describe steps for metabolic labeling of cells, cell harvesting for carrying out immunoprecipitations, subjecting immunocomplexes to SDS-PAGE, and transferring them to polyvinylidine flouride (PVDF) membranes. We then detail detection of labeled target proteins by exposing PVDF membranes to phosphor screens and using a phosphor imager machine.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Sameer Ahmed Bhat, Rachel Ferguson, Ritika Adhikari, Shafi Kuchay

kuchay@uic.edu

Highlights

Cellular protein prenylation and palmitoylation detection with tritiated-metabolite labeling

Highly sensitive and reproducible protocols

Protocols adaptable for endogenous and exogenously expressed proteins

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Detection of membrane-anchoring lipid modifications of proteins in cells by radioactive metabolic labeling

Sameer Ahmed Bhat,[1,2] Rachel Ferguson,[1] Ritika Adhikari,[1] and Shafi Kuchay[1,2,3,*]

[1]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, MBRB #1252, Chicago, IL 60607, USA

[2]Technical contact

[3]Lead contact

*Correspondence: kuchay@uic.edu
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SUMMARY

Prenylation and palmitoylation are two major lipid modifications of cellular proteins that anchor proteins to cell membranes. Here, we present a protocol for detecting these modifications in cellular proteins by radioactive metabolic labeling. We describe steps for metabolic labeling of cells, cell harvesting for carrying out immunoprecipitations, subjecting immunocomplexes to SDS-PAGE, and transferring them to polyvinylidine flouride (PVDF) membranes. We then detail detection of labeled target proteins by exposing PVDF membranes to phosphor screens and using a phosphor imager machine.

For complete details of this protocol, please refer to Liang et al.[1]

BEFORE YOU BEGIN

The protocols below describe the detection of two major lipid modifications, prenylation and palmitoylation, by metabolic labeling.[2–9] This procedure is adaptable to both endogenous and exogenously expressed proteins. Here, we have used HEK293T and MCF10CA1a cells for radioactive metabolic labeling. For exogenously expressed tagged proteins, immunoprecipitations are carried out using tag-specific resins. For endogenous proteins, protein-specific antibodies can, alternatively, be used for immunoprecipitation. Detection of the labeled target is done with the use of phosphor imager machine.

Institutional permissions

Authorization for radioactivity use and training of personnel should be obtained through relevant institutional mechanisms. We are permitted for usage by the Radiation Safety Section of Environmental Health & Safety Office of University of Illinois at Chicago (UIC), USA. At UIC, authorized users attend an initial radiation safety lecture followed by an annual training refresher.

Cell culture and transfection

1. Maintain HEK293T cells in DMEM containing 10% FBS and 1× Pen-Strep in a humidified incubator at 37°C and 5% CO_2 concentration.

2. Maintain MCF10CA1a in DMEM/F12 medium containing 5% horse serum, 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin and 1× Pen-Strep.

   Note: These growth conditions are specific to HEK293T and MCF10CA1a cells. Cell culture growth conditions should be optimized depending on the cell type used for labelling.

3. Prepare transfection reagents (e.g., PEI Max in our protocol, details below) and high-quality plasmid DNA (e.g., by using QIAGEN® Plasmid Mini or Midi Kit).

Radioactivity, inhibitors, and buffers

4. Evaporate the solvent of the radiolabel (e.g., ethanol in the case of H^3-mevalonolactone and H^3-palmitic acid) on the day of labeling (details below).
5. For prenylation protocol, make lovastatin at a concentration of 10 mM (details below).
6. Prepare 50× stock solution of protease inhibitor cocktail (PIC) (details below).
7. For palmitoylation protocol, prepare fresh defined labeling medium before the experiment (details below).
8. Prepare 1× lysis buffer, 1× running buffer, 1× transfer buffer, and 2× sample buffer (recipe details below).

⚠ CRITICAL: H^3-mevalonolactone and H^3-palmitic acid stock solutions contain ethanol at high concentrations as solvent. If not evaporated before use, ethanol may kill the cells while labeling (see below for removal details).

Radioactivity testing and discard

Radioactive solid and liquid wastes were discarded according to UIC's Radiation Safety section guidelines. Swipe tests using Liquid Scintillation Counting were performed to check for any possible H^3 contamination.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-FLAG-M2 affinity gel	Millipore Sigma	Cat#A2220
Rabbit polyclonal anti-FLAG (1:1000)	Millipore Sigma	Cat#F7425-.2MG
Chemicals, peptides, and recombinant proteins		
Mevalonolactone RS-[5-3H]	ARC	Cat#ART-0315A
Palmitic Acid, [9,10-^{3}H(N)]	PerkinElmer	Cat#NET04300
Lovastatin	Millipore Sigma	Cat#M2147-25MG
DMEM	Millipore Sigma	Cat#D5796
DMEM/F12	Gibco	Cat#11330-032
Horse serum	Gibco	Cat#16050-122
Hydrocortisone	Millipore Sigma	Cat# H-0888-1G
Cholera toxin	Millipore Sigma	Cat#C-8052-2MG
Insulin	Millipore Sigma	Cat# I1882-100MG
Fetal Bovine Serum	Corning	Cat#35-010-CV
Fetal Bovine Serum (Dialyzed)	Gibco	Cat#26400-036
Penicillin-Streptomycin-L-Glutamine (100×)	Corning	Cat#30-009-CI
Sodium Pyruvate (100mM)	Millipore Sigma	Cat#S8636-100ML
Opti-MEM	Gibco	Cat#31985-070
rProtein G Agarose	Invitrogen	Cat#15920010
Nonidet P-40 Substitute	Roche	Cat#11332473001
Complete EDTA-free Protease Inhibitor Cocktail	Roche	Cat#04693132001
LDS Sample Buffer (4×)	Novex	Cat#B0008
Bolt 4%–12% Bis-Tris Plus	Invitrogen	Cat#NW04127BOX
20× Bolt MES SDS Running Buffer	Invitrogen	Cat#B0002-02
Ponceau S	Fisher Scientific	Cat#BP103-10
Experimental models: Cell lines		
HEK 293T	ATCC	Cat#CRL-3216

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
MCF10CA1a	Hay Lab, UIC	
Software and algorithms		
ImageJ		https://imagej.nih.gov/ij/
Adobe illustrator		https://www.adobe.com/
BioRender		https://www.biorender.com/
Other		
Sonic Dismembrator	Fisher Scientific	FB705
Phosphor screen	Cytiva	Cat#28-9564-82
Phosphor imager	Amersham Typhoon, GE	
Immobilon-P Transfer membrane	Millipore Sigma	Cat#IPVH00010
CO2 incubator	Fisher Scientific	HERACELL VIOS 160i
Biological Safety Cabinet	Fisher Scientific	Cat#1385
Centrifuge (use for 15 mL tubes)	Fisher Scientific	Sorvall ST16R
Centrifuge (use for 1.5 mL tubes)	Fisher Scientific	Sorvall Legend Micro 21R
Vacufuge Plus Centrifuge Concentrator	Eppendorf	Cat#022820109
Heat block	Fisher Scientific	Cat#88870001
Chemiluminescence Imager	GE Healthcare	ImageQuant LAS 500
Mini Gel Tank and Blot Module Set	Invitrogen	Cat#NW2000
Microcentrifuge	Benchmark	Cat#C1012
Vortex	Fisher Scientific	Cat#88880017
Tube rotator with 1.5/2 mL rotator drum	Fisher Scientific	Cat#88861049/ Cat#88861121
Power Supply	Fisher Scientific	Cat#FB300Q
Platform Rocker	Corning	Cat#6705

MATERIALS AND EQUIPMENT

Note: Refer to key resources table for vendor and catalogue numbers of commercially available reagents and equipment used in our protocols.

- Prepare PEI Max transfection reagent by dissolving the powder at 1 mg/mL in ddH_2O and adjust the pH to 7.0. Sterile filter through 0.22 μm filter (we have optimized PEI Max for use with HEK293T cells).
- Evaporate the ethanol from H^3-mevalonolactone and H^3-palmitic acid solutions. This can be done in a centrifuge concentrator at room temperature to reduce ethanol to a level that can be tolerated by the cells. Keep the lid of the microcentrifuge tube open while evaporating. Alternatively, ethanol can be evaporated by putting a relevant amount of label to dry in a 6 cm dish with the lid removed (5–10 min, under tissue culture conditions). Reconstitute the label by adding the labeling medium (details below) to the dish and keeping the dish at 37°C in the CO_2 incubator for 5 min.

Note: Vacufuge Plus Centrifuge Concentrator (Eppendorf) was used for evaporating ethanol.

- Prepare 10 mM lovastatin for prenylation protocol[8] by dissolving 40 mg of lovastatin in 0.72 mL of ethanol at 55°C. Add 0.36 mL of 0.6 M NaOH and 7.2 mL of ddH_2O. Incubate for 30 min at room temperature. Adjust the pH to 8.0 with HCl and make up the volume to 10 mL with ddH_2O. Aliquot and store at −20°C.
- Palmitoylation labeling medium for HEK 293T: DMEM containing 10% dialyzed FBS, 5 mM sodium pyruvate (to inhibit β-oxidation of palmitate) and 3.6 mg/mL fatty acid-free BSA. For making 10 mL, weigh 36 mg of fatty acid-free BSA and add 1 mL of dialyzed FBS, 0.5 mL of sodium pyruvate (from 100 mM stock), and 8.5 mL of DMEM. Mix and sterile filter using 0.45 μm filter.[1,10]
- Palmitoylation labeling medium for MCF10CA1a: DMEM/F12 containing 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, 5% dialyzed FBS, 5 mM sodium pyruvate and 3.6 mg/mL fatty acid-free BSA.

Lysis buffer		
Reagent	Final concentration	Amount
Tris pH 7.4 (1 M)	50 mM	2.5 mL
NaCl (5 M)	150 mM	1.5 mL
NaF (0.5 M)	50 mM	5 mL
EDTA (0.5 M)	1 mM	0.1 mL
NP-40 substitute (10%)	0.1%	0.5 mL
ddH_2O	N/A	40.4 mL
Total	**N/A**	**50 mL**
Store at 4°C. It can be stored at this temperature for a few months.		

Transfer buffer		
Reagent	Final concentration	Amount
Tris	25 mM	3.03 g
Glycine	192 mM	14.4 g
ddH_2O	N/A	500 mL
Methanol	20%	200 mL
Total	**N/A**	**To 1 L with ddH_2O**

- A 50× stock of PIC can be made by dissolving one tablet in 1 mL ddH_2O. Store the PIC solution at −20°C for long-term use. Add to lysis buffer as 1× immediately before use.
- Running buffer (1×): Dilute 20× Bolt MES SDS Running Buffer (Invitrogen B0002-02) to 1× (50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3) with ddH_2O.
- Sample buffer (4×/2×): Dilute 4× LDS Sample Buffer (Novex-B0008) to 2×. Both 4× and 2× sample buffers are used in the protocol. Add 50 mM DTT from 1 M stock freshly before using the sample buffer.

Note: The composition of 1× LDS sample buffer is 141 mM Tris base, 106 mM Tris HCl, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G, 0.175 mM Phenol Red pH 8.5.

- Ponceau S staining solution: 0.1% (w/v) Ponceau S, 5% acetic acid (v/v). For preparing 500 mL, add 25 mL of glacial acetic acid to 400 mL of distilled water. Dissolve 0.5 g of Ponceau S powder and make the final volume to 500 mL with distilled water.

⚠ CRITICAL: Radioactive materials should be handled with care. Store the radioactive material in a locked cabinet. Wear a lab coat and thick gloves while handling radioactive material. Dispose the waste according to the guidelines. Perform swipe tests after using the radioactivity. Evaporation of ethanol from the label is critical for proper metabolic labeling without killing cells.

Alternatives: Depending on compatibility with cell systems, other transfection reagents such as Lipofectamine 3000 may also be used for cellular transfections, cell lysis may be performed using 1% NP-40 in the lysis buffer or using RIPA buffer for proteins that are difficult to extract.

STEP-BY-STEP METHOD DETAILS

Transfection and radioactive labeling

Timing: 1 day

For efficient labeling of exogenously expressed protein, transfection of vehicle (e.g., a plasmid carrying the protein of interest) and radioactive metabolite addition is concomitantly carried out in

mammalian cells. To monitor labeling of endogenously expressed protein, skip the transfection steps 3–6, and 9. Below are step-by-step details for transfection and radioactive metabolic labeling optimized for HEK293T cells.

1. Split the cells a day before transfection into 6 cm dishes so that they reach 70% confluency on transfection day.

   Note: A 6-well plate format also works for HEK293T cells if the expression of the gene of interest is good.

2. For prenylation assays, incubate the cells in 4 mL DMEM containing 5% dialyzed serum and 15 μM lovastatin[8] for 2 h.

   Note: Skip this step for palmitoylation assays.

   ⚠ CRITICAL: Treatment with lovastatin (or compactin[11]) inhibits HMG-CoA reductase to suppress production of endogenous unlabeled mevalonate from HMG-CoA, increasing the probability to incorporate H^3-mevalonate label derivatives into intended target protein.

3. Thaw the plasmids and PEI Max at room temperature.
4. Put optiMEM (100 μL/μg DNA) into 1.5 mL centrifuge tubes and add plasmids (1–5 μg/6 cm culture dish).

   ⚠ CRITICAL: The plasmid amounts depend on the expression levels for the genes of interest and should be optimized for dish formats used.

5. Add PEI Max at a ratio of 3:1 (ratio of PEI to DNA) and mix by flicking or alternatively brief careful vortexing.
6. Incubate the tubes at room temperature for 10–15 min.
7. For prenylation labeling, add 2 mL of fresh DMEM containing 5% dialyzed serum, 400 μCi of concentrated H^3-mevalonolactone (0.2 mCi/mL)[11] and 15 μM lovastatin to the 6 cm dishes.

   Note: For cost-effectiveness, we labeled the cells with 400 μCi of concentrated H3-mevalonolactone (0.2 mCi/mL) in minimum possible volume enough to cover the cells. Tritiated-labeling metabolites are rather costly reagents.

   Note: To distinguish between geranylgeranylation and farnesylaion use specific inhibitors (GGTi vs FTi) during labeling and/or use cell systems lacking specific prenyltransferase (GGTase 1/2/3 or FTase).

8. For palmitoylation labeling, change the medium of the 6 cm dishes to 2 mL of labeling medium (see materials and equipment for the composition) containing 0.2 mCi/mL of concentrated H^3-palmitic acid.[1,10]

   Note: The labeling medium composition varies depending on the cell line used for the assay.

9. Add the transfection mixture dropwise to cells. Mix well by back-and-forth dish shaking.
10. Incubate the cells overnight at 37°C in the CO_2 incubator.

Harvesting, lysis, and immunoprecipitation

Timing: 6–24 h

In these steps the cells are harvested, lysed, and the protein of interest is immunoprecipitated.

Note: To immunoprecipitate endogenously expressed protein, use protein-specific antibodies in steps where FLAG-M2 antibody is used in this protocol.

11. Remove the medium from the plates and dispose according to the liquid radioactivity disposal guidelines.
12. Add 1 mL PBS to the plates and scrape the cells.
    a. Pipette up and down and transfer the cells in a 15 mL centrifuge tube.
    b. Wash the plate once with 1 mL PBS to recover residual cells.
    c. Combine the 1 mL wash with scraped cells in the same 15 mL tube.
13. Centrifuge the tube at 200 × *g* for 5 min at 4°C to pellet down the cells.
14. Aspirate the supernatant and resuspend the cell pellet in 1 mL PBS.
15. Transfer the mixture into a fresh 1.5 mL centrifuge tube.
16. Centrifuge at 200 × *g* for 5 min at 4°C and aspirate the supernatant.
17. Resuspend the pellet in 500 μL lysis buffer containing 1× PIC and incubate on ice for 30 min with intermittent vortexing (we used NP-40 based lysis buffer).
18. Sonicate the lysate for 3 min (pulse on: 10 s, pulse off: 10 s, and Amplitude: 30%) in Sonic Dismembrator with Cup Horn (Fisher Scientific, FB705).

Note: All liquid washes should be disposed of according to the liquid radioactivity disposal guidelines. For sonication, it is better to use Cup Horn instead of a probe to avoid contamination of the probe, cross contamination between the samples, and loss of sample. Use cold distilled water in the Cup Horn to decrease sample degradation.

19. Centrifuge the tubes at 21000 × *g* for 20 min and transfer the supernatants to separate 1.5 mL microcentrifuge tubes.
20. Aliquot 10% of the samples as input in a separate 1.5 mL tube.
    a. Add 4× sample buffer containing freshly added DTT (50 mM) to a final concentration of 1×.
    b. Heat at 70°C for 10 min.
    c. Cool at room temperature.
    d. Give a short spin (full speed) in a tabletop centrifuge, and store at −20°C.
21. Wash rProtein G Agarose and M2-FLAG-Agarose Gel in lysis buffer without PIC 2–3 times, each time centrifuging at 350 × *g* for 3 min and aspirating the supernatant.
22. Resuspend the pelleted beads in lysis buffer as 50% mixture (1:1 mixing).

Note: M2-FLAG- Agarose Gel can also be centrifuged at 5000 g for 30 s. M2 Agarose Gel is used for immunoprecipitation of FLAG-tagged proteins. In case of performing endogenous IPs for a target protein, specific antibodies can be used either conjugated to resin or rProteinA/G resin can be used after the primary incubation period to pull down the target immunocomplex.

23. Aliquot 40 μL of the washed rProtein G Agarose into fresh 1.5 mL microcentrifuge tubes.

Note: For homogenous aliquoting of washed rProtein G Agarose and washed M2-FLAG resin, cut the front portion of a 200 μL pipette tip with clean scissors to make a wider bore.

24. Add labeled protein samples to microcentrifuge tubes containing aliquoted rProtein G Agarose and mix by flicking gently to preclear the samples.

Optional: The lysates can be quantified by BCA or Bradford assay to add equal amounts of protein samples to rProtein G Agarose resin.

Note: 1–5 μg/μL of protein concentration and a total of 1–3 mg of protein is optimal for an IP.

25. Rotate the samples for 1–2 h at 4°C (~16 rpm).

Note: Tube rotator with 1.5/2 mL rotator drum (Fisher Scientific) was used for rotating the samples.

26. While the samples are preclearing, aliquot 40 μL of the washed M2-FLAG-Agarose Gel into fresh 1.5 mL microcentrifuge tubes.
27. Spin the samples from step 25 at 350 × *g* for 3 min and add the supernatants into the microcentrifuge tubes containing aliquoted M2-FLAG-Agarose Gel. Mix by flicking gently.
28. Rotate samples for 1–16 h at 4°C (~16 rpm).

Note: (a) In this step, samples can be rotated between 1–16 h depending on the expression levels of the target protein. As a rule of thumb, endogenous IPs may be carried out for longer periods than exogenously expressed tagged proteins. *It is recommended to optimize this step for the target protein beforehand, preferably without radiolabeling.* (b) If the antibody used to IP endogenous protein is not conjugated to resin, add 30–50 μL of washed rProtein A/G Agarose resin (50% mixture of resin and buffer) for additional 1–2 h before next step.

29. Spin the samples and wash 3 times with lysis buffer (1 mL/wash) without PIC, each time centrifuging at 350 × *g* for 3 min and aspirating the supernatant.

Note: All liquid washes should be disposed of according to the liquid radioactivity disposal guidelines.

30. Remove the remaining supernatant with a 200 μL tip.
31. Add 20–30 μL of 2× sample buffer and denature at 98°C for 10 min. Cool the tubes at room temperature and spin for a short time at full speed. Samples can be stored at −20°C or −80°C before loading.

Optional: The samples can alternatively be eluted with 3X-FLAG peptide in TBS (concentration of 3X-FLAG peptide and volumes used for elution should be optimized) and then reconstituted with the sample buffer.

Running and transfer to PVDF membrane

Timing: 3 h

32. Run the samples on Bolt™ 4%–12% Bis-Tris Plus gel at 150 V for 50 min in an Invitrogen Mini Gel Tank. Include a calorimetric or radioactive molecular weight marker.

Note: (a) As a routine lab practice, we usually run 5%–10% of the sample to confirm IPs and for normalization of baits among various samples by Western blotting (anti-FLAG) before proceeding to run final gels for the autoradiography. (b) In our protocols, we used gradient gels. Bolt™ 4%–12% Bis-Tris Plus gels provide a wide range of resolution for sample targets, particularly for those with higher molecular weights, and the run times are shorter. Tris-glycine-based gels and manually casted gels were not used in our protocol, and if used, should be checked beforehand for the target protein.

33. Activate the PVDF membrane by wetting it in methanol for a few seconds and equilibrating in the transfer buffer until it sinks.
34. Transfer the samples at 20 V for 1 h in an Invitrogen™ Mini Gel Tank.

Note: Our protocol SDS-PAGE run time, transfer voltage and time are optimized for Bolt™ gradient gels with regards to protein of interest. The running and transfer conditions should be empirically optimized for manually cast gels.

35. Stain the PVDF membrane with Ponceau S to confirm the proper transfer of proteins.
    a. Wash the membrane with TBST (0.1% Tween-20).
    b. Stain with Ponceau S until color develops.
    c. Wash with distilled water to remove the background.
    d. De-stain with TBST.
    e. Wash with PBS/TBS once before next step.
36. Air dry the PVDF membrane on filter paper, carefully monitoring the orientation of top vs. bottom sides.

PVDF exposure to phosphor screen and detection using phosphor imager

Timing: ~2 weeks

37. Cover the PVDF membrane with the phosphor screen in a cassette.
    a. Use low adhesion tape to keep the phosphor screen in place.
    b. Close the cassette tightly.
    c. Place the cassette at room temperature in dark to expose the phosphor screen for 2–3 weeks.

Note: Phosphor screens are rather expensive reagents. Accordingly, portions of the phosphor screen, enough to cover the PVDF membrane, should be cut with clean scissors to minimize costs.

Alternatives: Kodak BioMax MS film for autoradiography can be used instead of phosphor screen. In this case, exposure is done at −80°C which increases the sensitivity of the film.[10]

38. Remove the phosphor screen from the cassette and detect the H^3 signal in a phosphor imager.

Note: 2–3 weeks of exposure are generally sufficient to detect the signal.

39. Take a colorimetric image of the PVDF membrane if using a colorimetric molecular weight marker.

Note: Markers on PVDF are used to correctly align and locate the positions of the bands in the autoradiograph.

40. Process the same PVDF membrane for Western blotting.
    a. Block the PVDF membrane in 5% nonfat dry milk.
    b. Immunoblot for the transfected target protein with rabbit anti-FLAG antibody.

Note: Alternatively, Western blotting can also be done prior to exposure to the phosphor screen. Moreover, portions of the samples can be used for Western blotting (see Note (a) in step 32).

EXPECTED OUTCOMES

If metabolic labeling is successful, prenylated and palmitoylated proteins will be detected upon exposure of PVDF membranes containing transferred proteins from labeled cell lysates to the phosphor screen. In case metabolic labeling fails, then prenylated and palmitoylated proteins should not be detected. Numerous proteins are palmitoylated and prenylated in the cells (Figures 1A and 1B).

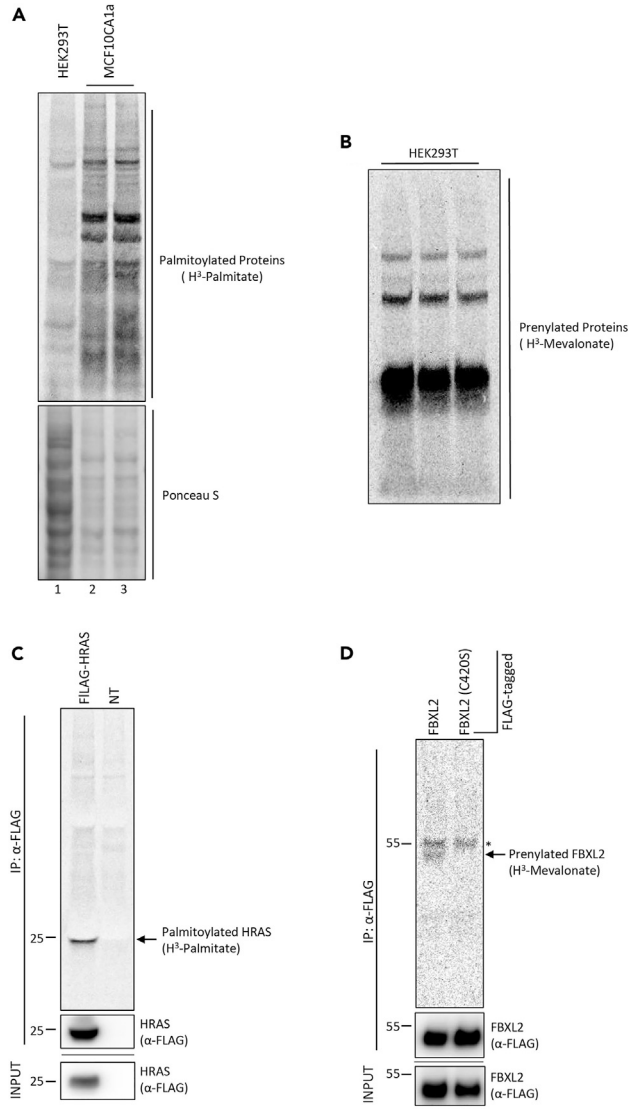

Figure 1. Detection of palmitoylation and prenylation by radioactive metabolic labeling

(A) HEK293T (lane 1) and MCF10CA1a (lanes 2 and 3 as replicates) cells were labeled overnight with H^3-palmitic acid using the protocol described in this manuscript. Post-labeling, cells were harvested, and whole cell lysates were processed to obtain soluble protein fractions using the protocol. Soluble protein fractions were subjected to SDS-PAGE using pre-cast gradient Bolt™ 4%–12% Bis-Tris Plus gels and transferred to a PVDF membrane. Subsequently, the PVDF membrane was exposed to a phosphor screen for about 2–3 weeks and H^3-labeled proteins were detected with the phosphor imager. Top: The autoradiograph shows the profile of palmitoylated proteins in HEK293T and MCF10CA1a cell lines. Bottom: PVDF membrane was stained with ponceau S to visualize the inputs in the lanes.

(B) HEK293T cells (3 replicates) were labeled overnight with H^3-mevalonolactone according to the protocol described in this manuscript. Post-labeling cells were processed as in (A). The autoradiograph, developed after 2 weeks exposure to phosphor screen, shows that various proteins are prenylated in HEK293T cells.

(C) FLAG-tagged HRAS construct was transfected in HEK293T cells and concomitantly labeled with H^3-palmitic acid overnight using the protocol described in this manuscript. Non-transfected (NT) control cells were also labeled for the same time. Post labeling and cell lysis, HRAS was immunoprecipitated using anti-FLAG-resin from soluble protein fractions. Immunocomplex was subjected to SDS-PAGE and transferred to a PVDF membrane. PVDF membrane was exposed to the phosphor screen for about 3 weeks before detection of the label with the phosphor imager (details described in the protocol). Top panel: The autoradiograph shows specific detection of palmitoylated FLAG-HRAS, but not in non-transfected control (NT). Refer to Liang et al.[1] wherein palmitoylation-deficient HRAS control is included under similar experimental settings. Portions of the immunoprecipitated fractions (middle panel) and inputs

Figure 1. *Continued*
(bottom panel) were probed with anti-FLAG antibody to detect the corresponding FLAG-HRAS compared to NT control.
(D) FLAG-tagged FBXL2 and FBXL2 (C420S) constructs were transfected in HEK293T cells and concomitantly labeled with H^3-mevalonolactone using the protocol described in this manuscript. FLAG-FBXL2 (C420S), a prenylation-deficient mutant, serves as a control. Post-labeling and cell lysis, anti-FLAG immunoprecipitations were carried out using anti-FLAG resin and processed as in (B) and the label was detected after 2-week exposure with the phosphor imager. Top panel: The autoradiograph shows the prenylation of FLAG-FBXL2, but not FLAG-FBXL2 (C420S). The same PVDF membrane was probed with anti-FLAG antibody (middle panel) to detect corresponding FLAG-FBXL2 and prenylation deficient FLAG-FBXL2 (C420S). Bottom panel shows inputs probed with anti-FLAG antibody. * Non-specific bands (Refer to Liang et al.[1] wherein non-transfected control is included under similar experimental settings).

Interestingly, we found that the profile of palmitoylated proteins varied among cell lines used for labeling (Figure 1A). To detect specific protein labeling, immunoprecipitations from labeled cell lysates with protein-specific antibodies or protein-tag-specific antibodies should be performed first. Next, transfer of immunocomplexes to PVDF membranes should be performed before exposing to the detection procedure for corresponding radiolabeled target proteins (palmitoylation and prenylation). For example, we immunoprecipitated exogenously expressed FLAG-HRAS, a well-known palmitoylated protein, with anti-FLAG resin from H^3-palmitate labeled cell lysates and detected its palmitoylation (Figure 1C). Please refer to Liang et al.[1] wherein the specificity of HRAS palmitoylation is shown with its palmitoylation deficient mutant (C181S/C184S) under similar experimental setting. Similarly, to detect prenylation from H^3-mevalonate labeled cell lysates, we used exogenously expressed FLAG-FBXL2, a well-known CAAX motif containing prenylated protein, and its variant that lacks prenylation due to mutation in cysteine residue in its CAAX motif (Figure 1D). To make sure immunoprecipitations are successful and equalized baits are exposed for detection, we performed Western blotting in parallel (bottom panels, Figures 1C and 1D).

LIMITATIONS

- Although very sensitive, these are time-consuming protocols. It takes 2–3 weeks to obtain results.
- Cost: the H^3-mevalonolactone and H^3-palmitic acids are rather expensive reagents. It is particularly costly if the target protein expression is very low. The experiment must be scaled up to detect the immunoprecipitated protein and its labeling.
- If not careful, tritiated samples can be hazardous.

TROUBLESHOOTING

Problem 1

No bands or weak bands detected on the autoradiograph (see example, Figures 2A and 2B).

Potential solution

- Increase the exposure time of PVDF membranes to phosphor screen (see and compare, Figures 1A and 1C vs. Figures 2A and 2B).
- Check by immunoblotting whether the protein of interest is properly transferred after immunoprecipitation. This should be done on the same PVDF membrane which does not show autoradiography results.
- Make sure during the setup of exposure for PVDF membrane to phosphor screen that the side of the PVDF membrane with the transferred ladder (and hence proteins) directly faces the white side of the phosphor screen.

Problem 2

The quality of immunoprecipitations and immunoblotting is not satisfactory (while optimizing the IP).

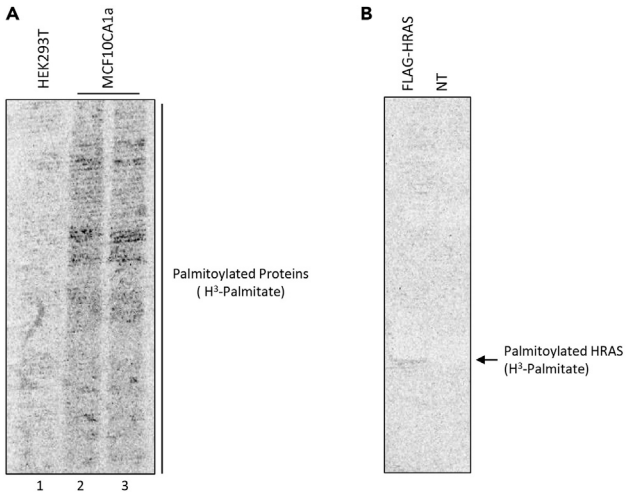

Figure 2. Problem associated with less exposure time of PVDF membranes to phosphor screen

(A) Samples (as in Figure 1A) incubated with phosphor screen for about 1 week. The autoradiograph shows that less exposure time resulted in very weak bands.

(B) Samples (as in Figure 1C) incubated with phosphor screen for about 1 week. The autoradiograph shows a very weak band for HRAS due to less exposure time.

Potential solution

- Check the quality of the plasmids used for transfections on an agarose gel and by 260/280 absorbance measurements.
- Use higher amounts of the expression plasmid during the transfection if expression of target protein is low.
- Optimize the transfection by choosing the appropriate transfection reagents for the cell system that work best.

Problem 3

Metabolic labeling of proteins may not work under certain culture conditions.

Potential solution

- Cell culture conditions including pH should be checked. Optimal pH is ~7.4.
- Cell condition: check that cells are not dying upon labeling.
- Check labeling reagents for expiry.

Problem 4

- Phosphor imager used for the detection is not properly used.

Potential solution

- Prior training in phosphor imager usage should be done.
- Training manual should be consulted and properly followed.

RESOURCE AVAILABILITY

Lead contact

Additional information and requests for reagents and resources should be directed to the lead contact, Shafi Kuchay (kuchay@uic.edu).

Materials availability

This study did not report or generate new cell lines or other new reagents. Any inquiries or requests can be directed to the lead contact.

Data and code availability

- These protocols do not report original code.
- The lead contact can provide all data reported, upon request.
- More information, upon request, to reanalyze the data reported in this protocol is available from the lead contact.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.A.B., S.K.; methodology, S.A.B., R.A.; validation, S.A.B.; investigation, S.A.B.; writing - original draft, S.A.B.; visualization, S.A.B., R.F.; reading and editing the manuscript, S.A.B., R.F.; resource procurement, R.A., S.A.B.; supervision, S.K.; writing - review & editing, S.K.; project administration, S.K.; funding acquisition, S.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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