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Report

Stearoyl CoA Desaturase Is Required to Produce Active, Lipid-Modified Wnt Proteins

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SUMMARY

Wnt proteins contain palmitoleic acid, an unusual lipid modification. Production of an active Wnt signal requires the acyltransferase Porcupine and depends on the attachment of palmitoleic acid to Wnt. The source of this monounsaturated fatty acid has not been identified, and it is not known how Porcupine recognizes its substrate and whether desaturation occurs before or after fatty acid transfer to Wnt. Here, we show that stearoyl desaturase (SCD) generates a monounsaturated fatty acid substrate that is then transferred by Porcupine to Wnt. Treatment of cells with SCD inhibitors blocked incorporation of palmitate analogs into Wnt3a and Wnt5a and reduced Wnt secretion as well as autocrine and paracrine Wnt signaling. The SCD inhibitor effects were rescued by exogenous addition of monounsaturated fatty acids. We propose that SCD is a key molecular player responsible for Wnt biogenesis and processing and that SCD inhibition provides an alternative mechanism for blocking Wnt pathway activation.

INTRODUCTION

Wnt proteins are a family of secreted signaling glycoproteins that play major roles in coordinating tissue development and cell fate determination during embryogenesis as well as tissue homeostasis and oncogenesis in adults (Clevers, 2006; Logan and Nusse, 2004). Activation of the canonical Wnt signaling pathway stabilizes the transcriptional coactivator β-catenin, which translocates to the nucleus, where it binds the T cell factor family of transcription factors and activates expression of Wnt target genes. In order to signal correctly, Wnt proteins need to be processed, modified and secreted. All Wnt ligands contain a signal sequence at the N terminus, several N-glycosylation sites, and a cysteine-rich domain. In addition, Wnts undergo a unique and essential lipid modification: the cis-Δ9-monounsaturated fatty acid, palmitoleate (C16:1$^{\Delta 9}$) is attached to a highly conserved serine residue, corresponding to Ser 209 on Wnt3a (Takada et al., 2006). Lipid modification is required for Wnt secretion, as mutants lacking the Ser modification site are retained in the endoplasmic reticulum (ER) (Takada et al., 2006) and are unable to interact with Wntless (Wls) (Coombs et al., 2010; Herr and Basler, 2012), a conserved membrane protein dedicated to the secretion of Wnt proteins. In addition, palmitoleic acid plays a major structural role in mediating the interaction of Wnt with its receptor Frizzled (Janda et al., 2012; Kurayoshi et al., 2007). Thus, Wnt fatty acylation is necessary in order to produce secreted, fully active Wnt protein.

Genetic (Kadowaki et al., 1996; Tanaka et al., 2000; van den Heuvel et al., 1993) and biochemical (Chen et al., 2009; Takada et al., 2006) studies have identified Porcupine (Porcn) as the acyltransferase responsible for lipid modification of Wnts. Porcn is a member of the membrane-bound O-acyltransferase (MBOAT) family (Hofmann, 2000) and is predicted to modify all Wnt family members containing the conserved Ser 209 equivalent (Takada et al., 2006). Wnt signaling is tightly linked to and fine tuned by Porcn expression (Proffitt and Virshup, 2012), placing Porcn as an attractive target for the development of drugs that modulate Wnt pathway activity in Wnt-driven diseases (Chen et al., 2009; Dodge et al., 2012; Proffitt et al., 2013). A small-molecule inhibitor of Porcn, LGK974 (commercially available as WntC59), has been developed and is currently in early phase clinical trials.

Although a role for Porcn as a Wnt acyltransferase has been established, it is not known how Porcn recognizes its fatty acid substrate and why a monounsaturated fatty acid (MUFA) is attached to Wnt proteins. All studies of Wnt acylation to date have relied on labeling cells with the saturated fatty acid (SFA) palmitate (Chen et al., 2009; Doubravska et al., 2011; Komekado et al., 2007; Takada et al., 2006; Willert et al., 2003), but mass spectrometric analysis indicates that, under these conditions, palmitoleate is the major fatty acid attached to Wnt (Takada et al., 2006). Thus, a mechanism must exist to convert the SFA to a MUFA, either prior to or after transfer to Wnt proteins. We hypothesized that stearoyl-CoA desaturase (SCD) is responsible for generating the MUFA substrate for Porcn. SCD, an ER-resident protein, is the rate-limiting enzyme in the biosynthesis of MUFAs from SFA precursors. It introduces a *cis* double bond at position 9 of palmitoyl-CoA (16:0) and stearoyl-CoA (18:0) to generate palmitoleoyl-CoA (16:1) and

oleoyl-CoA (18:1), respectively (Miyazaki et al., 2006; Ntambi and Miyazaki, 2004). MUFAs synthesized by SCD are then used as major substrates for the synthesis of phospholipids, triglycerides, and cholesteryl esters.

We assessed the role of SCD during Porcn-mediated Wnt palmitoylation using a cell-based palmitoylation assay system. Here, we show that SCD activity is required to generate a MUFA substrate for Porcn and that Porcn transfers MUFAs, but not SFAs, to Wnt3a. Furthermore, SCD inhibition blocks Wnt3a secretion and renders Wnt inactive. These findings establish a role for SCD as an essential intermediate in Wnt protein biogenesis and processing.

RESULTS

^{125}I-Iodo-pentadecanoate Serves as a Probe for Porcn-Dependent Wnt Fatty Acylation

To understand the mechanism by which Porcn transfers fatty acids to Wnt proteins, cells were metabolically labeled with ^{125}I-iodo-pentadecanoate (^{125}I-IC15:0), a radio-iodinated analog of palmitate. COS-1 cells were cotransfected with complementary DNAs (cDNAs) encoding Myc-tagged Wnt3a and either empty vector (pcDNA3.1) or FLAG-tagged Porcn and labeled with ^{125}I-IC15:0 for 6 hr, a time window chosen to avoid potential degradation of the radiolabeled fatty acid. Cells were lysed, Wnt3a was immunoprecipitated, and radiolabel incorporation was detected by phosphorimaging analysis after SDS-PAGE. Wnt3a incorporated radiolabel when Porcn, but not empty vector, was coexpressed (Figure 1A). A similar result was obtained in mouse L cells stably expressing Wnt3a (L-Wnt3a) (Figure 1B). Wnt3a was radiolabeled when L-Wnt3a cells were transfected with a vector expressing wild-type Porcn, but not with H341A Porcn, an active site mutant (Figure 1B). Radiolabel incorporation into Wnt3a was detectable in cells transfected with 200 ng of Porcn plasmid, but was maximized when higher amounts of Porcn were coexpressed. It is likely that, when Wnt3a is overexpressed, higher levels of Porcn expression are required for efficient radiolabel incorporation during the 6 hr labeling time period. Porcn-dependent radiolabeling of Wnt3a was inhibited when Ser209 was mutated, but mutation of Cys77 had no effect (Figure 1C), consistent with the model that Wnt3a is lipidated only at Ser209 and not at Cys77 (Janda et al., 2012; Willert et al., 2003). Moreover, treatment of cells with the Porcn inhibitor WntC59 resulted in a 90% decrease in label incorporation into Wnt3a (Figure 1D), indicating that fatty acylation was Porcn dependent.

To determine the nature of the linkage between the fatty acid and Wnt3a, we tested the lability of the modification to hydroxylamine treatment. Thioester-linked fatty acids are sensitive to NH_2OH treatment at neutral pH, whereas oxyester linkages are resistant. NH_2OH released ^{125}I-IC15:0 from the Src family kinase Fyn, a thio-ester-linked palmitoylated protein, but not from Wnt3a (Figure 1D), consistent with Wnt3a fatty acid attachment occurring via an oxyester linkage.

Transfer of Fatty Acids onto Wnt3a by Porcn Is Sensitive to Acyl Chain Length

Our laboratory has extensively used ^{125}I-iodo-hexadecanoate (IC16:0) as a probe to monitor protein palmitoylation in cells (Alland et al., 1994; Buglino and Resh, 2008). Surprisingly, we failed to detect ^{125}I-IC16:0 incorporation into Wnt3a when Porcn was coexpressed (Figure 1E). The ^{125}I-iodine atom is equivalent in size to a methyl group, making IC16 resemble a 17-carbon fatty acid. We hypothesized that Porcn is sensitive to acyl chain length and tested whether shorter fatty acids were substrates. When COS-1 cells were incubated with the ^{125}I-iodo-fatty acids decanoic acid (IC10), dodecanoic acid (IC12), tridecanoic acid (IC13), or pentadecanoic acid (IC15), radiolabeling of Wnt3a occurred (Figure 1E). However, no incorporation of the longer fatty acids stearic acid (IC18) or oleic acid (IC18:1) into Wnt3a was evident, whereas Fyn was radiolabeled with these fatty acids (Figure S1).

Although IC16:0 was not incorporated into Wnt3a, low levels of IC16:1 were detected in the presence of Porcn (Figure 1E). One explanation for this finding is that the kink introduced by the *cis* double bond makes the fatty acyl chain shorter and able to fit into the active site of Porcn. Of note, ^{125}I-iodo-pentadecenoic acid (IC15:1) labeling of cells yielded a strikingly strong signal (Figure 1E). These data suggest that MUFAs might be better substrates for Porcn than their saturated fatty acid cognates, and imply that a cellular fatty acid desaturase is required to generate a suitable fatty acyl CoA substrate for Porcn.

SCD Inhibition Blocks ^{125}I-IC15:0 Incorporation into Wnt3a

SCD is the major desaturase responsible for generating 16:1 and 18:1 MUFAs in the cell. We next tested the hypothesis that SCD activity is required to produce the appropriate substrate for Porcn-mediated acylation of Wnt. When L-Wnt3a cells expressing Porcn were labeled with ^{125}I-IC15:0 in the presence of the SCD inhibitors CAY10566 or A939572, Porcn-mediated label incorporation into Wnt3a was dramatically reduced (Figures 2A and 2B). A similar result was obtained in COS-1 cells coexpressing Wnt3a and Porcn (Figure 2C). Treatment with CAY10566 also blocked Porcn-mediated acylation of Wnt5a, a noncanonical Wnt ligand (Figure 2D), suggesting that the requirement for SCD activity is conserved across Wnt family members. SCD inhibition had no effect on the levels of Wnt3a, Wnt5a, or Porcn protein expression (Figures 2A–2D). Moreover, CAY10566 did not alter fatty acylation of Sonic hedgehog (Shh), which is palmitoylated by the MBOAT family member Hhat, or Fyn (Figures 2E and 2F), indicating that these reactions do not depend on SCD activity.

SCD is expressed as multiple isoforms (SCD1,2,3,4 in mice; SCD1,5 in humans), which share >80% sequence identity but are differentially expressed and regulated (Flowers and Ntambi, 2008; Paton and Ntambi, 2009). Quantitative PCR analysis revealed that mouse L-Wnt3a cells primarily express SCD1 and SCD2 messenger RNAs (mRNAs) (average mRNA expression/HPRT ± SEM: SCD1, 3.07 ± 0.04; SCD2, 10.12 ± 0.16; SCD3, 0.002 ± 0.07; SCD4, 0.001 ± 0.05). Treatment of L-Wnt3a cells with SCD1 siRNA had no effect on Wnt3a fatty acylation, whereas addition of both SCD1- and SCD2-directed siRNAs reduced Porcn-mediated labeling of Wnt3a with ^{125}I-IC15:0 (Figure S2). Since both SCD1 and SCD2 are capable of generating 16:1 from 16:0, it is likely that SCD1 and SCD2 are responsible for Wnt3a acylation in L-Wnt3a cells. These findings demonstrate that SCD activity is required for Wnt acylation by Porcn,

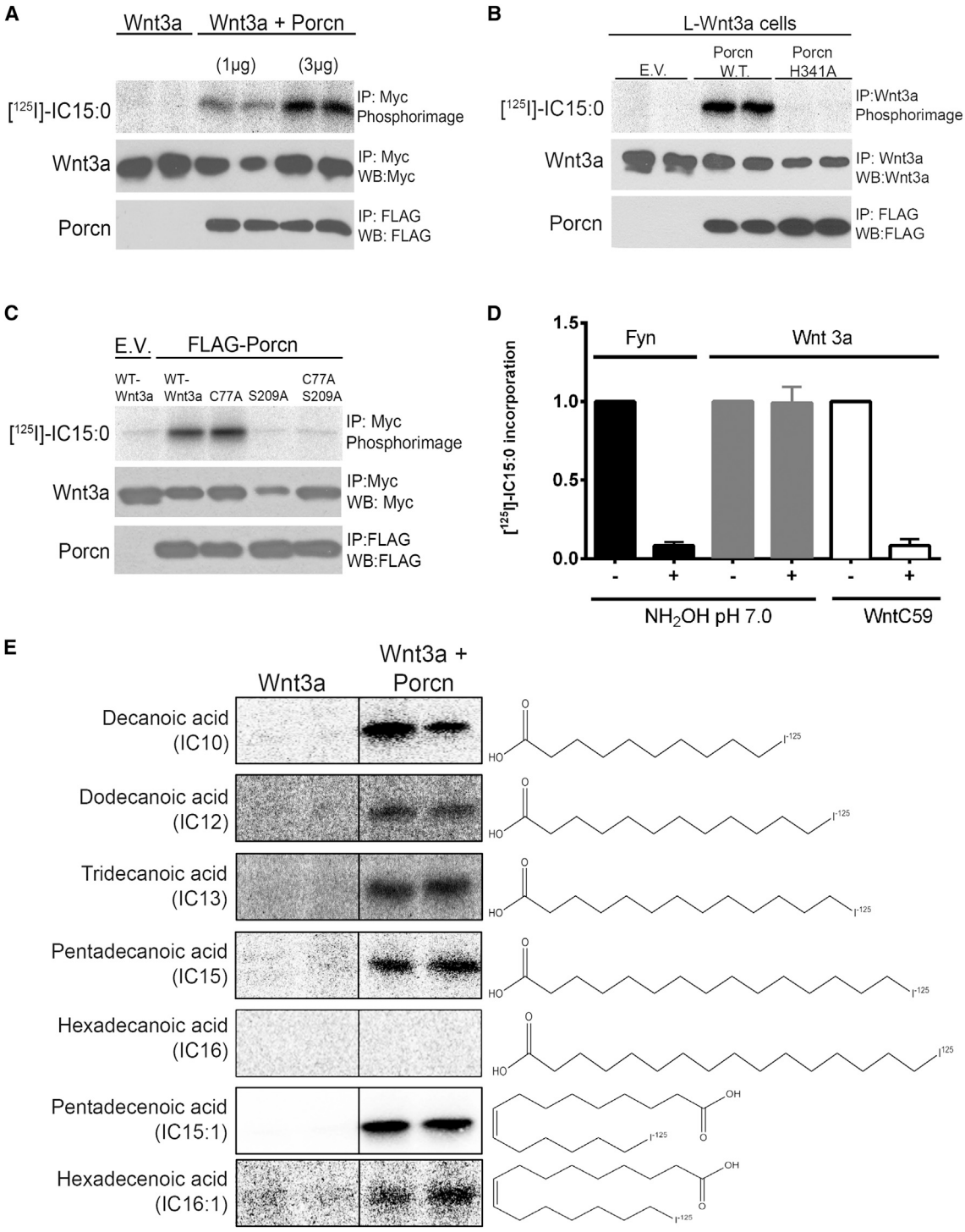

Figure 1. Reconstitution of Wnt3a Palmitoylation by Porcn in Cells

(A and C) COS-1 cells were cotransfected with plasmids encoding Myc-tagged Wnt3a constructs and either empty vector (pcDNA3.1) or FLAG-tagged Porcn and labeled with 40 μCi of ^{125}I-IC15:0 for 6 hr. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody and analyzed by SDS-PAGE and phosphorimaging (top panels) or western blotting (WB) with anti-Myc antibodies (middle panels). Porcn expression was detected by IP and WB with anti-FLAG antibodies (lower panels). (A) The amount of each plasmid transfected (1 μg or 3 μg) is indicated. Gel images represent duplicate samples.

(B) L-Wnt3a cells expressing FLAG-tagged Porcn constructs were treated and analyzed as in (A). E.V., empty vector.

(D) COS-1 cells were labeled as in (A). Wnt3a and Fyn immunoprecipitates containing ^{125}I-IC15:0 were treated with 1 M Tris or NH_2OH (pH 7.0) for 1 hr at room temperature and analyzed as in (A). For WntC59 experiments, cells were treated with DMSO or 10 nM of drug 3 hr prior to label addition. Phosphorimaging signals

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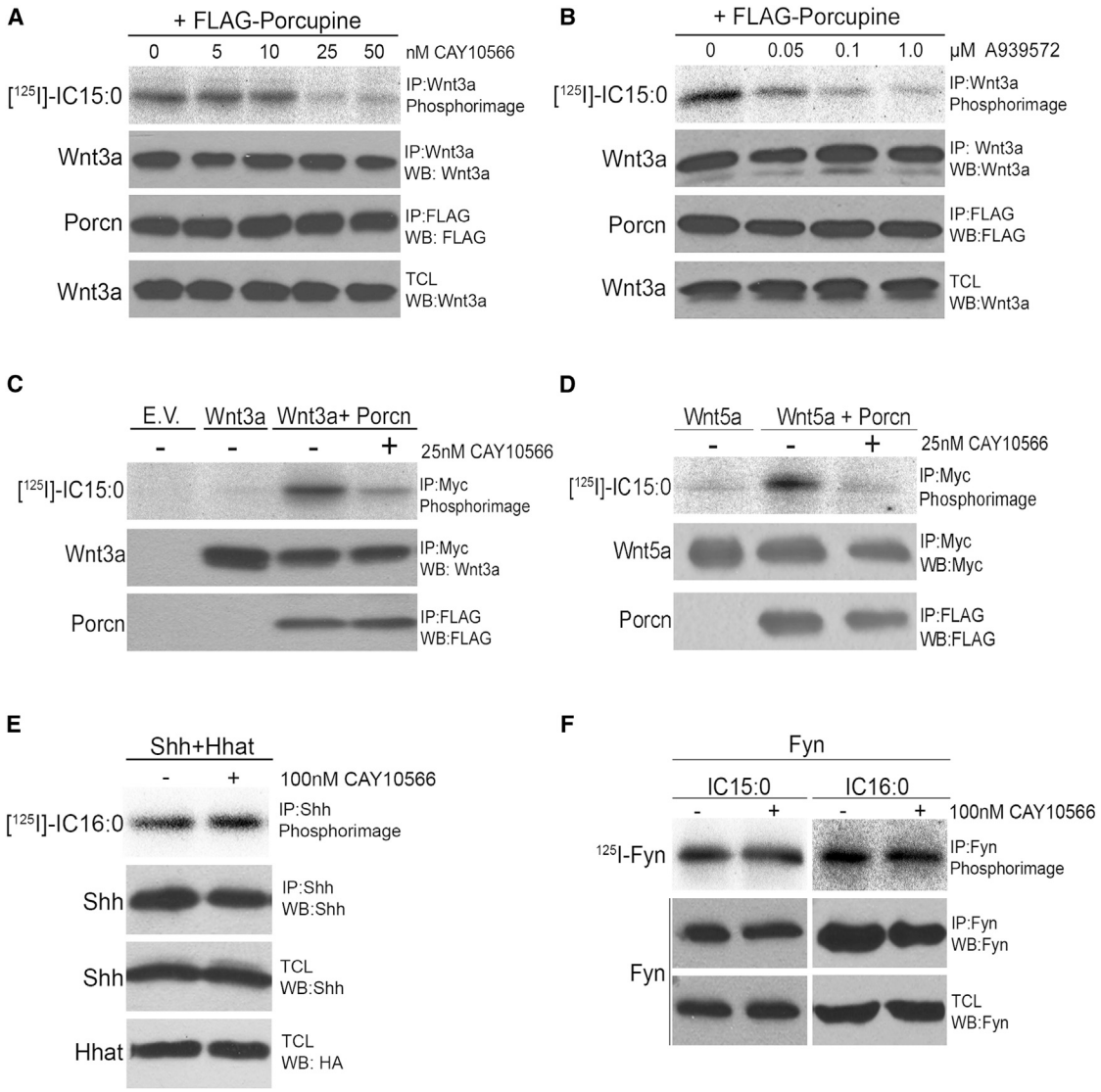

Figure 2. SCD Activity Is Required for Wnt Fatty Acylation

L-Wnt3a cells (A and B) or COS-1 cells (C–F) expressing the indicated constructs were incubated for 3 hr in media containing CAY10566 or A939572 and then labeled with 40 μCi of ^{125}I-IC15:0 (A–D and F) or ^{125}I-IC16:0 (E and F) for 6 hr. Cell lysates were analyzed as in Figure 1. In the lower panels (A, B, E, and F), total cell lysates (TCL) were analyzed directly by WB using the indicated antibodies. See also Figure S2.

and suggest that Porcn transfers MUFAs, but not saturated fatty acids, to Wnt family members.

Exogenous Addition of MUFAs Bypasses the Requirement for SCD

To test the hypothesis that the authentic cellular substrate for Porcn is a MUFA, cells were labeled with the *cis*-Δ9-MUFA iodo-pentadecenoate (IC15:1). ^{125}I-IC15:1 was incorporated into wild-type, but not S209A, Wnt3a (Figure 3A). No radiolabeling of Wnt3a occurred in cells expressing H341A Porcn or Hhat, the Shh acyltransferase (Figure 3B). These data indicate that Wnt3a acylation with the IC15:1 MUFA is Porcn dependent. In stark contrast to the results with IC15:0, treatment of COS-1 or L-Wnt3a cells expressing Porcn with CAY10566

were normalized to protein levels; NH_2OH and WntC59 sensitivity are expressed as percentage of Tris- or DMSO-treated controls ± SEM. The experiment was performed two times in duplicate.

(E) COS-1 cells were cotransfected with the indicated plasmids, labeled with 40 μCi of the indicated radio-iodinated fatty acids for 6 hr, and analyzed as in (A). Gel images represent duplicate samples.

See also Figure S1.

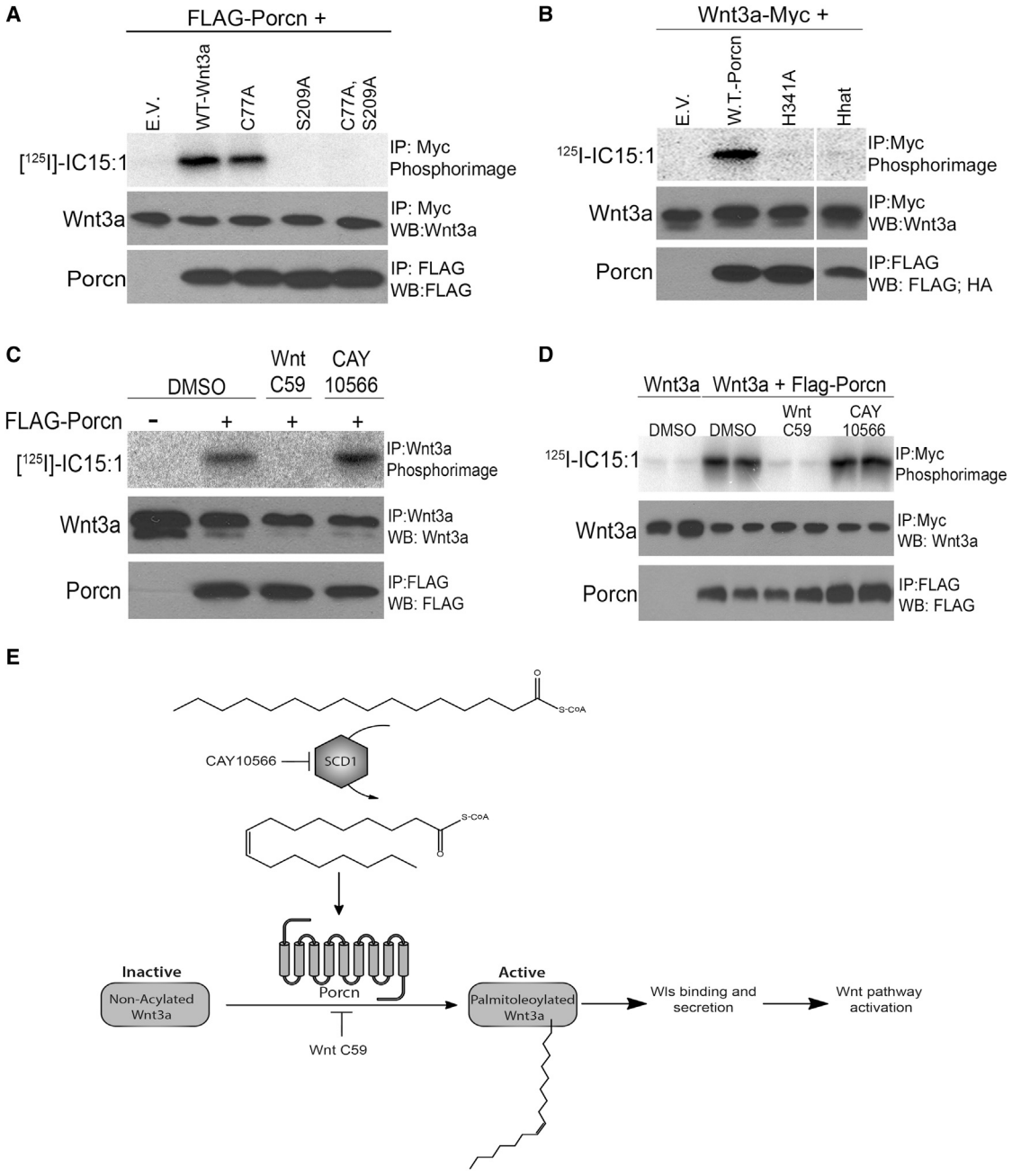

Figure 3. The SCD Requirement Is Bypassed by Exogenous Addition of MUFAs

(A–D) COS-1 cells expressing Myc-tagged Wnt3a and/or FLAG-tagged Porcn or hemagglutinin-tagged Hhat constructs (A, B, and D) or L-Wnt3a cells expressing FLAG-tagged Porcn constructs (C) were labeled with ^{125}I-IC15:1 ± 100 nM CAY10566 or WntC59. Samples were analyzed as in Figure 1. (D) Gel images represent duplicate samples.

(E) A schematic model illustrating successive Wnt processing by SCD and Porcn.

See also Figure S3.

or with SCD-1 and SCD-2 directed small interfering RNAs (siRNAs) had no effect on incorporation of either ^{125}I-IC15:1 (Figures 3C and 3D; Figure S2) or ^{125}I-IC16:1 (Figure S3) into Wnt3a. Thus, the requirement for SCD can be bypassed by directly providing a MUFA substrate to cells expressing Porcn and Wnt3a.

SCD Inhibition Reduces Wnt3a Secretion and Signaling Activity

If SCD is required for Porcn-mediated attachment of endogenous MUFAs to Wnt proteins, defects in Wnt3a secretion and signaling should occur in cells treated with SCD inhibitors. When L-Wnt3a cells were treated with CAY10566 or Wnt C59,

Wnt3a secretion into the media was decreased by 50% and 90%, respectively, compared to DMSO-treated controls (Figure 4A). Treatment with CAY10566 had no effect on secretion of Shh (Figure S4), indicating that gross aberrations in the secretory pathway were not occurring. Decreased Wnt3a levels in the media were confirmed using a luciferase-based reporter system of Wnt signaling, Super TOP-FLASH (STF). L-Wnt3a cells were treated with 10 nM or 25 nM CAY10566 or 10 nM WntC59. The conditioned media (CM) was used to stimulate HEK293T cells coexpressing STF and Renilla luciferase (RL) reporter plasmids. L-Wnt3a CM from DMSO-treated cells caused ~100-fold induction in signaling activity, whereas CM from cells treated with either CAY10566 or WntC59 yielded 60%–90% lower activity (Figure 4B).

The STF system was also used to examine the effect of SCD inhibition on paracrine and autocrine Wnt signaling activity. To measure paracrine signaling, L-Wnt3a cells were cocultured with HEK293T cells coexpressing STF and RL. Treatment with CAY10566 resulted in a ~50% decrease in Wnt signaling activity compared to DMSO-treated controls (Figure 4C). Of note, this signaling defect was rescued by supplying exogenous palmitoleic acid to the cocultures, whereas the inhibitory effect of Wnt C59 could not be rescued (Figure 4C). Autocrine signaling activity, as measured in HEK293T cells expressing STF, RL, and Wnt3a, was also inhibited when cells were treated with CAY10566 or Wnt C59 (Figure 4D). To exclude the possibility that CAY10566 impairs the ability of reporter cells to respond to Wnt proteins, the drug was added directly to the CM from L-Wnt3a cells, and then CM containing drug was added to HEK293T cells expressing STF and RL. Signaling activity was not reduced by the presence of CAY10566 in the CM (Figure 4E). Finally, treatment of cells with either CAY10566 or WntC59 inhibited Wnt signaling pathway components, as evidenced by decreased levels of cytosolic β-catenin and phosphorylated Dishevelled (Figure 4F). Taken together, these data indicate that SCD inhibition blocks Wnt secretion as well as autocrine and paracrine signaling activity.

DISCUSSION

The data presented in this study strongly suggest that SCD is responsible for generating the MUFA substrate for Porcn and that conversion to an unsaturated fatty acid occurs prior to transfer by Porcn. Depletion of SCD1 and SCD2 or treatment of cells with two different, highly specific SCD inhibitors blocked acylation of Wnt3a when cells were incubated with radiolabeled saturated fatty acids. Moreover, the inhibitory effect of CAY10566 on Wnt3a labeling could be completely rescued when cells were directly supplied with the MUFAs C15:1 or C16:1. These findings are consistent with the following series of events: (1) palmitate enters the cell and is converted to palmitoyl CoA; (2) SCD converts palmitoyl CoA to palmitoleoyl CoA; and (3) Porcn uses palmitoleoyl CoA as a substrate and transfers the unsaturated fatty acid to Wnt (Figure 3E).

Iodo-fatty acids have served as versatile tools to monitor a wide variety of fatty acylation reactions, including N-myristoylation, S-palmitoylation, and N-palmitoylation (Alland et al., 1994; Buglino and Resh, 2008; Liang et al., 2004). A notable feature of these reagents is their limited metabolic interconversion to longer or shorter fatty acid species (Peseckis et al., 1993). We have shown that palmitoylated proteins such as Src family kinases, GAP43, and Shh are efficiently acylated with ^{125}I-IC16:0. However, this fatty acid was not a substrate for Porcn, presumably because it is sterically similar to a 17-carbon fatty acid. Longer-chain fatty acids such as IC18:0 and IC18:1 were not incorporated into Wnt3a, but fatty acids with shorter chains were transferred. It is important to note that cell-based labeling assays provide only a qualitative measure of fatty acid substrate specificity, since we do not know the size of the nonradioactive pool of these fatty acids in the cytosol or in the ER lumen, where Wnt protein acylation occurs. Thus, it is not possible to quantitatively compare the stoichiometry of incorporation of fatty acids of varying lengths into Wnt. Nonetheless, it is reasonable to conclude that Porcn exhibits chain-length sensitivity, as it is unable to transfer fatty acids longer than 16 carbons to Wnt proteins. The data presented in Figure 3 suggest that metabolic labeling with ^{125}I-IC15:1 provides a robust, accurate, and direct readout of Porcn activity in the cell.

Palmitoleic acid is a rare fatty acid and its distribution in cells is highly regulated (Cao et al., 2008). It is not known why Wnt proteins are modified with palmitoleate instead of palmitate, which is more abundant and is the fatty acid typically attached to "palmitoylated" proteins (Resh, 2012). The bent conformation of palmitoleate may provide an appropriate three-dimensional conformation for interaction of Wnt with Wls (Herr et al., 2012), thereby regulating Wls-dependent packaging of Wnt into exosomes and Wnt secretion. A bent conformation may also assist in insertion of palmitoleate into the hydrophobic groove of Frizzled that binds Wnt (Janda et al., 2012). Thus, the active site of Porcn may have evolved to accommodate a *cis*-unsaturated fatty acid. Our finding that shorter fatty acids, presumably via their *cis*-unsaturated forms, are incorporated into Wnt3a suggests that Porcn generates a varied population of Wnt proteins with different acyl groups. Differentially lipidated Wnts may exhibit different binding affinities for Frizzled and/or different stabilities and secretion, which could shape the morphogen gradient and thereby regulate signaling in vivo. We speculate that the intracellular pool of fatty acylCoAs and/or the MUFA/SFA ratio dictate the identity of the fatty acid attached to Wnt proteins. It is possible that when palmitoleate levels are limiting, Porcn utilizes shorter fatty acid substrates. This could link fatty acid metabolism and Wnt signaling activity and suggests that Wnt signaling might respond to changes in the metabolic state of the cell.

As a result of gene duplication during evolution, different species contain different numbers of SCD genes and express different numbers of SCD isoforms (Castro et al., 2011). In mice, all four SCD isoforms share >80% amino acid sequence identity, all contain the 8-histidine motif required for catalysis, and all exhibit desaturase activity (SCD1, SCD2, and SCD4 desaturate palmitoyl-CoA and stearoyl-CoA; SCD3 activity is restricted to palmitoyl-CoA; Miyazaki et al., 2006). Human SCD1 shares 85% homology with mouse Scd1–Scd4, whereas SCD5 shares limited sequence identity (65%); both isoforms exhibit desaturase activity. At least two isoforms, SCD1 and SCD2, have been shown to be inhibited by CAY10566 (Masuda

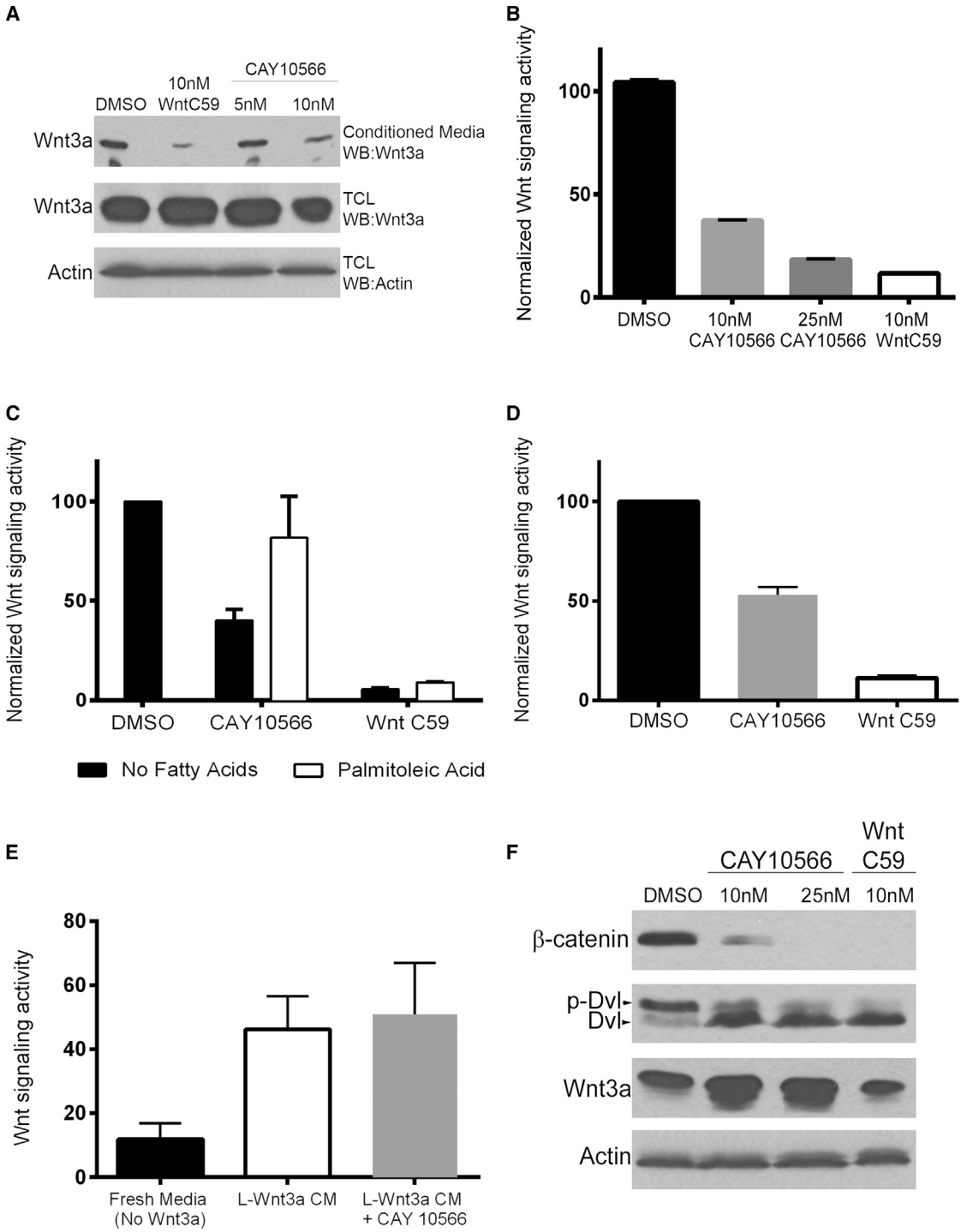

Figure 4. SCD Inhibition Reduces Wnt3a Secretion and Signaling

(A) L-Wnt3a cells were grown in the presence of the indicated drugs for 48 hr. The conditioned media was concentrated and analyzed along with the TCL by WB using anti-Wnt3a antibody. See also Figure S4.

(B) L-Wnt3a cells were cultured as in (A). The conditioned media was used to stimulate HEK293T reporter cells expressing STF and RL for 24 hr. The y axis represents absolute values for luciferase activity, after adjusting for RL and FOP-Flash activity. The experiment was performed two times in triplicate; a representative image is shown.

(C) L-Wnt3a cells were cocultured with HEK293T reporter cells and grown for 24 hr in the presence of DMSO, CAY10566, or WntC59. A total of 100 μM palmitoleic acid was added to the culture medium where indicated. Luciferase activity was measured 24 hr after stimulation, normalized to RL and FOP-Flash activity, and expressed as a percentage of DMSO-treated controls; bars represent mean ± SEM (n = 3).

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et al., 2012), but given the high degree of conservation, especially in the catalytic region, it is likely that all isoforms are susceptible to pharmacologic inhibition. SCD1 is ubiquitously expressed in the mouse, while the other isoforms exhibit predominant expression in brain (SCD2), skin (SCD3), and heart (SCD4). Human SCD1 is also ubiquitously expressed, while SCD5 is most abundant in brain and pancreas. However, many tissues express multiple SCD isoforms, and one isoform can partially compensate for loss of the other, which likely explains why, despite their metabolic defects, SCD1$^{-/-}$ and SCD2$^{-/-}$ mice are viable (Miyazaki et al., 2001, 2005). siRNA-mediated knockdown of both SCD1 and SCD2 was required to reduce radiolabel incorporation into Wnt3a in IC15:0 labeled cells, indicating that both isoforms generate MUFAs for Wnt acylation in mouse L-Wnt3a cells. It is possible that other SCD isoforms also contribute, especially in tissues where the other isoforms are differentially expressed.

SCD1 has recently gained prominence as a target for regulating stem cell and cancer cell growth. For example, a newly developed SCD1 inhibitor, PluriSIn #1, selectively eliminates human pluripotent stem cells while sparing progenitor and differentiated cells (Ben-David et al., 2013). SCD1 expression levels are upregulated in colon, breast, prostate, and lung cancers and inhibition of SCD blocks cell proliferation and tumor growth (Igal, 2010; Roongta et al., 2011). Potential mechanisms to explain how SCD1 inhibition blocks cancer cell proliferation include attenuation of AKT signaling and induction of senescence and apoptosis (Igal, 2010). Given the well-documented roles of Wnt in regulating stem cell and tumor cell proliferation and our findings on the requirement for SCD in Wnt processing, we suggest that inhibition of Wnt protein acylation and function might be responsible, in part, for the inhibitory effects observed in these studies. Two recent studies are consistent with this hypothesis. Mauvoisin et al. reported that depletion of SCD1 inhibits β-catenin accumulation and activity as well as the growth and invasiveness of breast cancer cells, leading these authors to suggest that SCD inhibition might impact signaling through Wnt acylation (Mauvoisin et al., 2013). In another study, constitutive overexpression of SCD5 in neuronal cells increased MUFA production, Wnt5a secretion into the media, and noncanonical Wnt5a signaling (Sinner et al., 2012). However, opposite effects on Wnt7a were observed: SCD5 overexpression decreased Wnt7b expression and signaling, findings that are not readily reconcilable with the effects we observed for Wnt3a. It is also possible that other signaling pathways are affected, directly or indirectly by alterations in SCD levels. In summary, the current report provides a biochemical mechanism documenting how SCD activity controls Wnt biogenesis and pathway activation. Our findings suggest that SCD inhibition may present an alternative method of blocking Wnt pathway activation in normal and disease states.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Rabbit polyclonal anti-Wnt3a and rabbit polyclonal anti β-catenin were purchased from Cell Signaling Technology. Rat polyclonal anti-Wnt3a, goat polyclonal anti-Shh (N-19), rabbit polyclonal anti-Shh (H-160), and protein A/G plus agarose were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-FLAG M2 and palmitoleic acid were purchased from Sigma-Aldrich. Anti-Fyn serum was generated as reported previously (Liang et al., 2001). Small molecule compounds were purchased from Cayman Chemical (CAY10566), BioVision (A939572), and Cellagen Technology (Wnt C59). SiRNA duplexes against mouse Scd1 were purchased from Origene (Cat# SR411990). A smart pool siRNA against mouse Scd2 was obtained from Dharmacon/Thermo Scientific (Cat# L-045507-01-0005).

Plasmids, Cell Culture, and Transfection

Cloning of *Wnt3a-Myc*, *Shh*, *Hhat-HA*, and *Fyn* cDNAs into mammalian expression vectors has been described elsewhere (Buglino and Resh, 2008). N-terminal FLAG-tagged *Porcn* cDNA was generated from a murine *Porcupine* clone in pcDNA3.1 (a gift from Dr. Joseph Goldstein, UT Southwestern). COS-1, HEK293T, L, and L-Wnt3a cells were obtained from ATCC. Super 8 × TOP/FOP FLASH and pRL-TK plasmids were a gift from Dr. Anthony MC Brown (Weill Cornell Medical College). COS-1, HEK293T, and L-cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS). L-Wnt3a cells were grown in DMEM/10% FBS and 0.5g/l Geneticin. Cells were transfected with Lipofectamine 2000 (Invitrogen) in 100 mm plates. When using siRNA, L-Wnt3a cells in 60 mm plates were cotransfected with 1 μg FLAG-Porcn plasmid and 20 nM siSCD1 and/or siSCD2.

Synthesis of ^{125}I-Iodo-Fatty Acids

Iodo-decanoate, iodo-dodecanoate, iodo-tridecanoate, iodo-pentadecanoate, iodo *cis*-9-pentadecenoic acid, and iodo *cis*-9-hexadecenoic acid were synthesized by the Sloan-Kettering Organic Synthesis Core Lab. Conversion into ^{125}I-iodo-fatty acids was performed using ^{125}I-NaI (Perkin Elmer) (Alland et al., 1994; Peseckis et al., 1993).

Metabolic Labeling of Cells with Radio-Iodinated Fatty Acid Analogs

COS-1 cells transfected with plasmids encoding Wnt3a-myc and FLAG-Porcn, or L-Wnt3a cells transfected with 6 μg FLAG-tagged Porcn plasmid ± 20 nM of siSCD1 and/or siSCD2, were incubated in DMEM containing 2% dialyzed FBS for 3 hr. When indicated, CAY10566, A939572, or WntC59 was added. 40 μCi of the indicated radio-iodinated fatty acid was added and cells were grown for 6hrs at 37°C. Cell lysates (Buglino and Resh, 2008) were immunoprecipitated with 5 μl of the indicated antibody and 60 μl of protein A/G-agarose for 16 hr at 4°C, eluted in sample buffer containing 60 mM dithiothreitol, and electrophoresed on 12.5% SDS-PAGE gels. When indicated, immunoprecipitates were treated with 40 μl of 1 M Tris or 1 M NH_2OH (pH 7.0) for 1hr at room temperature and eluted with 5× sample buffer. Radiolabel incorporation into Wnt3a, Wnt5a, Shh, or Fyn was detected on a Fuji FLA-700 phosphorimager and quantified using ImageGauge software. Protein expression was detected by western blotting (WB) using enhanced chemiluminescence (Thermo Scientific) and quantified with Image J software.

Secretion Assays

L-Wnt3a cells were transfected with 6 μg of FLAG-Porcn plasmid. Cells were switched to DMEM/2% dialyzed FCS and either DMSO, Wnt C59, or CAY10566 24 hr posttransfection and grown for 48 hr. Conditioned media

(D) HEK293T cells expressing Wnt3a, STF, and RL were grown ± 10 nM CAY10566 or 10 nM WntC59 for 24 hr. Luciferase activity was measured 24 hr after stimulation, normalized to RL and FOP-Flash activity, and expressed as a percentage of DMSO-treated controls; bars represent mean ± SEM (n = 3).
(E) DMSO or 25nM CAY10566 was added to L-Wnt3a CM collected from a 48 hr culture. This mixture was then added to HEK293T reporter cells. As a control, fresh media (not incubated with L-Wnt3a cells) was added. The y axis represents absolute values for luciferase activity, after adjusting for RL and FOP-Flash activity.
(F) HEK293T cells expressing Wnt3a were treated with CAY10566 or WntC59 for 24 hr. Lysates were collected and probed by WB with the indicated antibodies. The cytosolic fraction from a second set of cells was probed for β-catenin. All error bars are ± SEM.

was cleared by centrifugation at 500 × *g* for 5 min and concentrated to a final volume of 500 μl using 10,000 molecular weight cutoff centrifugal devices (Sartorius Stedim Biotech). The amount of Wnt3a in the media was analyzed by WB.

Wnt Signaling Activity Assays

To measure paracrine Wnt signaling activity, HEK293T cells in 100 mm plates were transfected with 3 μg of STF or Super FOP and 0.3 μg pRL-TK, and 24 hr posttransfection, cells were cocultured in six-well plates with L-Wnt3a cells or L cells expressing Wnt3a and Porcn at a 3:1 ratio (L-Wnt3a:293T) in the presence of the indicated drugs for 1–2 days. Wnt3a pathway activity was detected using a dual-luciferase reporter assay (Promega). FL and RL activity were recorded as relative luciferase units using a Veritas microplate luminometer (Promega). For autocrine Wnt signaling, HEK293T cells grown in 100 mm plates were cotransfected with plasmids encoding Wnt3a-myc, STF, or Super FOP Flash and RL. Then 24 hr posttransfection, cells were seeded in six-well plates at a density of 0.5 × 10^6 cells per well in DMEM/2% dialyzed fetal calf serum (FCS) and DMSO, CAY10566, or WntC59. Cells were grown for 24–48 hr and luciferase activity was measured.

To detect markers of Wnt pathway activation, HEK293T cells grown in 100 mm plates were transfected with 1 μg Wnt3a-myc plasmid. Then 24 hr posttransfection, cells were switched to DMEM containing 2% dialyzed FCS and either DMSO, 10 nM Wnt C59, or 25 nM CAY10566 and cultured for 24 hr. Cells were lysed in 1× RIPA containing protease and phosphatase inhibitors for 10 min at 4°C. Lysates were cleared by centrifugation at 14,000 rpm for 30 min and analyzed by SDS-PAGE and WB. A parallel set of plates was subjected to cell fractionation to obtain the cytosolic pool of β-catenin.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.027.

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