Stereoselective fatty acylation is essential for the release of lipidated WNT proteins from the acyltransferase Porcupine (PORCN)

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ABSTRACT

The maintenance of adult animal tissues is dependent upon highly conserved intercellular signaling molecules that include the secreted WNT proteins. Although it is generally accepted that lipidation of WNTs by the acyltransferase Porcupine (PORCN) and their subsequent recognition by the Wntless (WLS) protein is essential for their cellular secretion, the molecular understanding of this process remains limited. Using structurally diverse fatty acyl donor analogs and mouse embryonic fibroblasts (MEFs) expressing PORCN protein from different metazoan phyla, we demonstrate here that PORCN active-site features, which are conserved across the animal kingdom, enforce cis-Δ⁹ fatty acylation of WNTs. Aberrant acylation of a WNT with an exogenously supplied trans-Δ⁹ fatty acid induced the accumulation of WNT-PORCN complexes, suggesting that the fatty acyl species is critical to the extrication of lipidated WNTs from PORCN. Our findings reveal a previously unrecognized fatty acyl-selective checkpoint in the manufacturing of a lipoprotein that forms a basis for WNT signaling sensitivity to trans fats and to PORCN inhibitors in clinical development.

INTRODUCTION

Multicellular organisms that generate specialized tissues and organs must balance the needs of its constituent cells with those of the collective. Mechanisms that support the integration of metabolic processes and cell-cell communication provide a direct means for synchronizing efforts that meet these demands. For example, the immobilization of lipids onto proteins that function in cell signaling can dictate protein distribution in the intra- and extracellular milieu, or directly gate protein (1). The WNT proteins control cell fate decision-making in diverse tissues during development and homeostatic renewal (2,3). Animals typically express multiple WNTs that require lipidation for their secretory pathway transit and ultimate release into the extracellular milieu (4). Amongst the three characterized lipidated extracellular proteins that also include the Hedgehog (HH) and Ghrelin proteins, WNT proteins are unique in their subjection to this production checkpoint. This phenomenon involves a polytopic protein known as WLS, which fails to ferry WNT proteins to the extracellular space in the absence of PORCN activity (5,6). How WLS distinguishes between an acylated and naked WNT protein remains unknown.

PORCN is the founding member of the membrane bound O-acyltransferase (MBOAT) gene family which consists of 16 polytopic acyltransferases with established lipid and protein substrates (7-9). PORCN is the only enzyme that has been shown to covalently attach a
monounsaturated fatty acid (MUFA) onto proteins. Given the lipid adduct identity has only been determined in a single mammalian WNT protein, it remains unclear if it varies depending upon species-specific metabolic considerations or if evolutionary constraints have eliminated lipid diversity. Indeed, the mechanistic basis for WNT production dependency on MUFA acylation remains elusive.

The recent advance of PORCN inhibitors in clinical testing has galvanized efforts to gain structural insights into the basis of chemical specificity, and more broadly a mechanistic understanding of why loss of lipidation cripples WNT production (10-12). Here, using a cross-species PORCN interrogation platform and chemical probes that target the PORCN active site, we reveal PORCN harbors features conserved across diverse animals that enforce MUFA modification of WNTs. We further show this enforcement is coupled to the ability of lipidated WNT proteins to extricate from PORCN and transfer to WLS. Our studies reveal a previously unrecognized checkpoint for fatty acyl selectivity in WNT manufacturing that is the target of trans fats and synthetic inhibitors of PORCN.

RESULTS

Conservation of monounsaturated fatty acid (MUFA) labeling in WNT proteins

Despite the recognition that WNTs are found in all metazoans, unclear is whether or not this conservation extends to the nature of the covalently attached lipid moiety. To address this question, we assembled a collection of PORCN cDNAs from diverse metazoan phyla that in some cases reach >70% sequence divergence thus providing a means to broadly evaluate PORCN fatty acyl donor preferences within the animal kingdom (Fig. 1A, Supp. Fig 1). Next, we built upon an assay previously described using PORCN null mouse embryonic fibroblasts expressing a murine WNT3A protein to first evaluate the ability of various PORCN molecules to induce WNT signaling (13) (Fig. 1B). This approach affords a controlled experimental platform focused on a single WNT substrate while removing species- or cell line-specific lipid metabolic considerations from the observations. We then established that the PORCN proteins evaluated were indeed capable of promoting murine WNT3A activity as measured using either the synthetic WNT pathway reporter described above (Fig. 1B; Fig. 1C), or phosphorylation of Dishevelled 2 (DVL2), a Frizzled (FZD) receptor regulated signaling molecule (see Fig. 1B; Fig. 1D, Fig. 1E). A PORCN inhibitor WNT974 suppressed pathway activity mediated by PORCN from multiple sources, barring the activity mediated by Drosophila and Schistosoma PORCNs. These observations suggest a minimal WNT sequence requirement for maintaining an enzyme-substrate relationship. Indeed, PORCN is able to lipidate the disulfide-bonded β-hairpin that harbors the conserved acylated serine in WNT proteins when it is expressed in the context of a structurally distinct protein scaffold (14).

Having established a platform for measuring the activity of PORCN protein from diverse animals using the same cellular backdrop and a shared WNT substrate, we then evaluated the preference of various PORCN proteins for alkynylated palmitate (C16:0) or palmitoleate (C16:1n-7) (15). Observed for every PORCN protein tested was abundant labeling of the WNT protein with the palmitoleate but not the palmitate analogue (Fig. 1F). These observations that include PORCN from 4 out of the 9 animal phyla and representative animals that diverged ~1.2 billion years ago (between Nematoda and Chordata) (16) suggest that PORCN universally enforces a monounsaturated fatty acyl donor preference in metazoans. Fatty acid desaturation is crucial for the labeling of multiple WNT proteins as A939572, an inhibitor of stearoyl-CoA desaturase (SCD), which converts palmitate to palmitoleate, blocked WNT lipidation (Fig. 1G, Supp. Fig. 2). IWP2, a PORCN inhibitor structurally distinct from WNT974 (17,18), also inhibited WNT palmitoleation.

A universal carbon counting mechanism found in PORCN enforces WNT modifications with a MUFA

Although animals typically harbor multiple WNT genes, only a single WNT protein produced from an isolated cell line has been subjected to mass spectrometric analysis with the intention of identifying the adducted lipid (19). At the same time, inhibitors of stearoyl-CoA desaturase (SCD) which prevent WNT labeling with exogenously provided palmitate (20) are inconsistent in their activity against WNT signaling thus suggesting that lipids other than palmitoleic acid may be incorporated (Fig. 2A).
To directly probe the geometry of the acyl donor pocket in the PORCN active site, we labeled cells expressing a WNT3A fused with an Fc domain of immunoglobulin G (WNT3A-Fc) with various exogenously supplied lipid probes that differ with respect to desaturation position and length, and that would enable subsequent copper-assisted cycloaddition of a biotin-conjugated azide to the alkyne group (Fig. 2B, C). Whereas previous investigations into fatty acyl donor length preferences are consistent with those presented here showing PORCN favoring medium length fatty acyl chains (20-23), we also observed PORCN preference for the position of desaturation (Fig. 2C). Indeed, moving the double bond in palmitoleic acid to the Δ7 and Δ11 positions (in case of C16:1n-9 and C16:1n-5 respectively) greatly diminished the ability to label WNT protein. In contrast, the HHAT acyltransferase did not exhibit any fatty acyl donor selectivity for acylating SHH-Fc protein in cultured cells. This data suggests that determinants within the PORCN site measure the distance of the double bond relative to the coenzyme A (CoA) group (Fig. 2D).

Bypassing PORCN fatty acyl donor stereoselectivity using an exogenously provided trans fat

A challenge to interrogating the role of the WNT lipid adduct in WNT manufacturing and signaling has been an inability to experimentally examine the consequences of immobilizing alternate fatty acids (FAs) onto WNT proteins. Our studies using PORCN from diverse animals species failed to delineate a strategy for attaching other fatty acids onto WNT proteins given the conservation of active site features that enforce the use of a cis palmitoleic acid. A FA with a trans double bond at the Δ9 position in a gauche-C11/12 conformation is topologically similar to cis palmitoleate and may serve as a weak PORCN fatty acyl donor (Fig. 3A). Whereas HHAT did not distinguish between the C16:1n-7 FA isomers in agreement with its absence of a preference for a specific lipid, PORCN preferred the cis FA but nevertheless was able to make limited use of the trans molecule (Fig. 3A). A SCD inhibitor (A939572) blunted WNT acylation with a saturated fatty acyl probe (C16:0) consistent with previous observations (20) but not with the cis or trans fat probes suggesting that the trans fat labeling was not enabled by cellular isomerization (Fig. 3A, Supp. Fig 2) (24).

Using a pulse-labeling strategy with either cis/trans palmitoleic acid analogues, we observed a crippling effect of trans-Δ9 fatty acylation on WNT protein cellular release (Fig. 3B-D). Given the total amount of secreted WNT protein however was comparable between cis and trans fat treated cells, we assume the trans fat did not alter general protein secretion (Fig. 3C). This result also suggests that indeed a trans FA, and not a cis FA produced from an unknown cis-trans isomerase, was affixed onto the WNT protein. We also note that WNT974 inhibited trans fatty acylation of WNT protein thus confirming the role of PORCN in this biochemical event (Fig. 3C). Although previous results have established the importance of lipidation in moving the WNT protein to the extracellular milieu, these observations are the first to our knowledge that demonstrate the essentiality of a cis double bond to WNT protein manufacturing.

Trans fatty acylation compromises the ability of WNT proteins to extricate from PORCN

Substrate-enzyme interactions are oftentimes transient. However, we observed that WNT-PORCN complex are detectable using biochemical approaches and moreover this interaction is sensitive to PORCN and SCD inhibitors (Fig. 4A, B). Thus, immobilization of a lipid on WNTs likely stabilizes WNT-PORCN complexes. In order to determine the molecular basis for failures in trans fatty acylated WNT proteins to reach the extracellular milieu, we next measured the effects of exogenous cis and trans FA exposure on WNT-PORCN complexes. Cells fed trans-palmitoleate harbored more WNT-PORCN complexes than those exposed to cis-palmitoleate suggesting that trans fatty-labeled WNTs are hindered in their capacity to unload from PORCN (Fig. 4C, D).

Lipidation of WNTs is essential to their ability to interact with WLS and to exit the cell (25,26). WLS expression is controlled by WNT/β catenin signaling thus supporting a transcription-based feed forward signaling mechanism (27). Here, we show that forced expression of WNT proteins in HEK293 cells, including those that do not induce transcriptional responses in these cells, is matched by changes in the abundance of WLS protein thus suggesting the total levels of WNT production dictate the rate of WLS protein turnover (Fig. 4E). WLS in complexes with multiple WNT family members are eliminated in
cells treated with PORCN inhibitors (Fig. 4F-G). At the same time, these interactions were also sensitive to the presence of A939572 thus suggesting that the bulk of the palmitoleate used for WNT fatty acylation in this cell line is generated from cellular stores of palmitate (Fig. 4F). Given our observations that the act of lipidation results in WNTs becoming tethered to PORCN, it is conceivable that WLS requires this enrichment step to facilitate WNT-WLS interactions. Due to the entrapment of trans fatty acylated WNTs on PORCN, it is technically not possible to study the effect of WNT trans fatty acylation on its binding ability to WLS. However, we reasoned that similar to trans-labeled WNTs, we should also see increased WNT-PORCN complexes in cells devoid of WLS. We used CRISPR/Cas9 gene editing technology to generate WLS or PORCN knock-out (KO) subclones of the haploid cell line HAP1. Genomic DNA sequencing confirmed the presence of frame-shift inducing insertion/deletion mutations in the PORCN and WLS genes (Fig. 4H,I). Elimination of PORCN or WLS failed to activate a WNT pathway reporter in the presence of WNT3A (Fig. 4J) and inhibited WNT cellular release (Fig. 4K). Inhibition of PORCN by WNT974 blocked both the WNT reporter and WNT secretion in WT HAP1 cells. Elimination of PORCN likely inhibits endogenous WNT fatty acylation, thereby resulting in decreased WLS levels in PORCN KO HAP1 cells (Fig. 4K; Fig. 4E). The absence of WLS also halted the transfer of acylated WNTs from PORCN and resulted in an increased WNT-PORCN complex accumulation (Fig. 4L).

**DISCUSSION**

The dearth of salient features in aliphatic chains that could be exploited by proteins for selective engagement of lipids poses a physical challenge to the inclusion of FAs as intermediaries in signal transduction. Here, we demonstrate that a cis-Δ9 MUFA is the critical FA for WNT release from PORCN thereby extending the previously established importance of this lipid in the WNT protein lifecycle (summarized in Fig. 5). The cis palmitoleate with its kink in the Δ9 position may lower the energy requirements for either their diffusion away from PORCN before binding to WLS, or WLS-mediated extrication from the PORCN active site. The interaction of WLS and PORCN suggests the latter mechanism is more likely to be the case (28).

Post-WNT production, the kink in palmitoleate is likely responsible for the ability of WNT proteins to induce dimerization of the extracellular domains of FZD receptors (29). At the same time, the WNT deacylase NOTUM incorporates several residues within its active site as part of a steric gate that precludes the binding of FAs without a cis-Δ9 double bond (30). Our finding that conserved features in the active site of PORCN enforces the addition of a cis-Δ9 MUFA onto WNT proteins in likely all animals reinforces the importance of this molecular kink in WNT signaling. The delineation of a trans-lipid bilayer tunnel created by a ring of helices revealed in the first crystal structure of an MBOAT family member (DltB from Gram-positive bacteria) could help to identify potential residues that contribute to PORCN fatty acyl donor selectivity (31).

Yet, the influence of fatty acyl protein modifications on WNT signaling is pervasive and extends beyond the biosynthetic steps involved in the manufacturing of WNTs. For example, the ability of LRP6 to exit the ER is gated by palmitoylation of a juxtamembrane residue (32), and the activity of PORCN itself is controlled by palmitoylation by an unknown intracellular enzyme (22). The extensive interconnectivity of lipid metabolism and WNT signaling may have its roots from the coopting of a lipid sensing apparatus in unicellular organisms for the purpose of intercellular communication (33). At least in humans, palmitoleate is the second most abundant MUFA in adipose tissue and blood (34) and perhaps serves in the PORCN-WLS system as currency for synchronizing metabolic health and tissue renewal. Indeed, loss of SCD in induced pluripotent stem cells elicits a transcriptional response that includes the induction of many WNT genes suggesting a hardwiring of MUFA abundance with WNT signaling (35).

Based on our observations with a trans fatty acylated WNT molecule, we assume WNT proteins modified with a saturated FA similarly would not be readily extricated by WLS and thus destined for entrapment in the secretory pathway. Given that exogenously supplied lipids can influence octanoylation of the appetite controlling hormone ghrelin by its acyltransferase GOAT (36), we suspect that dietary contributions of saturated and trans fats might influence the efficiency of WNT production in adult cells.

Despite their chemical diversity, the majority of PORCN inhibitors likely target the PORCN...
active site based on their ability to successfully compete with a fluorescently labeled active site probe for PORCN binding (18). For antagonists such as GNF-1331 (the precursor of WNT974) and IWP2 that have also been evaluated for their activity against other MBOATs, there is evidence that these molecules exhibit selectivity for PORCN (18,37). Our observations described here provide insights into how this is achieved - namely determinants that support PORCN selectivity for cis-Δ9 fatty acyl donors can be exploited to achieve chemical control of PORCN. Additional support for this conclusion may be found in enantiomer-dependent activity of at least one class of PORCN inhibitors (38). The PORCN-WLS relationship likely extends throughout the animal kingdom based on our evidence for the conservation of determinants that enforce cis-Δ9 acylation of WNT proteins, and that mammalian WNT proteins expressed in Drosophila are not released in the absence of WLS (25). Thus, PORCN inhibitors disrupt an ancient partnership between PORCN and WLS that likely was forged to overcome biophysical and protein design constraints associated with the use of lipid intermediaries for signal transduction. At the same time, the inability of WNT974 to disable a subset of PORCN from the animal sources tested, such as Drosophila and Schistosoma (Fig. 1C), further suggests that some non-conserved features found in the PORCN active site could exploit for species-specific disruption of animal development for therapeutic development against parasites that afflict a variety of host animals including humans, livestock, and pets.

Experimental Procedures:
Cell culture and reagents
PORCN-/- MEFs were provided by Charles Murtaugh (University of Utah). HEK293, COS7 and HELA cells were purchased from the American Type Culture Collection (ATCC). HAP1 cells were purchased from Horizon Discovery. G. gallus PORCN construct was provided by Laura Burrus. C. elegans PORCN construct was provided by Rueyling Lin. X. tropicalis PORCN construct was purchased from Transgenic technologies. Neobiolab synthesized a humanized S. mansoni PORCN-myc construct with the following sequence:

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GCGGCCGCATGGACGAGCAGGAGGAGGTCT
GTTCGAGGAAGTGGACGAGGAGAACGGCG
ATAGCGCATCTGAGGCACAAGCCAAGGCGAGGAG
GCACCTGAAACCCGTGCTGAAGGCGAG
CGATTTCGAGACGTGTCCTCTGAGAACGCCAG
CTGTGGCACACCATGAACAAGTGGTACAAC
GTCGAGCTTCTGGTCCTCATCCCTCAGGCTACT
CCAGTCAGAAGGCAGTGACATCTCACTCATCA
ACAGCGACATCAGCAACAGCCACGGCCAG
AAGAACTTCTACAAGACCGTGAACAA
GGAGCCTTGGAAGTGGGAGCTGTTCGAGGACT
GGCCAAGGACACGCGCCACGCAGCTCTGGC
GGAGTTCAGGATCTTGGGCAGAGCTTCAACTGA
GCTGAGCTGGGACGCTTGCATGCTGAGGAG
TGATCTTATGGAACCTGCTCCACATGCACT
TGAGCTGGAGATGAGCTAGCTGAGAACAGG
GACACCTTGCAATGAGCTTGGGAAGTGGCAG
TGGGAACATACAGCCGAGCGCCAGCCCTG
ACCCGGCTAAGCTGAGCAGCCAGGCG
GACTGGAAGGCTGGGCTTGGAGGTGGTGTA
TGTCATCTTCATGCCAGTCTCATGGCTGAC
CGAGTACGTTCCGAGCGCAAGTGGCAG
GCTGGGAGGCTGGGCTGGAGGCTGGGCTT
CCTGGGCTACCTGGCAGTCTGCTGATAC
TGGTCACAGCAGCAATGCTGCTGAGGAG
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GCCAACTTCCGTCTACCTGACCTGCG
GCCGCTCGAGGTCACCCATTCGAACAAAA
ACTCATCAGAAGGAGGATCTGAATATGC
ATACCGGTCATCATCACCACCATCATTGA

**Chemicals:**
IWP2 was synthesized as previously described (17). WNT974 was purchased from Active BioChem and the SCD inhibitor A939572 was purchased from Biofine International. Biotin-azide and click chemistry reaction buffers were purchased from Invitrogen. Cis and trans ω-alkynyl fatty acid probes were synthesized by Dr. Chuo Chen’s group and is described in Supporting experimental procedures.

**Western blot analysis**
Cell lysates were generated with PBS/1% NP40 buffer supplemented with protease inhibitor cocktail (Sigma Cat. No. S8820). Protein sample loading buffer was added to cell lysates and proteins were separated on SDS-PAGE (BioRad Criterion TGX Precast Gel). Antibodies used for immunoblotting were purchased from the following sources: Cell Signaling Technology: DVL2 (Cat. No. 3216), WNT3A (Cat. No. 2721), MYC-Tag (Cat. No. 2272), SCD1 (Cat. No. 2794); Millipore: WLS/GPR177 (Cat. No. MABS87); Sigma: ACTIN (Cat. No. A2228); Santa Cruz Biotechnology: Fc-HRP (Cat. No. sc-2453); Biolegend: Strep-HRP (Cat. No. 405210).

**Luciferase reporter assay**
Reporter assay was executed as described using a Dual Luciferase kit (Promega). SuperTopFlash and control SV40 driven Renilla luciferase reporters used in reporter assays were previously described (39).

**Click chemistry assay**
HEK293 cells were transiently transfected with either the IgG-Fc, the WNT3A-Fc or the SHH N-Fc DNA expression constructs and treated with various ω-alkynyl fatty acid probes (100 μM final concentration) for 6 hrs. WNT-Fc and SHH-Fc proteins modified with the synthetic alkynylated fatty acid probes were then purified from the lysate using Protein A sepharose and subjected to copper catalyzed alkyne-azole cycloaddition reaction in the presence of biotin-azide. The biotinylated WNT3A-Fc and SHHN-Fc proteins were run on a SDS-PAGE gel and detected using Streptavidin-HRP. The expression of total IgG-Fc, WNT3A-Fc and SHHN-Fc proteins was evaluated using anti-Fc IgG crosslinked to HRP.

**WNT secretion assay**
HAP1 cells (WT, PORCN KO and WLS KO) transiently transfected with either the control or the WNT3A cDNAs were treated with DMSO or WNT974 (5 μM) for 48 hrs. Total secreted WNT3A proteins from the culture medium were enriched on Con A-Sepharose. WNT3A immobilized on the sepharose beads were extracted by adding sample loading buffer and heating the beads at 95°C for 2 mins. protein was separated on SDS-PAGE and detected using WNT3A antibody.

**Pulse-chase assay to monitor secretion of acylated WNT proteins.** HEK293 cells transiently transfected with WNT3A cDNA were labeled with either cis or trans alkynylated palmitoleic acid for 6 hrs. The cells were thoroughly washed with PBS to remove any remaining fatty acid isomers after the end of the labeling period and replenished with fresh culture medium. The WNT proteins labeled with alkynylated probes were collected after 24 hrs from the medium (immobilized on Con A-Sepharose) or total lysate (immobilized on Protein A-Sepharose using WNT3A antibody). Click chemistry reaction was performed on the sepharose-bound WNT3A proteins and the palmitoleated WNT proteins were detected using Streptavidin-HRP. The total WNT3A proteins isolated from either of the sources were detected using WNT3A antibody. The value for total acylated WNTs in lysate was used for normalizing the WNT secretion value derived from the medium.

**Binding assay for measuring WNT-PORCN interaction**
HEK293 cells were transiently transfected with WNT3A-Fc and PORCN-GL cDNA constructs. 24 hrs post-transfection, the cell culture medium was changed to low serum medium (1% FBS) along with either cis or trans alkynylated palmitoleic acid. To avoid isomerization of the fatty acyl probes, cells were also treated with 2.5 μM of a SCD inhibitor (A939572) along with the exogenously supplied FA probes. Post 24 hrs treatment, cell lysates were generated and incubated with protein A sepharose for 6 hrs in a vertical rotator. After several washes, gaussia
luciferase (GL) signal associated with the sepharose beads were determined. GL signal from the total cell lysate was used for normalizing the luciferase signal bound to WNT3A-Fc.

Statistics and reproducibility
The number of times an experiment was repeated is indicated in the figure legends. All experiments were repeated at least twice. Quantitative data are presented as mean ± standard deviation (s.d.) from three independent measurements. Statistical testing was performed using Student’s t-test, * P <0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001.

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Conflict of interest:
The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. A universal requirement for cis-A9 fatty acids in metazoan WNT production. (A) PORCN protein sequence divergence across metazoan phyla. Chordata [mouse (M. musculus), zebrafish (D. rerio), chicken (G. gallus), frog (X. laevis)], nematoda [roundworm (C. elegans)], platyhelminthes [flatworm (S. mansoni)], and arthropoda [fruitfly (D. melanogaster)]. Percentages of amino acid sequence similarities of various PORCN enzymes relative to human PORCN are indicated in parenthesis. Cladogram was generated using Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo/.

(B) An assay for monitoring the activity of exogenously provided PORCN proteins. Activation of canonical WNT pathway results in the nuclear accumulation of β-catenin, and the induction of TCF/LEF transcription factors-associated target gene expression. A firefly luciferase (FL)-based reporter that measures β-catenin/TCF activity [Super Topflash (STF)] is used to monitor the activity of introduced PORCN DNA in PORCN null MEFs stably expressing murine WNT3A (PORCN-/-; WNT3A; STF cells). (C) PORCN from diverse animals reconstitutes mammalian WNT/β-catenin transcription. PORCN inhibitor WNT974 (5μM) exhibits WNT pathway inhibitory activity. All error bars represent mean of triplicates ± s.d. The experiment was repeated three times with similar results. (D) WNT3A dependent DVL2 phosphorylation in MEFs devoid of PORCN expression was restored in cells transfected with a cDNA encoding a WT but not a catalytically inert (H330D) PORCN protein. WNT3A-(S209A) lacks the palmitoleation site. A PORCN inhibitor (WNT974, 5μM) blocks WNT3A acylation. The experiment was repeated twice with similar results. (E) Introduced PORCN from various animals promote WNT-mediated phosphorylation of DVL2 protein. The experiment was repeated twice with similar results. (F) Conservation of PORCN fatty acyl donor selectivity across metazoans. PORCN null MEFs expressing WNT3A-Fc and indicated PORCN proteins from various animals were treated with saturated alkynyl-palmitic acid (C16:0) or unsaturated alkynyl-palmitoleic acid alkenes (C16:1n-7). An IgG-Fc protein with a signal sequence serves as a control (IgG-Fc). IgG-Fc or WNT3A-Fc protein immobilized on Protein A Sepharose was subjected to copper catalyzed alkynyl-azide cycloaddition reaction in the presence of biotin-azide and lipidated proteins were detected using HRP conjugated streptavidin. The experiment was repeated twice with similar results. (G) A MUFA modification likely exists in all WNT proteins. A collection of WNT-Fc fusion proteins was used to determine the sensitivity of acylation (monitored using the click chemistry assay) in the presence or absence of a stearoyl-CoA desaturase inhibitor (A939572, 5 μM) or PORCN inhibitor (IWP2, 5 μM). The experiment was repeated twice with similar results.
Figure 2. The active site features of PORCN enforce cis-\(\Delta\)9 palmitoleation on WNT proteins. (A) A SCD inhibitor does not consistently block cell autonomous WNT signaling in different cell lines. The lung cancer-derived H23 and cervical cancer-derived HECA cell lines were treated with either IWP2 (5 \(\mu\)M) or A939572 (5 \(\mu\)M). Whereas in H23 cells both compounds were able to block WNT activity as indicated by the reduction of phosphorylated DVIL2 protein, A939572 was only active in H23 cells. The accumulation of SCD1 is presumably due to the pharmacoperone-like activity of SCD inhibitors. The experiment was repeated twice with similar results. (B) A collection of alkynylated FAs differing in carbon chain length and desaturation position. (C) Characterization of PORCN fatty acyl donor preferences using a click chemistry approach. Cells transfected with cDNA encoding either WNT3A or the N-terminal signaling domain of SHH fused with IgG Fc domain (WNT3A-Fc and SHH-N-Fc, respectively) were treated with various alkynylated FAs (illustrated in B) and subjected to cycloaddition reaction as described previously. SHH-N-Fc labeling serves as a control for alkynyl probe cellular availability. The experiment was repeated twice with similar results. (D) Active site models of HHAT and PORCN. The PORCN active site differs to HHAT in its ability to recognize the position of desaturation within the fatty acyl donor.
Figure 3. WNT molecules labeled with trans palmitoleic acid fail to leave the secretory pathway. (A) PORCN exhibits stereoselectivity for its fatty acyl donor. Cis and trans alkynylated palmitoleic acids (C16:1n-7) were used to label WNT-Fc or SHH-Fc proteins. Stearoyl-CoA desaturase inhibitor (A939572, 5 μM) was used to prevent cellular isomerization of alkynylated probes. SHH-Fc labeling serves as a control for alkynl probe cellular availability. The experiment was repeated thrice with similar results. (B) A click chemistry based strategy for investigating the influence of palmitoleic acid isomerization on WNT cellular release. (C) Cell medium was collected 24 hrs following pulse labeling of HEK293 cells with either cis or trans alkynylated palmitoleic acid. WNT proteins from the culture medium were enriched using concanavalin A (con A) sepharose beads and subjected to a cycloaddition reaction. A baseline labeling efficiency associated with each palmitoleate isomer was determined using a similar analysis of WNT protein isolated from total lysate. The experiment was repeated twice with similar results. (D) The values for total and released click chemistry-labeled WNT proteins were used to calculate normalized WNT secretion value for each palmitoleate isomer. In two separate experiments, WNT protein release is compromised when it is adducted to a trans palmitoleate.
Figure 4. **Trans** fatty acylation hinders WNT extraction from PORCN. (A) A quantitative assay for measuring PORCN and WNT interactions in cells treated with cis or trans palmitoleic acid alkylne. HEK293 cells transiently transfected with PORCN-GL and WNT3A-Fc DNA were treated with cis or trans palmitoleic acid for 24 hrs and then lysed. PORCN-GL activity associated with WNT3A-Fc bound to Protein A sepharose beads was then determined. PORCN-GL signal from the total cell lysate was used for normalizing the PORCN-GL signal bound to WNT3A-Fc. (B) A939572 inhibits the interaction between PORCN-GL and WNT3A-Fc in a dose-dependent manner. (C) Accumulation of WNT-PORCN intermediates with WNT trans fatty acylation. Results of assay described in “A” with cells treated with or without A939572 (2.5 μM) or WNT974 (2.5 μM) (D) Modification of WNTs with a trans fat increases WNT-PORCN complex formation in COS-7 cells. All error bars in the experiments B-D represent mean of triplicates ± s.d. Each experiment was repeated twice with similar results. (E) WLS protein abundance is gated by WNT ligands in a β-catenin/TCF-independent fashion. Expression of WNT-Myc proteins that induce β-catenin/TCF transcriptional response (WNT1, WNT3A) and that do not induce β-catenin/TCF transcriptional response (WNT5B, WNT7B, WNT9B) stabilize WLS protein. The experiment was repeated twice with similar results. (F) The WLS chaperone protein is a sensor for WNT protein abundance. Several WNT-Fc proteins bind to endogenous WLS protein in a PORCN inhibitor- and SCD inhibitor-sensitive manner. The experiment was repeated twice with similar results. (G) A PORCN inhibitor WNT974 disrupts the WNT-WLS interaction. (H) Generation of PORCN KO HAP1 cells using the CRISPR/Cas9 system. (I) Generation of WLS KO HAP1 cells using the CRISPR/Cas9 system. (J) PORCN and WLS KO HAP1 cells have compromised WNT/β-catenin pathway activity. WNT974 blocks WNT3A mediated transcriptional activation in WT HAP1 cells. The experiment was repeated three times with similar results. (K) WNT proteins fail to leave the secretory pathway in the absence of PORCN or WLS. The experiment was repeated twice with similar results. (L) An increase in WNT-PORCN complex abundance in HAP1 cells lacking WLS. PORCN-GL signal from the total cell lysate was used for normalizing the PORCN-GL signal bound to WNT3A-Fc. The experiment was repeated three times with similar results.
Figure 5. Fatty acyl species-selectivity check-points in WNT production. (A) PORCN active site features conserved across animals favor cis-Δ9 fatty acyl CoA substrates, an adduct on WNTs that is essential for their extrication from PORCN presumably by WLS. (B) Active site features that govern PORCN-mediated fatty acyl selectivity are likely attacked by PORCN inhibitors. (C) The addition of a trans fat to unacylated WNTs result in the accumulation of PORCN-WNT complexes presumably due to their inability to transfer to WLS.
Stereoselective fatty acylation is essential for the release of lipidated WNT proteins from the acyltransferase Porcupine (PORCN)
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