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In Vitro Analysis of Hedgehog Acyltransferase and Porcupine Fatty Acyltransferase Activities

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Abstract

Hedgehog and Wnt proteins are modified by covalent attachment of the fatty acids palmitate and palmitoleate, respectively. These lipid modifications are essential for Hedgehog and Wnt protein signaling activities and are catalyzed by related, but distinct fatty acyltransferases: Hedgehog acyltransferase (Hedgehog) and Porcupine (Wnt). In this chapter, we provide detailed methods to directly monitor Hedgehog and Wnt protein fatty acylation in vitro. Palmitoylation of Sonic hedgehog (Shh), a representative Hedgehog family member, is assayed using purified Hedgehog acyltransferase (Hhat) or Hhat-enriched membranes, a recombinant 19 kDa Shh protein or C-terminally biotinylated Shh 10-mer peptide, and ¹²⁵I-iodopalmitoyl CoA as the donor fatty acyl CoA substrate. The radiolabeled reaction products are quantified by SDS-PAGE and phosphorimaging or by γ -counting. To assay Wnt acylation, the reaction consists of a biotinylated, double disulfide-bonded Wnt peptide containing the sequence surrounding the Wnt3a acylation site, [¹²⁵I] iodo-cis-9-pentadecenoyl CoA, and Porcupine-enriched membranes. Radiolabeled, biotinylated Wnt3a peptide is captured on streptavidin coated beads and the reaction product is quantified by γ -counting.

Keywords

Hedgehog; Hedgehog acyltransferase; Porcupine; Wnt proteins; Fatty acylation

1 Introduction

Hedgehog and Wnt proteins are secreted signaling proteins that mediate growth, patterning, and differentiation during embryo-genesis as well as tumorigenesis in adults [1, 2]. In order for these proteins to signal effectively, they must be modified by covalent attachment of a fatty acid. The 16-carbon saturated fatty acid palmitate is attached via amide bond to the N-

terminal Cys of hedgehog proteins, a reaction catalyzed by the enzyme Hedgehog acyltransferase (Hhat) [3–5]. Wnt proteins contain the *cis*-9-monounsaturated fatty acid, palmitoleate (C16:1 9), attached to a conserved, internal Ser; this reaction is catalyzed by Porcupine (Porcn) [6–8]. Both Hhat and Porcn are multipass membrane proteins that reside in the endoplasmic reticulum (ER), and are members of the MBOAT (membrane bound *O*-acyl transferase) family of acyltransferases [9, 10].

Cell-based assays have been developed to monitor fatty acylation of Hedgehog and Wnt proteins [3, 11, 12]. Cells are typically grown in medium supplemented with radiolabeled (³H or ¹²⁵I) fatty acids or with fatty acid analogs amenable to click chemistry analysis. Fatty acids that enter the cell are converted to fatty acyl CoA, and then must enter the lumen of the ER, where Hhat and Porcn catalyze transfer of the fatty acid from the fatty acyl CoA to the protein substrate. Wnt protein acylation requires an additional step: Stearoyl CoA desaturase introduces a *cis*-double bond to convert palmitoyl CoA into palmitoleoyl CoA, the substrate for Porcn [13]. Given the essential role of fatty acylation in Hedgehog and Wnt signaling activities, it is important to understand the enzymatic mechanisms that underlie these reactions, and this requires the use of direct *in vitro* assays. Here we detail the reagents and methodology to monitor Hhat and Porcn fatty acylation activities *in vitro*. For Hhat, we outline several options for the source of enzyme (purified Hhat or Hhat in membranes from cells overexpressing Hhat) and protein substrate (recombinant protein or N-terminal peptide) [3, 14] (Fig.1). Sonic hedgehog (Shh), one of the 3 members of the mammalian family of hedgehog proteins, is used as it is the best studied of the family members. To date, there is only one option for monitoring Wnt acylation *in vitro*: membranes from cells overexpressing Porcn and a peptide modeled on the sequence surrounding the Wnt3a acylation site (Fig.1) [15].

2 Materials

2.1 Cell Growth and Transfection

1. 293FT cells.
2. Culture medium: DMEM, 10% fetal bovine serum, 50 units/mL penicillin, 50 µg/mL streptomycin, 500 µg/mL geneticin, 1 mM GlutaMAX, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids.
3. Flag-tagged Hhat cDNA cloned into a mammalian expression vector (e.g., HA-Flag-his-Hhat [3]).
4. Lipofectamine 2000 (Invitrogen) transfection reagent (*see* Note 1).

2.2 P100 Membranes

1. NTE: 100 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA. Store at 4 °C.
2. Hypotonic lysis buffer: 10 mM Tris–HCl (pH 7.4), 0.2 mM MgCl₂. Store at 4 °C.
3. 5× sucrose: 1.25 M. Add 21.39 g sucrose to 50 mL with dH₂O.
4. Sucrose–Tris–EDTA: 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA.

5. Hypotonic Lysis Buffer/0.25 M sucrose/1 mM EDTA/Thermo Scientific™ Halt™ Protease Inhibitor Single-Use Cocktail (AEBSF, aprotinin, bestatin, E64, leupeptin, pepstatin A).

2.3 Hhat Purification

1. NTE: 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA. Store at 4 °C.
2. HEPES lysis buffer: 10 mM HEPES pH 7.3, 0.2 mM MgCl₂.
3. 5× sucrose: 1.25 M. Add 21.39 g sucrose to 50 mL with dH₂O.
4. Solubilization buffer: 350 mM NaCl, 20 mM HEPES 7.3, 1% octylglucoside, 1% glycerol.
5. FLAG-M2 beads (Sigma-Aldrich).
6. Elution buffer: Solubilization buffer supplemented with 300 ng/mL 3× Flag peptide.

2.4 Biotinylated Shh and Wnt Peptides

1. Shh peptides: A 10-mer peptide containing the first 10 amino acids of the mature human Shh sequence (CGPGRGFGKR) with a C-terminal PEG-biotin. A control peptide with the N-terminal cysteine replaced by alanine (AGPGRGFGKR) with a C-terminal PEG-biotin. Both peptides can be synthesized using standard methods by most in-house and commercial peptide synthesis facilities. Purity should be assessed by reversed phase HPLC and the sequence confirmed by LC-MS analysis.
2. Wnt3a peptides: C-terminal biotinylated (Biotin-PEG Nova-Tag™) containing 21 amino acids upstream and downstream of the serine acylation site of Wnt3a (MHL KC(S)KC(S-)HG LSG SC(S-)E VKT C(S-)WW). The peptides must contain two sets of disulfide-bonded cysteine residues as indicated in Fig.1. This mimics the two-dimensional conformation of Wnt proteins as revealed by the crystal structure [16]. Synthesis must be performed by a lab with specific expertise in generating disulfide-bonded peptides. Purity should be assessed by reversed phase HPLC and the sequence confirmed by LC-MS analysis (*see Note 2*).

2.5 Iodo-Fatty Acyl CoA Synthesis ([¹²⁵I] IC16-CoA and [¹²⁵I] IC15:1-CoA)

1. Iodopalmitate (IC16) and iodo-cis-9-pentadecenoic acid (IC15:1): synthesized and radioiodinated with [¹²⁵I]NaI as described [3, 17] and in Subheading 3.4 (*see Note 3*).
2. 20 mM Lithium CoA, trilithium salt in dH₂O, pH to 5.0 with NaOH. Store in aliquots at −80 °C following reconstitution.
3. 50 mM ATP disodium salt hydrate in dH₂O, pH to 7.0 with NaOH. Store in aliquots at −80 °C following reconstitution.

4. 2 U/mL Acyl CoA Synthetase in 50 mM HEPES, pH 7.3. Store in aliquots at –80 °C following reconstitution.
5. 1 M HEPES pH 7.3.
6. 0.1% Triton X-100 (prepared from a 100% Triton X-100 stock).
7. 1 M DTT.
8. 1 M MgCl₂.
9. Grace™ Alltech™ Maxi-Clean™ Solid-Phase Extraction Cartridges, C18 (600 mg).
10. 50 mM ammonium acetate.
11. 25% acetonitrile in 50 mM ammonium acetate.
12. 45% acetonitrile in 50 mM ammonium acetate.

2.6 In Vitro Shh Peptide Fatty Acylation Assays

1. Reaction buffer: 167 mM MES, pH 6.5, 1.7 mM DTT, 0.083% Triton X-100.
2. RIPA buffer: 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA.

2.7 Recombinant Shh Protein Expression

1. Shh cDNA encoding amino acids 24–197 in pET19b plasmid.
2. Competent BL21DE3pLysS E. coli cells.
3. SOC chloramphenicol–ampicillin agar plates: 2% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, 25 µg/mL chloramphenicol, 40 µg/mL ampicillin.
4. LB medium: 1% tryptone, 0.5% yeast extract, 0.5% NaCl.
5. LB chloramphenicol–ampicillin medium: LB, 25 µg/mL chloramphenicol, 40 µg/mL ampicillin.
6. 1 M IPTG.
7. Resuspension Buffer: 50 mM Tris–HCl pH 8, 20% sucrose.
8. 5 M NaCl.
9. 1.43 M β-mercaptoethanol (1:10 dilution of the liquid stock supplied by the manufacturer).
10. 2 M imidazole.
11. 10% IGEPAL [0.1%].
12. Wash Buffer: 350 mM NaCl, 20 mM Tris–HCl pH 8.0, 10–20 mM Imidazole, 1 mM βME.

13. Elution Buffer: 350 mM NaCl, 250 mM Imidazole, 20 mM Tris-HCl pH 8.0, 1 mM β ME.
14. Dialysis Buffer: 350 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM β ME.
15. Storage buffer: 20 mM Tris-HCl pH 8.0, 1 mM β ME.
16. Enterokinase.

3 Methods

3.1 Cell Transfection

1. Grow cells in a 100 mm tissue culture dish until they are 70–80% confluent.
2. Add 6 μ g of either Hhat or Porcupine cDNA plasmid to 15 μ L Lipofectamine 2000 and incubate for 20 min at room temperature. Add DNA-lipid complex dropwise to cells and incubate overnight at 37 °C.
3. The following day, split the cells 1:2 and grow for 24–48 h.

3.2 P100 Membrane Preparation (Volumes per 1 $\times$ 100 mm Tissue Culture Plate)

1. Aspirate media from tissue culture plate.
2. Rinse plate with 5–10 mL cold NTE.
3. Scrape or pipette cells off the dish with 5–10 mL NTE. Centrifuge cells at 1000 $\times g$ for 5 min at 4 °C.
4. Add 0.8 mL Hypotonic lysis buffer to the cell pellet. Gently vortex and incubate on ice for 15 min.
5. Break open cells by 30 up and down strokes in a Dounce homogenizer, being careful not to cause foaming (see Note 4).
6. Add 200 μ L 5 $\times$ sucrose and 2 μ L 0.5 M EDTA.
7. Centrifuge cell suspension at 1000 $\times g$ for 10 min at 4 °C. Use a glass Corex tube so you can see the pellet clearly.
8. Carefully remove the supernatant (S1) and transfer to an ultracentrifuge tube. Avoid pulling up any of the nuclear pellet.
9. Resuspend the pellet in 1 mL Sucrose/Tris/EDTA and Dounce homogenize 10 strokes.
10. Centrifuge at 1000 $\times g$ for 10 min at 4 °C. Remove supernatant and combine with the S1 supernatant from **step 8** above.
11. Centrifuge the combined S1 fractions at 100,000 $\times g$ for 45 min at 4 °C.
12. Remove the supernatant. Resuspend the pellet (P100) in Hypotonic Lysis Buffer/0.25 M sucrose/1 mM EDTA/protease inhibitor, (Thermo Scientific™ Halt™ Protease Inhibitor Single-Use Cocktail [AEBSF, aprotinin, bestatin, E64, leupeptin, pepstatin A]) and Dounce homogenize.

13. Aliquot, quick freeze on dry ice, and store at 80 °C. Membrane aliquots should be thawed only once.

3.3 Hhat Purification

1. Start with 20 × 100 mm plates of 293FT cells transfected with Hhat-HA-Flag-His in pcDNA3.1. Remove plates from incubator and place on ice. All manipulations from this point on are performed on ice or in the cold room and all solutions and buffers are at 4 °C.
2. Aspirate media and wash each plate twice with 5 mL of NTE. 293FT are weakly adherent so wash as gently as possible.
3. Add 5 mL of NTE to each plate and scrape off cells with a rubber policeman. Collect and combine the suspensions into two 50 mL tubes.
4. Centrifuge at 1000 × *g* for 10 min at 4 °C.
5. Aspirate supernatant and resuspend each cell pellet in 4 mL of HEPES Lysis buffer. Keep tubes on ice for 15–30 min.
6. Combine both suspensions and lyse in a Dounce homogenizer, using 30–40 up and down strokes. Add 2 mL of 5 × Sucrose and homogenize 10–15 more strokes. Transfer homogenate to Beckman Ti 70.1 ultracentrifuge tubes. Spin in ultracentrifuge for 45 min at 45,000 rpm (186,000 × *g*), 4 °C.
7. Aspirate supernatant. Resuspend pellet in 8 mL Hypotonic Lysis Buffer +2 mL 5 × Sucrose and collect membranes by ultracentrifugation as above.
8. Aspirate supernatant. Resuspend pellet in 10 mL of solubilization buffer. If desired, the lysate can be stored at this point by freezing in liquid N₂ and storing overnight at –80 °C. The following day, thaw on ice and proceed to **step 9**.
9. Rock in cold room for 1 h to solubilize Hhat. Spin as in **step 6** to pellet insoluble material.
10. While the tube is spinning, prepare FLAG M2 Resin. Remove 1 mL from the 50% slurry and spin down at 850 × *g* in a 15-mL conical tube. Aspirate the supernatant (packing buffer). Resuspend the beads in 500 µL Solubilization buffer, mix, then collect resin by centrifugation. Repeat this wash **step 2** more times.
11. Transfer the supernatant containing solubilized Hhat from **step 9** to a 15 mL conical tube containing 1 mL of the prewashed resin (packed beads) in solubilization buffer.
12. Rock in cold room (4 °C) for 1 h.
13. Spin down the beads at 850 × *g*. Remove and save the supernatant as a precaution.
14. Wash beads 4 times with 5 mL of Solubilization buffer. Save washes as above.

15. After the last wash, remove as much wash buffer as possible without disturbing the beads. Then add 500 μ L of Elution Buffer and mix and spin as in **step 13**.
16. Remove and save supernatant. Repeat **step 15** two additional times for a total elution volume of 1.5 mL.
17. Remove a 50 μ L aliquot for Western blotting analysis.
18. Aliquot the remaining eluant, freeze in liquid nitrogen and store at -80°C (see Note 5).

3.4 Iodo-Fatty Acid Synthesis

1. Prepare a reaction vial by melting the tip of a Pasteur pipette. Set the temperature of a heating block to 55°C and place in a fume hood used for iodination. Place a crystallization dish containing mineral oil onto the heating block and insert a thermometer to monitor the temperature.
2. Add 200 μ L of IC16 or IC15:1 (10 μ M) resuspended in acetone into the reaction vial, dry under nitrogen and add 10 μ L of glacial acetic acid.
3. From this point on, work behind a lead-embedded plexiglass shield. Resuspend the NaI^{125} in 200 μ L of acetone and add to reaction vial. Rinse the container with another 200 μ L acetone and add to reaction vial.
4. Seal the pipette reaction vial with an 8 mm rubber septum. Insert a charcoal trap (sandwich activated charcoal between glass wool inside a 5 cc syringe with a 23 gauge needle) and incubate in the 55°C oil bath overnight or for 18 h.
5. The next day, remove the septum and trap and dispose. Add 0.5 mL water, and extract the aqueous layer twice with 1.2 mL chloroform. Transfer the combined chloroform (lower) layers to a glass 1-dram vial and dry under air or nitrogen. Dissolve the residue in 500 μ L ethanol.

3.5 Iodo-FattyAcyl CoA Synthesis

1. Set up a reaction with the following components: 100 μ L 1 M HEPES pH 7.3, 1 μ L Triton X-100, 250 μ L 20 mM Lithium CoA, 200 μ L 50 mM ATP, 1 μ L 1 M DTT, 100 μ L [^{125}I]IC16 or [^{125}I]IC15:1, 125 μ L 2 units/mL Acyl CoA synthetase, 10 μ L 1 M MgCl_2 , 213 μ L dH_2O . Incubate at room temperature for 3 h with stirring.
2. Preactivate a C18 reversed-phase column with 10 mL methanol, followed by a 5 mL H_2O wash and a 5 mL 50 mM ammonium acetate wash (see Note 6).
3. Add 5 mL Ammonium acetate to the column, and then add the reaction mix.
4. Wash the column with 15 mL 50 mM ammonium acetate.
5. Wash the column with 25 mL 25% acetonitrile in 50 mM ammonium acetate.
6. Elute in 5–8 mL 45% acetonitrile in 50 mM ammonium acetate.
7. Aliquot the eluate and dry down by rotary evaporating for 2.5–3 h.

8. Resuspend in 400–500 μL dH_2O .
9. Prepare a 1:1000 dilution in water and measure the activity of the preparation by determining counts per minute using a gamma counter.
10. Prepare a 1:200 dilution and measure the concentration of the preparation, using the extinction coefficient of palmitoyl CoA (*see* Note 7).

3.6 Recombinant Shh Protein Expression

1. Prepare SOC agar plates that contain both chloramphenicol [25 $\mu\text{g}/\text{mL}$] and ampicillin [100 $\mu\text{g}/\text{mL}$] (*see* Note 8).
2. Add 1 μL of plasmid DNA encoding Shh 24–197 in pET19b to 20 μL of competent BL21DE3pLysS *E. coli* cells.
3. Incubate on ice for 5 min.
4. Heat-shock for 30 s in a water bath set to 42 $^{\circ}\text{C}$.
5. Place cells on ice for 2 min.
6. Add 80 μL of SOC or LB media to each tube of cells.
7. Incubate cells for 1 h in a shaker set to 250 rpm and 37 $^{\circ}\text{C}$.
8. Plate the entire 80 μL of transformed cells and incubate plates for >16 h at 37 $^{\circ}\text{C}$ (*see* Note 9).
9. Pick one colony and grow overnight in 4 mL of LB containing 25 $\mu\text{g}/\text{mL}$ chloramphenicol and 100 $\mu\text{g}/\text{mL}$ ampicillin.
10. The next day, add 2.5 mL of the overnight culture to 100 mL of LB containing 25 $\mu\text{g}/\text{mL}$ chloramphenicol and 100 $\mu\text{g}/\text{mL}$ ampicillin.
11. Grow the culture in a shaker at 37 $^{\circ}\text{C}$, rpm ~250. Monitor the OD at 600 nm periodically.
12. When the culture reaches an OD of 0.8, induce protein expression by adding 100 μL of 1 M IPTG (Final concentration = 10 mM IPTG). Incubate for ~3 h in shaker.
13. Pour culture in a 50 mL falcon tube and spin down @ 1000 $\times g$ for 10 min. Discard the supernatant and spin the rest of the culture.
14. Remove the supernatant and flash-freeze the pellet in liquid nitrogen. Store at -80°C .
15. Put the pellet on ice and allow it to thaw.
16. Resuspend the pellet in ~ 1 mL of Resuspension Buffer, and add: 150 μL of 5 M NaCl (final concentration, 0.5 M), 1 μL of ten-fold dilution βME (1 mM), 15 μL of 100 mM PMSF (final conc., 1 mM), 7.5 μL of 2 M imidazole (final concentration, 10 mM), 15 μL of 10% IGEPAL (final concentration, 0.1%). Mix by pipetting up and down. AVOID BUBBLES.

17. Keeping the suspension on ice at all times, dissociate with six, 30 s pulses at 20% using a microtip on a sonicator. Allow the mixture to cool (for ~30 s) between pulses. Keep the sample on ice and make sure it does not get too hot (*see* Note 10).
18. Transfer the sonicated suspension to a clean tube.
19. Save a 10 μ L sample (label it “prespin”). Place it on ice.
20. Centrifuge the remainder of the sample for 30 min, $19,000 \times g$, at 4 °C.
21. Transfer the supernatant to a clean tube. Take a 10 μ L sample for the gel and label it “sup.”
22. Prepare the nickel resin by taking 100 μ L of Ni slurry using a wide-bore pipette tip, place it in a new microfuge tube, and spin in a microfuge ($6500 \times g$, 5 min, 4 °C). Remove the ethanol from the Ni beads, and add 1 mL of Wash Buffer. Vortex and spin in a microfuge ($6500 \times g$, 5 min, 4 °C). Repeat two more times.
23. Add 100 μ L of Ni slurry to the supernatant from step 21.
24. Place the sample on a nutator in the cold room and rock for 10 min (*see* Note 11).
25. Remove the sample from the nutator and spin down in a microfuge ($6500 \times g$, 5 min, 4 °C).
26. Remove supernatant. Save the supernatant and keep it on ice. Take a sample for the gel and label it “unbound.”
27. Wash the pellet with 1 mL Wash Buffer and centrifuge (8000 rpm, 5 min, 4 °C). Repeat two more times. Take gel samples from each wash, label each “Wash 1, 2, or 3.” Keep each wash and put them on ice.
28. Elute the protein from Ni beads: Add 100 μ L of Elution Buffer to the beads (1,1 ratio of elution buffer to Ni beads). Place sample on the nutator for 10 min. Spin sample down $6500 \times g$, 5 min, 4 °C. Remove supernatant from beads. Repeat the Elution step one more time and pool both Elution Fractions and label the pooled sample “Eluate.” Take a 10 μ L sample from the “Eluate” for the gel.
29. Flash-freeze the sample and store at -80 °C. All other samples that were taken from this prep can also be flash-frozen and stored at -80 °C.
30. Prepare Dialysis Buffer. Place buffer in the cold room in a beaker. Soak the dialysis cassette in dialysis buffer.
31. Defrost the eluate sample. Add ~8–10 units of enterokinase directly to the Eluate sample and mix by pipetting up and down (*see* Note 12).
32. Add the eluate plus enterokinase sample into the dialysis cassette using a syringe. Dialyze O/N in the cold room (4 °C).

33. The next day, remove the fraction from the dialysis cassette (take a sample for the gel). Filter the fraction through a 2 μ m filter (take a sample for the gel) (*see* Note 13).
34. Place the filtered sample over the Ni beads again (*see steps 24–29*).
35. Take the supernatant (which contains your cleaved purified protein) keep on ice (take sample for gel).
36. Elute the remaining protein off the beads using elution buffer (This elution contains uncleaved protein) (*see step 28*) (take sample for gel). Analyze all fractions by SDS-PAGE with Coomassie blue staining. The total yield of Shh protein from a 100 mL culture is 40–50 μ g. The culture can be scaled up accordingly for larger protein yield.

Concentrating Protein into Storage Buffer (Only do this for Large Scale Preps) (*see* Note 14).
37. Soak the membrane containing portion of a Micon Centriprep YM-10 10,000 MWCO concentrator in storage buffer.
38. Place the protein solution and the storage buffer in the designated compartments and spin (*see* Note 15).
39. Concentrate the protein down to ~3 mg/mL. Determine the concentration with the Bradford reagent or other protein determination kit.
40. Aliquot the protein into eppendorf tubes (~100 μ L/tube). Analyze by either Coomassie staining of an SDS-PAGE gel, or by Western blotting with anti-Shh antibody.

3.7 In Vitro Shh Pep Fatty Acylation Assay

1. In a 100 μ L Eppendorf tube, add 10 μ g of P100 membranes prepared from 293FT cells expressing Hhat, 100 μ M Shh peptide, 167 μ M [125 I]IC16 CoA, and 30 μ L reaction buffer.
2. Incubate for 1 h at room temperature.
3. Add 400 μ L RIPA buffer and 50 mL streptavidin-agarose beads. Incubate for 1 h at 4 $^{\circ}$ C with continuous mixing.
4. Centrifuge at 1000 $\times g$ for 5 min at 4 $^{\circ}$ C.
5. Wash the beads (pellet) three times with 500 μ L RIPA buffer.
6. Count 125 I cpm in a γ counter.

3.8 In Vitro Shh Protein Fatty Acylation Assay with purified hhat

1. Combine the following in a final volume of 50 μ L: 10 μ L Hhat-HA-Flag-His in 20 mM HEPES, pH 7.3, 350 mM NaCl, 1% octylglucoside and 1% glycerol, 10 μ L recombinant Shh (dilute purified prep to 0.2–0.4 mg/mL in 20 mM MES, pH

6.5, 1 mM EDTA, 1 mM DTT) and 30 μ L of reaction buffer containing 167 μ M [125 I]IC16 CoA (Subheading 2.6, item 1).

2. Incubate for 30 min at room temperature.
3. Stop with 50 μ L 2 $\times$ SDS-PAGE sample buffer.
4. Run samples on 12.5% SDS-PAGE; dry gel; expose to phosphorimager. Quantify 125 I cpm incorporation into the 19 kDa Shh protein band.

3.9 In Vitro Wnt Peptide Acylation Assay [15]

1. In a 100 μ L Eppendorf tube, add 10 μ g of P100 membranes prepared from 293FT cells expressing Porcupine, 100 μ M Wnt peptide, 167 μ M [125 I]IC15:1 CoA, and 30 μ L reaction buffer.
2. Incubate for 1 h at 37 $^{\circ}$ C.
3. Add 400 μ L RIPA buffer and 50 mL streptavidin-agarose beads and incubate for 1 h at 4 $^{\circ}$ C with continuous mixing.
4. Follow **steps 4–6** as described for Subheading 3.7 above.

4 Notes

1. Any transfection reagent suitable for protein expression in mammalian cells can be used.
2. Synthesis and purification of a double disulfide bonded peptide is technically challenging, time-consuming, and expensive. We used AnaSpec (Fremont, CA) to synthesize C-terminal biotinylated (Biotin-PEG NovaTagTM), disulfide-bonded Wnt3a WT (MHL KC(S-)KC(S-)HG LSG SC(S-)E VKT C(S-)WW) and C-terminal biotinylated (Biotin-PEG NovaTagTM) disulfide-bonded Wnt3a S209A (MHL KC(S-)K C(S-)HG LAG SC(S-)E VKT C(S-)WW) and then analyzed by HPLC to assess purity to be greater than 95%. The handling of the peptides is a critical aspect of the procedure as the peptides must be used immediately after reconstitution for optimal results. Additionally, because of the limited quantity that can be synthesized at one time, great care must be taken to use the peptides as efficiently as possible as synthesis can take up to 2 months.
3. The iodination procedure should be conducted in a fume hood designated for iodinations, and all manipulations should be behind lead-embedded shielding. Monitor the surrounding areas at all times using a Geiger counter. The temperature of the oil should be checked at least one additional time prior to overnight incubation. Personnel should have thyroid scans prior to and after the iodination reaction.
4. When lysing cells by Dounce homogenization, check for cell lysis under the microscope.
5. To check the purity of purified Hhat by silver-stained gel, the final Flag eluate fraction should be concentrated to a final volume of ~100–200 μ L.

6. We use Grace™ Alltech™ Maxi-Clean™ Solid-Phase Extraction Cartridges, C18 (5122340). Push solutions through the column slowly and remove column when removing the plunger to add solutions as to avoid damaging the column.
7. The concentration of Palmitoyl-CoA can be determined using an extinction coefficient of $15.4 \times 10^3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 260 nm.
8. Prepare plates at least 1 day before you plan to transform.
9. To avoid over growth, split the 80 μL and plate 40 μL on two plates.
10. The suspension should become less viscous after sonication. If there is no change in viscosity, use a stronger or longer pulse. Be careful not to oversonicate the suspension, as this may lead to denaturation of the protein.
11. The Ni beads should remain suspended at all times during the incubation.
12. One unit is defined as the amount of enterokinase required to cleave ~25 μg of the fusion protein at 25 °C for 16 h.
13. Affinity based removal of the residual enterokinase from the cleavage reaction mixture can be done at this stage. We use the enterokinase removal kit from Sigma and follow their protocol.
14. The purpose of this step is to bring the protein to a concentration suitable for the in vitro reaction, and to lower the salt concentration to ~80 mM–125 mM.
15. This step depends on the type of concentrator that you will use. See the directions for your concentrator of choice.

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Fig. 1.
Sequences of the Shh (top) and Wnt3a (bottom) biotinylated peptides