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Membrane targeting of palmitoylated Wnt and Hedgehog revealed by chemical probes

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ABSTRACT

Palmitoylation of the Wnt and Hedgehog proteins is critical for maintaining their physiological functions. To date, there are no reported studies that characterize the cellular distribution of the palmitoylated forms of these proteins. Here, we describe the subcellular localization of palmitoylated Wnt and Sonic Hedgehog by using a highly sensitive and non-radioactive labeling method that utilizes alkynyl palmitic acid. We show that palmitoylated Wnt and Sonic Hedgehog localize to cellular membrane fractions only, highlighting a role for palmitoylation in the membrane association of these proteins. The method described herein has the utility to validate inhibitors of Wnt and Hedgehog acyltransferases in drug discovery, and enables further investigations of the role of palmitoylation in the secretion and signaling of these proteins.

1. Introduction

The Wnt and Hedgehog (Hh) pathways are critical regulators of embryonic development and cancer stem cell differentiation [1]. Emerging evidence indicates that deregulation of Wnt or Hh signaling is implicated in a number of human diseases. In particular, Hh ligand overexpression is correlated with colon, pancreatic and ovarian cancers [2,3]. Furthermore, activating mutations in the Wnt pathway are associated with several cancer types, such as breast, lung, colon and pancreatic [4,5].

Posttranslational palmitoylation of Wnt and Hh proteins regulates their signaling activity. For example, palmitoylation of Wnt is critical for the protein's ability to bind to its cell surface Frizzled receptors and initiate downstream signaling events leading to stabilization of β-catenin [6,7]. To a lesser extent, palmitoylation of Hh, together with cholesterol, permits it to bind to the cell surface, which appears to mediate its interaction with the receptor Patched to trigger activation of the downstream Gli zinc-finger transcription factors [1]. While the palmitoylation sites of these proteins have been identified, how palmitoylation regulates their function and localization at the molecular level is less well understood.

Protein palmitoylation involves the enzyme-catalyzed addition of a 16-carbon saturated fatty acid to cellular proteins [8,9]. For example, it has been shown that Wnt3a is palmitoylated at Cys 77 [10] and palmitoleoylated at Ser 209 [11]. Autoprocessing of Shh leads to generation of an N-terminal fragment (Shh-N) that undergoes concomitant cholesterol addition to its C-terminus [12], and palmitoylation at its N-terminus [13]. To our knowledge, there are no studies reported to date that characterize the cellular distribution of the palmitoylated forms of these proteins. Earlier studies utilizing site-directed mutagenesis combined with antibody detection and detergent phase separation methods revealed that palmitoylation-defective mutants of Wnt and Hh partitioned to the aqueous phase, while the wild-type proteins were present in the detergent phase [6,10,13,14], though the nature of the membranes is as yet unclear.

Our understanding of palmitoylation and its role in Wnt and Hh signaling remains elusive. It is widely appreciated that significant challenges exist in detecting the palmitoylation status of these proteins. This is in part due to the lack of robust non-radioactive methods that allow rapid detection of distinct lipid modifications on Wnt or Hh in a routine manner. The use of radioactivity combined with subcellular fractionation is cumbersome because of safety hazards and long exposure times associated with autoradiography.

Abbreviations: Hh, Hedgehog; Shh, Sonic Hedgehog; Alk-C16, ω-alkynyl palmitic acid; BPA, bromopalmitic acid; PA, palmitic acid; IWP-2, inhibitor of Wnt production; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine

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Furthermore, mass spectrometry methods are not straightforward because of the technical complexities associated with the hydrophobic nature of lipid-modified peptides. To address these issues, our lab has recently developed ω-alkynyl fatty acid analogues as tools for monitoring the lipid modifications of cellular proteins [15]. Using this technique, which includes an ω-alkynyl palmitic acid probe (Alk-C16 [9,15], Fig. 1) combined here with immunoprecipitation and subcellular fractionation, we report the non-radioactive detection of palmitoylated Wnt and Sonic Hedgehog (Shh) and describe their membrane attachment. The method described herein is highly sensitive, specific, rapid and does not comprise the use of hazardous radioactive reagents.

2. Materials and methods

2.1. Cell culture

Mouse fibroblast L-cells stably overexpressing Wnt3a (ATCC no. CRL-2647) or human embryonic kidney (HEK) 293 cells stably overexpressing Shh (Genentech) were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.4 mg/ml Geneticin® and 2 mM Glutamax™. All cells used were incubated in a 5% CO_2 humidified incubator at 37 °C for 24 h before any experiments.

2.2. Metabolic labeling of lipid modified proteins

ω-alkynyl palmitic acid (Alk-C16) was synthesized as previously described [15]. It was dissolved in DMSO to a final stock concentration of 50 mM and stored at -80 °C. Before cell treatment, Alk-C16 was diluted to a final concentration of 100 μM in DMEM supplemented with 5% BSA (fatty acid-free). The Alk-C16-containing medium was sonicated for 15 min at room temperature and then allowed to precomplex for another 15 min. Cells were seeded onto a 10 cm culture dish and incubated for 24 h before treatment. After removal of the growth medium, cells were washed once with PBS before addition of Alk-C16-containing medium (5 ml). After a 6 h incubation at 37 °C/5% CO_2, cells were washed three times with cold PBS. For detection in total lysates, cell extracts were prepared in 400 μl of lysis buffer (100 mM sodium phosphate, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, Pierce protease and phosphatase inhibitor cocktail). Protein extracts (~2 mg/ml) were then subjected to the click labeling reaction in a 100 μl volume for 1 h at RT at final concentrations of the following reagents: 0.1 mM biotin-azide, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich) dissolved in water, 0.2 mM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, Sigma–Aldrich) dissolved in DMSO/*tert*-butanol (20%/80%) and 1 mM $CuSO_4$ in water.

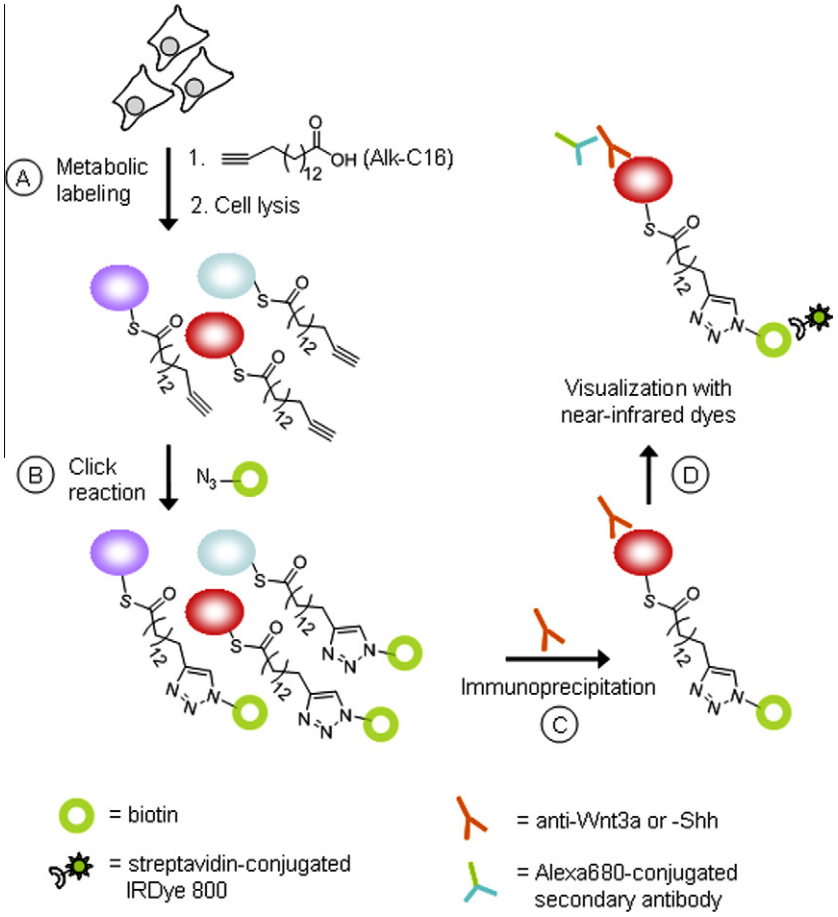

Fig. 1. Scheme for metabolic labeling and biochemical detection of palmitoylated Wnt and Hedgehog proteins. ω-Alkynyl palmitic acid (Alk-C16) is added to cultured cells and is incorporated into fatty acylated proteins, denoted by colored ovals (step A). After cell lysis, the alkynyl group is chemoselectively ligated to azide-tagged biotin by a Cu(I)-catalyzed [3+2] cycloaddition (click) reaction (step B). The biotin-conjugated target Wnt or Hedgehog proteins are then immunoprecipitated with specific antibodies (step C) and subsequently detected by streptavidin-conjugated infrared dye (step D), with optional detection of total Wnt or Hedgehog using fluorescent (Alexa680)-conjugated secondary antibodies..

2.3. Immunoprecipitation of Wnt3a and Shh

Cellular lysates were precipitated with 5 times volume of ice-cold acetone, vortexed and incubated overnight at –20 °C. The mixture was centrifuged at 16 000×*g* for 10 min at 4 °C and the supernatant was aspirated. The obtained pellet was air-dried for 5 min and then resuspended in 400 μl of lysis buffer. The lysates were sonicated for 10 min and then incubated with a final concentration of 5 μg/ml of well-characterized anti-Wnt3a (ab28472, Abcam) or anti-Shh (Murine 5E1 [16]; developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA) antibodies for 2 h at 4 °C with rocking, and subsequently immunoprecipitated with 40 μl of 1:1 mixture of protein-A (P2545, Sigma): protein-G (P3296, Sigma) sepharose beads with rocking for 1 h at 4 °C. The beads were washed six times with PBS containing 0.1% Tween-20 (PBST). They were then resuspended in 40 μl of lysis buffer containing SDS sample buffer and dithiothreitol (50 mM), heated for 5 min at 95 °C and resolved by SDS–PAGE.

2.4. Pull-down of Wnt3a from conditioned medium

Wnt3a conditioned medium was collected from L-Wnt3a cells that had been treated with Alk-C16 or DMSO for 48 h. The medium was incubated with 40 μl Blue Sepharose beads (17-0948-02, GE Healthcare Biosciences) on a rocking platform for 2 h at 4 °C. The beads were washed 6 times with PBST. Lysis buffer containing SDS sample buffer and 50 mM dithiothreitol (40 μl) was added to the beads and the mixture was heated at 95 °C for 5 min before being resolved on SDS–PAGE.

2.5. Cellular fractionation

Cells that had been metabolically labeled with Alk-C16 were fractionated as previously described [17]. Briefly, cells were washed twice with cold HEPES buffer (150 mM NaCl, 50 mM HEPES, pH 7.4), scraped off the tissue culture plates using a cell lifter, and collected. Cells were spun down at 1000×*g* for 10 min, resuspended in 10 ml of hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM HEPES pH 7.4 with EDTA-free complete protease inhibitors) for 20 min on ice, and then homogenized using a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 1000×*g* for 5 min at 4 °C and the postnuclear supernatant was collected. The pellet (nuclear fraction) was washed three times with HEPES buffer containing EDTA-free protease inhibitors (Pierce). The postnuclear supernatant was further centrifuged at 100 000×*g* for 30 min at 4 °C, yielding the cytosolic fraction (supernatant) and the membrane fraction (pellet). The pellet was washed three times with HEPES buffer containing EDTA-free complete protease inhibitors. Both the membrane and nuclear fractions were solubilized in HEPES buffer containing EDTA-free complete protease inhibitors and 1% SDS. The cytosolic fraction was also adjusted to 1% SDS. The protein concentration of all fractions was determined by the BCA assay (Thermo Scientific) according to manufacturer's recommendations. Protein extracts (0.7 mg/ml) were then subjected to the probe labeling reaction in a 100 μl volume, for 1 h at RT as described above.

2.6. Western blot

Labeled protein lysates or immunoprecipitated proteins were resolved by SDS–PAGE using 4–20% Tris-glycine gels. For immunoblotting of biotin-labeled proteins after electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was subsequently blocked with Odyssey Blocking Buffer overnight at 4 °C. The membrane was incubated with primary antibodies (anti-Wnt3a (ab28472), Abcam, 1:500; anti-Shh H-160 (sc-9024), Santa Cruz Biotechnology, 1:500; anti-Tim23 (611222), BD Biosciences, 1:2000; anti-GAPDH (2118) and anti-Lamin A/C (2032), Cell Signaling, both 1:500) in Odyssey Blocking Buffer for 1 h at RT. The membrane was washed three times with PBST (10 min each) and incubated with Alexa680-conjugated secondary antibodies in Odyssey Blocking Buffer containing 0.1% Tween-20 and 0.01% SDS. The membrane was then washed three times with PBST (10 min each). For detection of biotin-labeled proteins, the membrane was incubated with streptavidin-conjugated IRDye800 in Odyssey Blocking Buffer containing 0.1% Tween-20 and 0.01% SDS, then washed three times with PBST (10 min each). Total proteins were visualized in the 700 nm channel and biotin-labeled proteins were visualized in the 800 nm channel on an Odyssey Infrared Imager.

2.7. Cell-based Hh reporter assay

Hh signaling was measured using stably transfected *Gli*-luciferase reporter S12 cells as described previously [18]. Briefly, 10 000 S12 cells/well were plated on a white-walled clear-bottomed 96-well plate (Costar 3610) in regular growth medium (DMEM, 10% (v/v) FBS, 2 mM L-glutamine) for 24 h, and 20 000 HEK 293 cells stably overexpressing Shh were then co-cultured on top for 24 h, followed by 24 h of serum starvation (0.5% (v/v) FBS) in the presence of Alk-C16 (100 μM) or an equivalent volume of DMSO, prior to luciferase measurement (HTS SteadyLite kit, Perkin–Elmer Life Sciences).

2.8. Cell-based Wnt signaling activity assay

Wnt3a signaling was reported by measuring β-catenin levels in L- and L-Wnt3a cells as described previously [19]. Briefly, L or L-Wnt3a cells (5000 cells/20 μl/well) were seeded in a clear bottomed, black walled 384-well plate and grown for 24 h. Alk-C16 (20 μl), diluted in DMEM supplemented with 5% BSA (fatty acid-free), was added to a final concentration of 100 μM. After overnight incubation, the cells were fixed in 4% PFA by adding 20 μl of 12% PFA directly to the wells for 1 h at room temperature, permeabilized with PBS/0.1% Triton X-100 for 5 min, then blocked in Odyssey Blocking Buffer (50 μl/well) for 2 h at room temperature. The wells were incubated with mouse anti-β-catenin antibody (610154, BD Biosciences, 1: 200) in Odyssey Blocking Buffer for 2 h at room temperature (20 μl/well) and subsequently washed with PBST. Infrared anti-mouse IRDye800CW secondary antibody (1: 200) and DRAQ5 (1:10 000, Biostatus) in PBS/0.5% Tween-20 were then added (20 μl/well). The plates were incubated for 1 h at room temperature, and the wells were washed with PBST and incubated in PBS (50 μl/well). The plates were covered with black seals and imaged on an Odyssey infrared scanner.

3. Results and discussion

To probe palmitoylation of Wnt or Shh proteins, mouse fibroblast L-cells that stably express Wnt3a or human embryonic kidney (HEK) 293 cells that stably express Shh were incubated for 6 h with Alk-C16, a probe established earlier for the metabolic labeling of all cellular palmitoylated proteins [15,20–22]. The cells were then lysed, and the alkynyl-labeled palmitoylated proteins were chemoselectively ligated to azide-tagged biotin by a Cu(I)-catalyzed alkyne-azide [3+2] Huisgen cycloaddition reaction [15,23]. Total proteins were then precipitated to remove excess chemical reagents and the pellets were resuspended in lysis buffer. Wnt3a or Shh were subsequently immunoprecipitated using

Wnt3a- or Hh-specific antibodies and their biotin-tagged palmitate groups were detected by western blotting using streptavidin-conjugated IRDye800 near-infrared dye (Fig. 1).

Several major bands corresponding to Wnt3a were detected around 39 kDa, in agreement with its expected molecular weight (Fig. 2A). The multiple bands reflect the complex nature of the multiple post-translational modifications that exist on Wnt3a (see below). No labeling was observed in samples treated with DMSO, thereby yielding an excellent signal to background ratio. Moreover, the palmitate labeling of the Wnt3a bands with Alk-C16 was lost in the presence of a 5-fold molar excess of unlabeled palmitic acid, illustrating the specificity of the labeling reaction (Fig. 2A). More importantly, we find that Wnt3a labeled with Alk-C16 is secreted properly and detected in conditioned media to the same degree as the DMSO control (Fig. 2B). Consistent with this notion, and using a cell-based Wnt signaling activity assay [19], we further demonstrate that Alk-C16 treatment has no adverse effects on Wnt3a-mediated stabilization of β-catenin (Fig. 2C), indicating that Alk-C16-labeled Wnt3a retains its signaling activity.

We observe that most, if not all, of the immunoprecipitated Wnt3a bands are labeled with Alk-C16, indicating the presence of multiple palmitoylated species of Wnt3a (Fig. 2A). This can be explained in light of previous findings that clearly showed that Wnt3a can be glycosylated at several sites [6]. Given that N-linked glycosylation of Wnt3a appears to be necessary for its palmitoylation [6,24], it is conceivable that some of the non-palmitoylated Wnt3a bands contain few or no glycan modifications. Overall, the global biochemical profile revealed here provides insight into the diverse population of palmitoylated Wnt3a that exists in the cell. To further interrogate the scope of this novel labeling method,

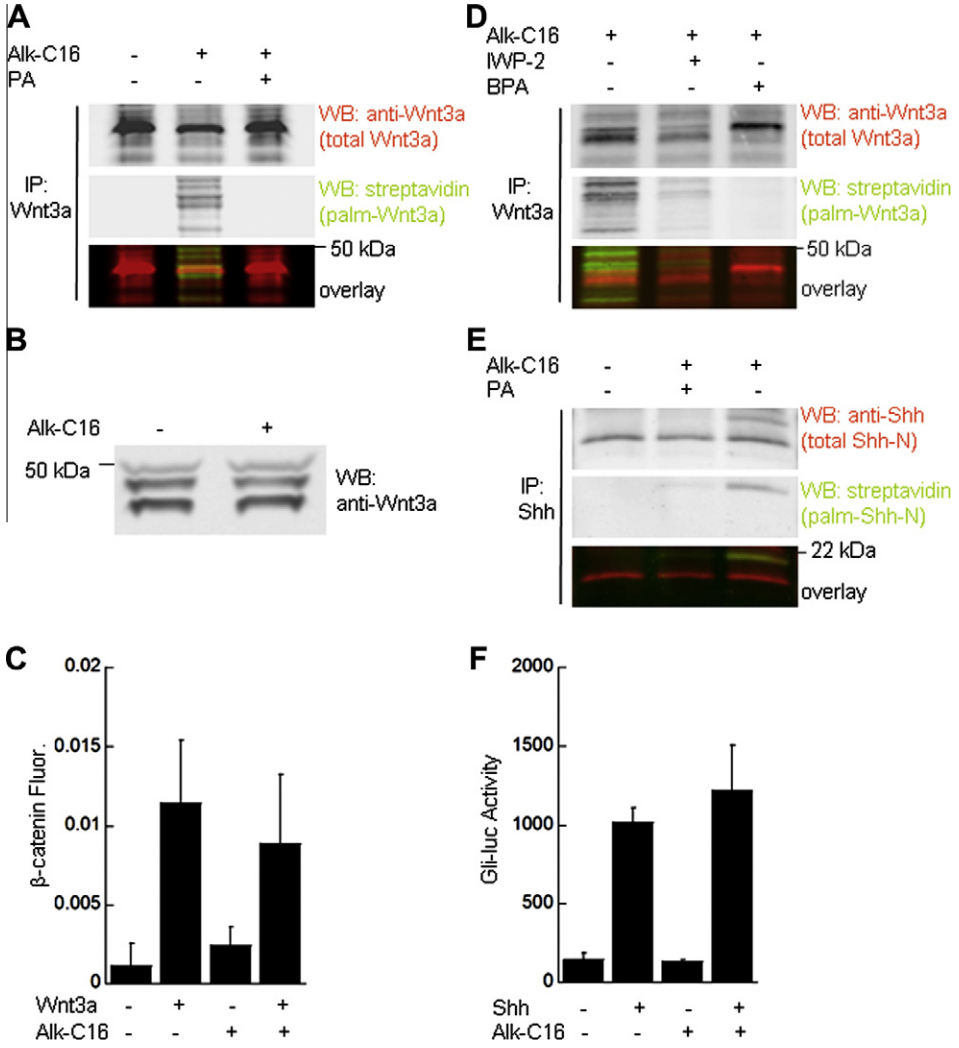

Fig. 2. Detection of the palmitoylated forms of Wnt3a and Sonic Hedgehog. Mouse L-cells stably expressing Wnt3a (A) or HEK293 cells stably expressing Shh (E) were treated with either DMSO or Alk-C16 in the absence or presence of a five-fold molar excess of unlabeled palmitic acid (PA) for 6 h. After preparation of the cell lysates and click reaction, Wnt3a or Shh were immunoprecipitated, resolved by SDS–PAGE and immunoblotted with anti-Wnt3a (A) or anti-Hh (E) primary antibodies, followed by Alexa680-conjugated anti-rabbit secondary antibodies and streptavidin-conjugated IRDye800 (for detection of biotin-conjugated palmitoylated Wnt3a and Shh). Multiple Wnt3a bands are detected at ~39 kDa, while a band corresponding to Shh-N is detected at ~19 kDa in all samples. For palmitoylated Wnt3a or Shh-N labeled with Alk-C16, see lane 2 (A) and lane 3 (E), respectively. (B) Alk-C16 labeled Wnt3a is secreted properly into conditioned media. Wnt3a was purified from conditioned media containing either DMSO or Alk-C16 using Blue Sepharose. The same level of secreted Wnt3a was detected from Alk-C16 or DMSO containing media. (C) Treatment with Alk-C16 does not interfere with Wnt3a signaling. Wnt3a signaling activity was reported by measuring the total level of β-catenin in the presence of DMSO or Alk-C16 in L- and L-Wnt3a cells. No significant difference between DMSO and Alk-C16 treatment is observed ($n = 8$). To account for minor differences in cell numbers, the β-catenin signal is normalized to DNA signal intensity. (D) Palmitoylation of Wnt3a is inhibited by IWP-2 or BPA treatment. L-Wnt3a cells were incubated overnight with Alk-C16 in the absence or presence of BPA (500 μM) IWP-2 (10 μM). (F) Treatment with Alk-C16 does not interfere with Shh signaling. Hh signaling activity was measured using stably transfected *Gli*-luciferase reporter S12 cells, co-cultured with HEK293 cells stably overexpressing Shh, in the presence of DMSO or Alk-C16, prior to luciferase measurement. No significant difference between DMSO and Alk-C16 treatment is observed ($n = 6$).

we took a pharmacological approach to assess whether it can be used to identify Wnt palmitoylation inhibitors. IWP-2 is an inhibitor of Wnt palmitoylation that functions by targeting Porcupine, an acyltransferase specifically responsible for Wnt3a palmitoylation [25], while bromopalmitic acid (BPA) is an inhibitor of total cellular palmitoylation. Treatment with either IWP-2 or BPA abolished palmitoylation of Wnt3a (Fig. 2D), as expected, further confirming the specificity of our labeling method. Together these data indicate that this method has the potential to be used for developing and validating inhibitors of Wnt acyltransferases, and also for dissecting the temporal dynamics of palmitoylation of Wnt and its role in signaling.

In cells expressing Shh, we detect a 19 kDa band that corresponds to the cleaved N-terminal fragment of Shh (Shh-N, Fig. 2E) but no ~45 kDa band corresponding to full-length Shh (data not shown). Treatment with Alk-C16 leads to the appearance of a single band that corresponds to palmitoylated Shh-N and is shifted to a higher molecular weight of approximately 22 kDa (Fig. 2E). This band is absent in the DMSO control and can be competed with excess palmitic acid (Fig. 2E), demonstrating specificity of the metabolic labeling reaction. However, only a fraction of the total immunoprecipitated band shifted, indicating that the palmitate labeling reaction is incomplete. This is most likely because Shh is unique in being palmitoylated via an irreversible amide bond rather than a thioester linkage [26], thus only non-palmitoylated Shh should be a substrate for Alk-C16. Additionally, there was no shift in the presence of excess unlabeled palmitate, indicating that it is the biotin (244 Da) conjugation rather than the palmitate (238 Da) per se that increases the apparent molecular weight. Furthermore, using a cell-based Hh reporter assay [18], we demonstrate that treatment with Alk-C16 has no adverse effects on Hh signaling (Fig. 2F). Overall, our data show that there exist two distinct populations of Shh, one that is represented by the palmitoylated upper band (labeled with Alk-C16) and the other represented by the lower band that is not labeled with Alk-C16. It is noteworthy that the current labeling method offers the advantage of visualizing both palmitoylated and total protein bands on the same gel, making it easy to differentiate superimposed or closely migrating bands.

The physiological function of protein palmitoylation is still not well understood. Acyltransferases that regulate palmitoylation of Wnt and Shh have been shown to be likely present in the lumen of the endoplasmic reticulum and/or Golgi apparatus [24,26]. These interesting observations prompted us to investigate the subcellular distribution of the palmitoylated forms of Wnt and Shh. To address this, we performed cellular fractionation of samples treated with Alk-C16. The cytosolic, membrane, and nuclear fractions obtained by differential centrifugation were probed with markers that are specific to these fractions such as GAPDH, Tim23, and Lamin A/C, respectively (Fig. 3). We find that total Wnt3a is highly enriched in the membrane fraction, with a minor subpopulation present in the cytosolic and nuclear fractions (Fig. 3A). More importantly, the palmitoylated form of Wnt3a is only found in the membrane fraction (Fig. 3A). The absence of palmitoylated Wnt3a from the cytosolic fraction reveals the pivotal role that palmitoylation plays in directing the association of Wnt3a with membrane-bound compartments. Collectively, our studies enable further investigation into the distribution of the palmitoylated form of Wnt3a across cellular membrane compartments.

We find that total Shh-N and palmitoylated Shh-N were present in the cellular membrane but not cytosolic fractions (Fig. 3B). It is striking that the lower Shh-N band not labeled with Alk-C16 is present in the membrane fraction. This could be either because the lower band was already irreversibly palmitoylated (and hence not accessible to the Alk-C16 labeling), or because palmitoylation is dispensable for Shh targeting to this compartment, as expected from the ability of the C-terminal cholesterol group to mediate membrane tethering [12]. The latter is consistent with earlier genetic studies demonstrating that a palmitoylation-defective mutant of Shh (Cys25Ser) is associated with lipid raft fractions in HEK293 cells [14]. All together, our data further support the existence of mechanisms other than palmitoylation, such as cholesterylation, that target Shh to membrane-bound compartments.

In summary, we report here the development of a facile and highly sensitive non-radioactive method to detect palmitoylation of Wnt and Hedgehog proteins. Furthermore, we characterized the unique subcellular distribution patterns of palmitoylated Wnt3a and Shh. The strong preference of palmitoylated Wnt3a and Shh towards localizing to cellular membrane compartments highlights membrane anchoring as one of the central functions of palmitoylation in these signaling pathways. While this manuscript was in preparation, a method for probing cholesterol modification

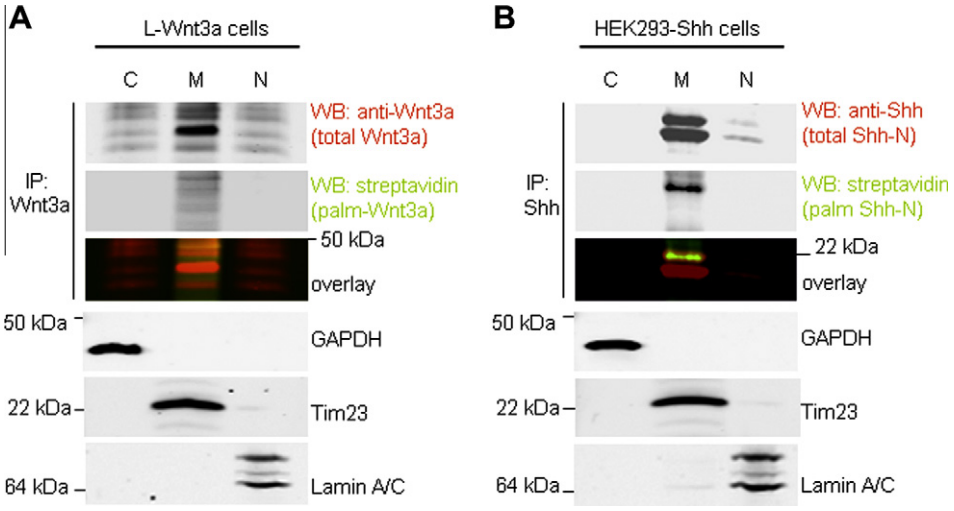

Fig. 3. Palmitoylated Wnt3a and Shh are exclusively detected in the cellular membrane fraction. (A) Cell extracts of L-cells stably expressing Wnt3a and treated with Alk-C16 were fractionated into cytosolic (C), membrane (M), and nuclear (N) fractions. Each fraction was subjected to immunoprecipitation followed by immunoblotting, as well as being probed with markers specific for these compartments, GAPDH, Tim23 and Lamin A/C, respectively. 3% of each fraction was loaded in each lane. Total Wnt3a is predominantly enriched in the membrane fraction and palmitoylated Wnt3a is exclusively detected in the membrane fraction. (B) Cellular extracts of HEK293 cells stably expressing Shh were processed as in (A), immunoprecipitated and detected by immunoblotting. Both Shh-N and palmitoylated Shh-N are detected in the membrane but not cytosolic fractions.

of Shh by using a bio-orthogonal cholesterol analogue was reported [27]. The study did not describe the membrane distribution of cholesterylated Shh; however this promising technique could allow for similar studies to examine the importance of cholesterol for directing Shh localization.

Wnt and Hh are attractive targets for the development of anticancer therapeutics [28,29]. Recent reports show that inhibition of Wnt3a palmitoylation by targeting the acyltransferase activity of Porcupine may be a viable strategy to counteract aberrant Wnt signaling [25]. Given its highly sensitive and non-radioactive nature, the method described herein has the utility to validate the mechanism of small molecule inhibitors of palmitoylation in drug discovery. Also, it facilitates investigation of the subcellular distribution of palmitoylated Wnt and Shh, and it enables further studies of the role of palmitoylation in the secretion and signaling activity of these proteins.

Author contributions

Xinxin Gao, Natalia Arenas-Ramirez, Suzie Scales and Rami Hannoush performed experiments. Xinxin Gao and Rami Hannoush designed and analyzed experiments and wrote the paper.

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