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Divergent effects of Porcupine and Wntless on WNT1 trafficking, secretion, and signaling

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Abstract

Loss-of-function studies have identified Porcupine (PORCN) and Wntless (WLS) as essential mediators of Wnt secretion and signaling. Whereas PORCN is thought to palmitoylate Wnt proteins, WLS is believed to transport palmitoylated Wnt proteins to the cell surface. However, little is known about how these two proteins cooperate to regulate Wnt palmitoylation, trafficking, secretion, and signaling. We first investigated possible interactions between PORCN, WLS, and WNT1, by carrying out co-immunoprecipitation studies. These studies demonstrate the existence of a complex containing PORCN and WLS. They further show that PORCN and WLS compete for binding to WNT1. Then, we used gain-of-function studies to investigate the cooperation between PORCN and WLS as well as possible biochemical interactions between PORCN, WLS, and WNT1. Consistent with the proposed roles for PORCN and WLS, we show that overexpression of PORCN promotes palmitoylation of WNT1 while overexpression of WLS does not. Overexpression of PORCN enhances the ability of WLS to promote WNT1 trafficking to the cell surface as well as secretion, but decreases the ability of WLS to activate WNT1 signaling in target cell. These observations suggest that the levels of WNT1 on the cell surface and in the media are not the sole determinants of the activation of Wnt signaling in target cells.

Keywords

WNT1; Porcupine; Wntless; palmitoylation; secretion; β-catenin dependent signaling

INTRODUCTION

Wnts are poorly secreted signaling proteins that are required for the coordination of growth and patterning in embryos and are essential for the regeneration and repair of damaged tissues in adults. The ability of Wnts to act as morphogens suggests that delivery of proper

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concentrations of ligand to appropriate target cells is key for normal function [1–5]. Aberrant regulation of this process can contribute to embryonic abnormalities or cancer [6–14]. As such, understanding the mechanisms underlying Wnt secretion is relevant for the elucidation the molecular underpinnings of birth defects and disease as well as the production of recombinant Wnt proteins for scientific and therapeutic use.

Wnts undergo several post-translational modifications, including fatty acylation, glycosylation, sulfation and disulfide bond formation [15–19]. Though all of these modifications influence secretion [15–17, 19–23], we are particularly interested in the role of palmitoylation in Wnt secretion. All Wnt family members, with the exception of *Drosophila* WNTD, are thought to be palmitoylated on a conserved serine residue (S224 for mammalian WNT1) [24, 25]. For WNT3A and WNT5A, it has further been shown that the palmitate moiety is mono-unsaturated [19, 26]. Substitution of the conserved serine residue with an alanine abolishes Wnt palmitoylation while causing a 90% reduction of secretion [17, 19, 20]. Consistent with the reported importance of Wnt palmitoylation for receptor binding [21, 27], lipid-deficient Wnts exhibit no detectable biological activity [17, 20]. By contrast, Wnt proteins bearing a threonine in place of the serine can still be palmitoylated and retain biological activity [17]. Glycosylation promotes, but is not required for, Wnt palmitoylation [17, 28].

The identification of Porcupine (PORCN) and Wntless (WLS) as upstream regulators of Wnt secretion has provided some insights into the role of palmitoylation in Wnt trafficking and secretion. For instance, PORCN is thought to promote the addition of palmitate to Wnt proteins on a conserved serine residue [17, 19, 29]. WLS is then thought to carry palmitoylated Wnt to the cell surface for secretion [30–32]. Data from a proximity ligation assay suggest that PORCN and WLS interact in the endoplasmic reticulum (ER) [33]. Loss-of-function studies have established that both PORCN and WLS are required for Wnt trafficking and secretion [19, 20, 22, 30–32, 34]. Consistent with these observations, PORCN or WLS deficient mice arrest at gastrulation, when Wnts are first required for development [12–14, 35]. Despite these insights, the specific role of each protein in regulating the trafficking of Wnt proteins through the secretory pathway remains poorly understood, as does the cooperation between PORCN and WLS.

Here, we use gain-of-function studies to investigate the effects of PORCN and WLS when overexpressed alone or in tandem. First, we show that PORCN and WLS can be detected in a complex in the absence of exogenous WNT1. When WNT1 is overexpressed, PORCN and WLS compete for binding to WNT1. Then, we demonstrate the roles of PORCN and WLS in WNT1 palmitoylation, lipid raft association, cell surface localization, secretion and signal activation. Our data confirm that PORCN, but not WLS, can promote the palmitoylation of WNT1. We further show that PORCN cooperates with WLS to regulate WNT1 trafficking to the cell surface and secretion, but not activation of signaling in target cells. In sum, these data show that the presence of WNT1 protein on the cell surface or in the media is not fully correlated with biological activity. We further show that overexpression of WLS alone increases the activation of a β-catenin dependent Wnt reporter more than overexpression of PORCN and WLS.

EXPERIMENTAL PROCEDURES

Many thanks to Dr. Rami Hannoush (Genentech) for supplying ω-alkyne palmitic acid. Thanks also to Dr. Randy Moon (University of Washington) for providing Super8xTopflash, Super8xFopflash and RL-CMV, Dr. Elena Frolova (University of Alabama – Birmingham) for a partial chick WNT1 cDNA, Dr. Tsutomu Nohno (Kawasaki Medical University) for a partial chick WNT3A cDNA, Dr. Tatsuhiko Kadowaki (Xi'an Jiaotong-Liverpool University) for mPORCND and HA-tagged mPORCND, and Dr. Roel Nusse for LSL cells (L cells stably transfected with TOPFLASH and LacZ constructs).

Materials and their respective vendors are as follows

TX-114 (ACROS Organics); Blue Sepharose (GE); Fetal Bovine Serum (Hyclone or Atlanta Biologicals); Lipofectamine 2000, Biotin Azide (Life Technologies); Dual Luciferase Reporter Assay System, Fugene HD Transfection Reagent, Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Promega); Sulfo-NHS-LC-Biotin, Sulfo-NHS-SS-Biotin, NeutrAvidin Agarose Resin, Halt protease and phosphatase inhibitor cocktail, A/G agarose (Pierce, ThermoScientific); CHAPS, BSA (fatty acid free) (Sigma); and TX-100 (Roche).

Constructs

The full-length sequence for chick *WNT1* has not been reported. Therefore, we used a construct in which the mouse *WNT1* signal peptide was appended to the available chick *WNT1* sequence [20]. As the mature proteins should be almost entirely chick derived, we refer to this construct as chick *WNT1*. Similar modifications were made for chick WNT3A [36].

Sequences encoding Chick WNT1, chick WNT3A, GFP, chick WLS, mouse PORCN (variant D) and HA:PORCN were subcloned into pcDNA3.1(−)A for overexpression in HEK293T cells. Chick WLS Y425A was generated from wild-type WLS using overlap extension [37]. MYC/H6-tagged versions of chick WLS and WLS Y425A were also subcloned into pcDNA3.1 without a stop codon such that the C-terminus was tagged with MYC and H6. Sequences encoding a chick WNT1:F_c fusion were also subcloned into pcDNA3 for use in the palmitoylation assay. To generate an ER-resident protein, we generated a fusion protein with the WNT1 signal peptide fused to GFP bearing a C-terminal KDEL tag. spGFP:WNT1 fusion proteins have been previously described [17, 20].

Antibodies

Mouse anti-PCNA (1:1000), mouse anti-β-Tubulin (1:400) (Santa Cruz Biotechnology); mouse anti-chick Wnt1 5F1-G11-D1 was made in the Burrus lab (1:10); rabbit anti-human PORCN was made in collaboration with Prosetta Biosciences (1/5000); anti-mouse Alkaline Phosphatase (1:1500), anti-rabbit Alkaline Phosphatase (1/1500) anti-mouse Dylight 649 (1/200)(Jackson Immunoresearch); anti-Flotillin-1 (1:1500) (BD Biosciences); Alexa Fluor® 680 Goat Anti-Mouse (1/4000) (Invitrogen); IRDye800 conjugated streptavidin (1/5000) (Licor); mouse anti-HA (1/1000) (Upstate, Millipore); mouse anti-H6 (1/500)

(Roche); mouse anti-MYC (1/10) (conditioned media made from cell line from ATCC CRL-1729).

Cell Culture

COS7 and HEK293T cells were grown in standard medium (DMEM with 10% fetal bovine serum, 4mM L-glutamine, and 1× penicillin/streptomycin) on 100mm plates in humidified incubators set to 10% CO_2.

Co-immunoprecipitations

HEK293T cells were transfected with Fugene HD transfection reagent according to manufactures protocol with pCDNA3.1 expression constructs encoding WNT1, GFP, WLS-MYC/H6 or HA-PORCN in a 6 well plate with a total of 3.3 ug DNA (1.1 ug of each DNA construct). Cells were incubated overnight and lysed in 300ul of 100mM sodium phosphate pH7.5 containing 150mM NaCl, 1% NP40, and 1× HALT protease inhibitor cocktail. Anti-HA or anti-H6 antibodies (2ul each) were added to 270ul of cleared lysate and rotated for 1hr at 4°C. A/G agarose resin (50ul) was added and rotated overnight at 4°C. Beads were washed 3× in 100mM sodium phosphate pH7.5 containing 150mM NaCl and 1% NP40, resuspended in 50ul of 1.5× loading buffer, heated to 100°C or 60°C (for PORCN) for 5 minutes, and analyzed by Western blot. Western blots were probed with anti-WNT1, anti-HA or anti-H6 followed with goat Alexa Fluor® 680-conjugated anti-mouse secondary antibody. Blots were scanned and analyzed on an Odyssey CLX scanner.

TX-114 phase separation assay

HEK293T cells were transfected with Lipofectamine 2000 according to manufacturer's protocol with pCDNA3.1 expression constructs encoding chick WNT1, GFP, WLS or PORCN in a 12 well plate with 1.6 ug of each vector. Cells were incubated overnight, washed once with PBS, lysed with 500ul of 100mM Hepes pH7.4 + 100mM NaCl and triturated with 25 gauge needle. An equal volume of ice cold 10mM Tris pH7.4 + 150mM NaCl + 4.5% TX-114 was added to the lysate, vortexed, incubated on ice for 5 minutes, incubated at 31–35°C for 5 minutes and centrifuged at 2000 $\times g$ at 31–35°C for 5 minutes. After transferring 450ul of the aqueous phase to a new tube, the remaining aqueous phase was removed by aspiration. The Tx-114 phase (50 ul) was transferred to a new tube along with 400ul of 100mM Hepes pH7.4 + 100mM NaCl. Aqueous and detergent fractions were acid precipitated and analyzed by Western blot. Western blots were probed with anti-Wnt-1 or anti-β-tubulin. Alkaline phosphatase-conjugated secondary antibodies were used for detection. Quantifications were performed in ImageJ.

Palmitoylation assay

HEK293T cells were transfected with Fugene HD transfection reagent according to manufacturer's protocol with pCDNA3.1 expression constructs encoding GFP, chick WLS or mouse PORCND and pcDNA3 expression construct with Wnt1:F_c fusion protein in a 6 well plate with a total of 3.3ug of DNA (1.65ug of each vector). 100 uM ω-Alkyne palmitic acid (Alk-C16) was diluted in DMEM containing 5% BSA (fatty acid free) and 4mM L-Glutamine and used to metabolically label HEK293T cells for 20–24 hrs [28, 38]. After

metabolic labeling of cells with Alk-C16, F_c containing proteins were immunoprecipitated with Protein A/G beads [39]. Proteins retained on the beads were subjected to click chemistry with biotin azide before separation by SDS-PAGE and analysis by Western blot [28, 38]. Western blots were probed with IRDye800 conjugated streptavidin, and anti-WNT1 antibodies followed by goat Alexa Fluor® 680-conjugated anti-mouse secondary antibody. Western Blots were scanned and analyzed on an Odyssey FC scanner.

Association of WNT1, PORCN, and WLS with Detergent Resistant membranes (DRMs)

HEK293T cells were transfected with Lipofectamine 2000 or Fugene HD according to manufacturer's protocol with pCDNA3.1 expression constructs encoding chick WNT1, GFP, chick WLS, chick WLS-MYC/H6, mPORCND, HA-mPORCND, or empty vector in a 6 well plate. Cells were incubated overnight and lysed in 100mM Tris pH 7.5, 150mM NaCl, 2mM EGTA, 1% TX-100 and protease inhibitors. Lysates were triturated with a 25 gauge needle 10× and centrifuged at 5000 $\times g$ for 5 minutes at 4°C. Cleared lysates were transferred to a new tube and 60% Optiprep was added for a final concentration of 40% Optiprep. A 40% (bottom) to 5% (top) Optiprep gradient was created by overlaying 400 ul of the lysates (in 40% Optiprep) with 1.2 ml 30% Optiprep and 400 ul 5% Optiprep. Samples were centrifuged at 100,000 × g for 5 hours at 4ºC. Fractions were collected, precipitated, resuspended in 1× SDS-PAGE loading buffer, heated to 100°C or 60°C (PORCN only) for 5 minutes, subjected to SDS-PAGE and analyzed by Western blot. Western blots were probed with anti-WNT1, anti-Flotillin, anti-GFP, anti-PCNA, anti-HA, anti-H6 or anti-PORCN. Alkaline phosphatase-conjugated secondary antibodies were used for detection. Quantification was performed using NIH ImageJ.

Detection of cell surface proteins

COS7 or HEK293T cells were transfected with Fugene HD transfection reagent according to manufactures protocol with pCDNA3.1 expression constructs encoding chick Wnt1, GFP, chick WLS, chick WLS-MYC/H6, chick WLS Y425A-MYC/H6, mPORCND or HA-mPORCND in a 12 well plate. Cells were incubated overnight and washed once with ice-cold PBS, pH8. The cells were labeled with 3mM Sulfo-NHS-LC-biotin or 3mM Sulfo-NHS-SS-biotin in PBS, pH8 or just PBS, pH8 for 15 minutes at 4°C. The reaction was stopping by washing cells with 100mM Glycine and lysing them TENT buffer (20mM Tris pH8, 150mM NaCl, 2mM EDTA, 1% Tx-100, 0.1% SDS + protease inhibitors). Lysates were cleared by centrifugation, transferred to a new tube, and incubated with NeutrAvidin Agarose overnight at 4°C on a rotator. Beads were washed 3× with TENT lysis buffer, resuspended in 1.5× protein loading buffer and heated for 5 minutes at 100°C or 60°C for PORCN. Samples were separated by SDS-PAGE and analyzed by Western blots probed with anti-WNT1, anti-HA, anti-H6, anti-β-tubulin and/or IRDye800 conjugated streptavidin. Alkaline phosphatase-conjugated secondary antibodies or goat Alexa Fluor® 680-conjugated anti-mouse secondary antibodies were used for detection. Quantification was performed using NIH ImageJ or an Odyssey CLX scanner.

Quantification of WNT1 secretion

HEK293T cells were transfected with Fugene HD transfection reagent according to manufactures protocol with pCDNA3.1 expression constructs encoding WNT1, GFP, WLS

or PORCN in a 12 well plate. Cells were incubated for 48hrs, media was collected and cells were lysed in 1ml of 1xCHAPS lysis buffer (20mM Tris pH7.3, 50mM KCl and 1%CHAPS). Lysates and media were cleared by centrifugation and transferred to a new tube. Blue sepharose beads were prepared by washing twice and resuspending in 1× CHAPS lysis buffer. Beads were added to lysates and media and rotated overnight at 4°C. Beads were washed three times in 1× CHAPS lysis buffer, resuspended in 40ul of 1.5× SDS-PAGE loading buffer and heated for 5 min at 100°C. Samples were subjected to SDS-PAGE and analyzed by Western blots probed with anti-WNT1 or anti-β-tubulin. Alkaline phosphatase-conjugated secondary antibodies were used for detection. Quantification was performed using NIH ImageJ.

Autocrine 8xSuperTopFlash Assay

HEK293T cells were transfected in a 24 well plate with Lipofectamine 2000 or FUGENE HD, according to manufacturer's protocol with pcDNA3.1 expression constructs encoding chick WNT1, GFP, chick WLS, chick WLS-MYC/H6 or mouse PORCN. DNA quantities used in transfections are as follows: pcDNA.GFP, pcDNA.WNT1, pcDNA.WLS, and pcDNA.PORCN were used at 0.25μg/well; Super8xTopFlash or Super8xFopFlash were used at 0.01μg/well; and RL-CMV was used 0.01ng/well. Cells were incubated overnight, lysed and measured as per Promega Dual-Luciferase Reporter Assay protocol. Luciferase measurements were carried out in a TD- 20/20 luminometer.

Paracrine 8xSuperTopFlash Assay

HEK293T cells were transfected 24 well plates with Fugene HD according to manufactures protocol with pCDNA3.1 expression constructs encoding WNT1, WNT3A, GFP, WLS or PORCN and incubated overnight. For transfections with "high" concentrations of DNA, we used 183 ng/construct. For those with "low" concentrations of DNA, we used 10 ng/construct. The cells were then split to white 96 well tissue culture assay plates and mixed with LSL cells (L cells stably harboring the TOPFLASH and LacZ constructs) at 25,000 cells/well of each and incubated overnight. The cells were then lysed and measured as per the Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System. Luciferase measurements were carried out in a MicroLumatPlus LB96V Microplate Luminometer.

Data analysis

The statistical significance of our data was analyzed using one-way analysis of variance (ANOVA) with a post-hoc Tukey HSD test.

RESULTS

HA-PORCN and WLS-MYC/H6 are properly localized and retain biological activity

For the studies below, we used tagged versions of PORCN and WLS to facilitate detection. Because the addition of epitope tags can influence subcellular localization and activity, we sought to validate our HA-PORCN and WLS-MYC/H6 fusions prior to use. Consistent with previous reports [33, 40, 41], our data show that HA-PORCN is primarily localized to the ER while WLS is localized to the ER as well as other compartments (Supp Fig 1A, C). Importantly, both retain biological activity (Supp Fig 1B, D).

PORCN hands off WNT1 to WLS

Current models suggest that PORCN-dependent palmitoylation is required for the binding of Wnt to WLS. Thus, we sought to determine whether PORCN, WLS, and/or WNT1 exist in a complex. To do this, we co-transfected HEK293T cells with different combinations of GFP (control), PORCN, WLS, or WNT1. The association of PORCN with WLS was probed by carrying out co-immunoprecipitations with anti-HA (PORCN) and anti-H6 (WLS) antibodies. As a positive control, we first immunoprecipitated lysates from PORCN-transfected HEK293T cells with anti-HA (PORCN) and blotted with the same antibody. HA-PORCN is specifically detected as a single band, migrating around 45 kDa (Fig 1A). Importantly, no PORCN is detected in lysates or beads from cells transfected with only GFP or WLS (and not PORCN) (Fig 1A). These data also show similar levels of PORCN expression/immunoprecipitation amongst all PORCN transfected cells (Fig 1B). Similar results were obtained in parallel experiments in which lysates from WLS transfected cells were immunoprecipitated with anti-H6 antibodies and blotted with the same antibody (Fig 1B). WLS migrates as a doublet around 50 kDa (Fig 1B). Chick WLS has two consensus sites for the addition of N-linked sugars. Experimental data also show that mouse WLS is glycosylated [42]. Thus, it is likely that the observed bands represent differentially glycosylated isoforms (Fig 1B). As above, similar levels of WLS were immunoprecipitated in all samples (Fig 2B).

HEK293T cells transfected with PORCN and WLS were then immunoprecipitated with anti-H6 (WLS) and probed with anti-HA (PORCN) (Fig 1C). The presence of a band at 45 kDa indicates that PORCN immunoprecipitates with WLS (Fig 1C, 2C). The co-expression of WNT1 along with PORCN and WLS had a significant positive effect on the amount of PORCN associated with WLS (Fig 2C, $p<0.01$). However, we cannot deduce from these studies whether WNT1 was actually present in the complex. In sum, these results indicate that PORCN and WLS can associate and that this association is augmented by the presence of WNT1.

We next asked whether overexpression of PORCN (or WLS) could affect the interaction of WLS (or PORCN) with WNT1. To do this, we first co-transfected cells with PORCN and WNT1 or with PORCN, WLS and WNT1 (Fig 1D–E, 2D–E). In experiments in which we immunoprecipitated PORCN and immunoblotted for WNT1, we found that significantly more WNT1 was co-immunoprecipitated from cell lysates transfected with WNT1 and PORCN than from those with WNT1, PORCN and WLS ($p < 0.01$) (Fig 1D, 2D). Thus, excess WLS disrupts the association of WNT1 and PORCN. In parallel experiments in which we immunoprecipitated WLS and immunoblotted for WNT1, we also observed that more WNT1 was co-immunoprecipitated in the presence of WLS than in the presence of PORCN and WLS (Fig 1E, 2E). Thus, the association of WNT1 with WLS is also disrupted by the addition of PORCN. Together, these data suggest that PORCN and WLS compete with each other for binding to WNT1 and are consistent with a model in which PORCN hands WNT1 off to WLS.

Overexpression of PORCN or WLS causes an increase in WNT1 hydrophobicity

It has previously been shown that PORCN promotes the hydrophobicity of WNT1 and WNT3A in a TX-114 phase separation assay [43]. As a first step toward understanding the potential interplay between PORCN and WLS, we performed a side by side comparison of the effects of PORCN or WLS overexpression on the partitioning of WNT1 and WNT1 S224A in a TX-114 phase separation assay. In this assay, partitioning of a molecule, such as WNT1, to the TX-114 detergent phase implies that it is hydrophobic while partitioning to the aqueous phase indicates that it is hydrophilic [44]. Upon overexpression of GFP (control), only a small fraction of the WNT1 protein partitioned to the detergent fraction (Fig 3A). Differential glycosylation accounts for the range of apparent molecular masses observed [18, 43, 45, 46]. By contrast, overexpression of PORCN caused a striking increase in the levels of WNT1 associate with the detergent fraction (Fig 3A). Consistent with previous reports, the predominant isoforms observed in the detergent fraction are highly glycosylated [43]. Although more subtle, WLS also elicited a reproducible increase in the partitioning of WNT1 to the detergent phase (Fig 3A). These results are consistent with models in which 1) both PORCN and WLS promote the lipid modification of Wnt proteins and/or 2) PORCN and WLS promote the apparent hydrophobicity of Wnts by binding to them and targeting them to the detergent phase (as both PORCN and WLS are membrane proteins and presumably quite hydrophobic themselves).

PORCN, but not WLS, promotes the palmitoylation of WNT1

To directly test whether WLS promotes the palmitoylation of WNT1, we took advantage of click chemistry to measure the incorporation of alkyne-palmitate into a WNT1:F_c fusion protein [47]. Overexpression of WNT1: F_c along with GFP (control) revealed no detectable palmitoylated WNT1 when cells are cultured in the presence of DMSO (vehicle) or alkyne-palmitate (Alk-C16) (Fig 3B,C). Upon overexpression of PORCN, we observed a fifteen fold increase in WNT1 palmitoylation (Fig 3B,C). By contrast, overexpression of WLS did not promote the palmitoylation of WNT1 (Fig 3B,C). In combination with the data from the TX-114 phase separation assay, these data are most consistent with a model in which WLS promotes the apparent hydrophobicity of WNT1 by binding to it. However, we cannot rule out the possibility that WLS promotes another type of lipid modification to WNT1.

PORCN and WLS promote the association of WNT1 with lipid rafts

It has previously been shown that Wnt proteins are associated with DRMs (Detergent Resistant Membranes) [20, 34]. Enrichment of a protein, such as Wnt, in DRMs indicates that it is likely to associate with lipid rafts [48]. The requirement of Flotillin2, an important lipid raft protein, for Wnt secretion suggests that lipid rafts are critical for Wnt secretion [20, 34, 49]. We used density gradient centrifugation through an Optiprep gradient to compare the role of PORCN and WLS in the association of WNT1 with lipid rafts. As controls, we assessed the fractionation of Flotillin1, a lipid raft marker, and PCNA, a soluble non-raft protein (Fig 4) [34]. We found that both PORCN and WLS promoted the association of WNT1 with lipid rafts (as compared to GFP) (Fig 4). While our finding that PORCN promotes the association of WNT1 with lipid rafts was expected [20, 34], the ability of WLS to promote the association of WNT1 with lipid rafts has not been previously reported.

PORCN and WLS are both localized to lipid rafts

To determine whether PORCN and WLS are also localized to lipid rafts, we fractionated PORCN (with or without HA tag) and WLS-H6 by density gradient centrifugation. Strikingly, PORCN and WLS were predominantly localized to fractions 2 and 3, where lipid rafts are found (Fig 5). The relative levels of PORCN and WLS found in lipid rafts (fractions 2 + 3/total) are similar. Thus, these data are consistent with a model in which PORCN and WLS are localized to lipid rafts.

PORCN and WLS cooperate to regulate the transport of WNT1 to the cell surface

As lipid rafts have predicted roles in membrane trafficking [50–52], we next tested the effects of PORCN or WLS overexpression on the transport of WNT1 to the cell surface. To do this, we transiently transfected HEK293T cells with WNT1 and PORCN, WLS, or GFP (control) and labeled cell surface proteins with Sulfo-NHS-LC-biotin. After cell lysis, labeled cell surface proteins were precipitated using NeutrAvidin beads. WNT1 protein in lysate and bead fractions was quantified using Western blot analysis. As cells were not washed with high salt or heparin prior to addition of Sulfo-NHS-LC-biotin, labeled WNT1 could represent WNT1 that is tethered to the membrane via its palmitate group or WNT1 that is bound to receptors, co-receptors, or heparan sulfate proteoglycans on the cell surface [53].

As a control to show that intracellular proteins are not detected using this method, we first showed that β-tubulin is not present on the cell surface (Fig 6). Our data show that when expressed with GFP (control), very little WNT1 is detected on the cell surface (Fig 6). Overexpression of either PORCN or WLS causes a slight increase in the amount of WNT1 found on the cell surface (Fig 6). Consistent with cooperative interplay between PORCN and WLS, overexpression of both PORCN and WLS caused the most robust increase in the levels of cell surface WNT1.

PORCN and WLS are both localized to the cell surface

We then asked whether PORCN and WLS are also found on the cell surface. Here, we used sulfo-NHS-SS-biotin to label cell surface proteins. Labeled proteins were precipitated with NeutrAvidin beads and separated via SDS-PAGE before immunoblotting with appropriate antibodies. Consistent with previous results [54], we found substantial levels of WLS on the cell surface (Fig 7A). We also detected overexpressed PORCN on the cell surface, albeit at a level roughly 30 fold below (bead/lysate) than WLS (Fig 7A). Loading controls demonstrate equal levels of β-tubulin in all samples and equal labeling of sulfo-NHS-SS treated samples (Fig 7B). To assess whether the presence of WLS on the cell surface contributes to the modest accumulation of PORCN, we overexpressed a WLS variant (WLS Y425A) with a mutated internalization motif [54]. As expected, the levels of the variant on the cell surface were roughly six fold higher than wild-type WLS (Fig 7 A,C–D). However, PORCN levels on the surface were indistinguishable in the presence of overexpressed WLS or WLS Y425A (Fig 7A, C). Thus, it seems unlikely that WLS promotes the transit of PORCN to the cell surface. We then tested whether PORCN influenced the accumulation of WLS or WLS Y425A on the cell surface (Fig 7A, D). Again, no significant differences were observed (Fig 7D). Thus, we think it unlikely that PORCN and WLS are transported to the surface in a

complex. As a control, we show that β-tubulin, an intracellular cytoskeletal protein, was not labeled with sulfo-NHS-SS-biotin (Fig 7E). Because PORCN is considered to be a resident ER protein, we were concerned that the detection of PORCN on the cell surface might be an artifact of overexpression. To test this possibility, we overexpressed a fusion protein comprised of a signal peptide linked to GFP with a C-terminal ER retention KDEL sequence. Because the generation of the spGFP fusion resulted in the inclusion of a single glycosylation site [20], we were not surprised to detect 2 bands in the cell lysates (Fig 7E). The fusion protein was not detected in the bead (cell surface) fractions (Fig 7E). The absence of overexpressed spGFP-KDEL on the cell surface further supports the idea that a small fraction of PORCN is indeed localized to the cell surface. As neither PORCN nor WLS affected the levels of the other on the cell surface, it seems unlikely that they require each other for transport to the surface (Fig 7A–D).

PORCN and WLS cooperate to regulate WNT1 secretion

We then tested whether the observed effects of PORCN and WLS on WNT1 transport to the cell surface correlate with effects on secretion. To do this, HEK293T cells were co-transfected with PORCN, WLS, or GFP (control) along with WNT1. WNT1 protein in cell lysates was quantified using Western blot analysis [43]. WNT1 protein in the media was concentrated using Sepharose-blue beads prior to Western blot analysis [44]. Consistent with previous reports, Western blot analysis showed that the levels of soluble WNT1 protein in the media are exceedingly low as compared to total cell lysates (Fig 8A) [46, 55, 56]. Whereas, overexpression of PORCN caused a 50% reduction in the levels of secreted WNT1, overexpression of WLS caused a modest 50% increase in secreted WNT1 (Fig 8B). Overexpression of PORCN along with WLS caused a significant increase in the level of secreted WNT1 as compared to overexpression of PORCN alone ($p < 0.01$). As expected, analysis of WNT1 in cell lysates showed the opposite effect as was observed in the media (Fig 8C). Overexpression of PORCN increased the retention of WNT1 in cells, while WLS decreased it. Overexpression of PORCN along with WLS caused a significant decrease in the level of cell associated WNT1 as compared to overexpression of PORCN alone ($p < 0.01$). In sum, these data suggest that PORCN alone cannot promote secretion, but that it can cooperate with WLS to promote secretion.

Overexpression of WLS, but not PORCN, has a striking effect on WNT1 signaling in autocrine and paracrine assays

Thus far, we have shown that overexpression of PORCN has a positive effect on WLS function with respect to trafficking and secretion. Do these results also extend to the activation of signaling in target cells? Here we use gain of function studies to test whether overexpression of PORCN promotes the ability of WLS to promote WNT1 signaling in two different assays. For the first assay, we tested the effects of PORCN, WLS, or GFP expression on WNT1 signaling in HEK293T cells, which were also transfected with the 8xSuperTopFlash reporter. This reporter drives the expression of luciferase in a β-catenin dependent manner [57]. As HEK293T cells function as the Wnt-producing and Wnt-receiving cells, activation of the 8xSuperTopFlash reporter should represent both autocrine and paracrine Wnt signaling. As expected, overexpression of WNT1 along with GFP causes a marked increase in signaling as compared to GFP alone (Fig 9, note that the value for GFP

alone (red) is roughly zero and thus, is not easily visible on the graph). To demonstrate specificity of the 8xSuperTopFlash reporter, we transfected cells with WNT1, GFP, and 8xSuperFopFlash (a reporter construct in which the TCF sites have been mutated) [57]. No activation of the mutated reporter was observed (data not shown). Consistent with the negative impact of PORCN on WNT1 secretion, our data show that overexpression of PORCN caused a 50% reduction of WNT1 signaling. Also consistent with our secretion data, overexpression of WLS significantly promoted WNT1 signaling by 2.5 fold (Fig 9, $p<0.01$). By contrast, we found that whereas co-expression of PORCN enhanced the positive effects of WLS on WNT1 secretion, the opposite trend was seen for WNT1 signaling. Overexpression of PORCN along with WLS significantly reduced WNT1 signaling as compared to overexpression of WLS alone (Fig 9, $p<0.01$).

Because PORCN and WLS have known roles in paracrine signaling, we also carried out a second assay in which we exclusively measured paracrine signaling. For this assay, PORCN, WLS, or GFP (control) was transiently expressed in HEK293T cells along with WNT1. Transfected HEK293T cells were then mixed in a 1:1 ratio with LSL cells, which were stably transfected with the 8xSuperTopFlash reporter [58]. As above, the expression of WNT1 along with GFP causes a substantial increase in the expression of luciferase as compared to expression of GFP alone (Fig 10A). Co-expression of PORCN along with WNT1 had little effect while co-expression of WLS caused a robust (14 fold) increase in Wnt signaling as compared to WNT1 alone ($p < 0.01$). Thus, overexpression of WLS alone has a far more dramatic effect on the activation of signaling than it does on secretion. As with the autocrine assay, co-expression of PORCN along with WLS and WNT1 decreased WNT1 signaling as compared to WLS alone; however, this difference was not significant (Fig 10A). Previous studies with PORCN and WLS indicate that the level of expression is critically important for proper Wnt signaling [25, 59]. To ensure that the results from our paracrine were not skewed by the expression of non-physiological levels of WNT1, PORCN, and WLS, we repeated the same experiment, using 18 fold less DNA for transfections (Fig 10B). Though the magnitude of the activation of WNT1 signaling is predictably less, the effect of PORCN and WLS on WNT1 signaling remained the same.

Lastly, we sought to determine whether PORCN and WLS would have similar effects on WNT3A, which is co-expressed with WNT1 throughout the developing spinal cord [60, 61]. Surprisingly, our results show that although the magnitude of the response for WNT3A alone was greater than that for WNT1 (raw data not shown), the effect of WLS on WNT3A signaling is quite different than what was observed for WNT1. Specifically, depending on the level of overexpression, WLS either decreased (Fig 10C) or had no effect (Fig 10D) on WNT3A signaling. When expressed at high levels, WLS and PORCN did show some cooperation with respect to WNT3A signaling (Fig 10C); however, this effect was not observed when expressed at lower levels (Fig 10D).

In sum, these results indicate that overexpression of PORCN alone has a nominal effect on WNT1 autocrine and paracrine signaling while overexpression of WLS significantly promotes signaling. For autocrine signaling, co-expression of PORCN along with WLS significantly reduces the positive effects of WLS. However, for paracrine signaling, this difference is not significant. Taken together, these data suggest that the levels of WLS, but

not PORCN, are rate limiting for autocrine and paracrine signaling when WNT1 is overexpressed. We further show that the effect of PORCN and WLS cannot be generalized to all Wnt ligands as substantially different results were obtained in parallel experiments with WNT3A.

Taken together, our data indicate that PORCN and WLS work together to promote the trafficking and secretion of WNT1, but only WLS promotes autocrine or paracrine WNT1 signaling. Thus, the secretion of WNT1 is at least partially uncoupled from the activation of signaling.

DISCUSSION

The experiments presented here present a detailed picture of the interplay between PORCN and WLS, from the initial hand-off of WNT1 from PORCN to WLS in the ER to cell surface and beyond.

PORCN and WLS form a complex

PORCN and WLS both reside in the ER [33, 41]. Our co-immunoprecipitation studies show that they associate even in the absence of exogenous WNT1. We do not know whether the PORCN and WLS association observed in our experiments reflects a Wnt-independent interaction or is dependent on low levels of endogenous Wnts. What we do know is that overexpression of WNT1 significantly promotes this interaction (see Fig 2B).

Our co-immunoprecipitation results further demonstrate that the association of WNT1 with PORCN can be disrupted by WLS, and that the association of WNT1 with WLS is also diminished by PORCN. The most parsimonious interpretation of these results is that PORCN and WLS compete for binding to WNT1. How do we reconcile the observation that WNT1 apparently promotes the association of PORCN and WLS yet PORCN and WLS appear to compete for binding to WNT1. While there is no simple explanation for this apparent conundrum, it is important to remember that these experiments do not demonstrate the existence of a ternary complex, nor can we distinguish between unpalmitoylated and palmitoylated subpopulations of WNT1.

Given that PORCN and WLS are in lipid rafts and promote the association of WNT1 with lipid rafts, we further propose that the handoff of WNT1 from PORCN to WLS takes place in lipid rafts. If the PORCN/WNT1/WLS complex represents a stable interaction, we would expect PORCN, WNT1, and WLS to traffic to the cell surface together. Conversely, if the complex represents a transient interaction, we would not necessarily expect the PORCN to be transported with WNT1 and WLS. In our cell surface labeling experiments, we did detect some PORCN on the cell surface. However, the relative levels were quite low as compared to WLS. In combination with data from the Virshup and Basler labs [62, 63], our data are most consistent with a model in which WNT1 is handed off from PORCN to WLS in the ER. Our studies, showing that overexpression of PORCN has no effect on the levels of WLS or WLS Y425A on the cell surface (and vice versa), reinforce this notion [54].

PORCN and WLS cooperate to regulate transport to the cell surface and secretion, but not Wnt signaling

Lastly, studies in which we co-expressed PORCN and WLS along with WNT1 reveal that PORCN and WLS cooperate to promote WNT1 trafficking and secretion, but not signaling. Rather than seeing the expected increase in signaling when PORCN and WLS are overexpressed together, we observed that PORCN inhibits the effects of WLS on WNT1 signal activation, especially in the autocrine assay. The lack of cooperation with respect to signaling tells us that getting WNT1 out of the cell is only part of the story. Interestingly, a rather different picture was obtained in parallel experiments with WNT3A, where the effects of WLS were nominal compared to those for WNT1. Given that all *Drosophila* Wnt family members (with the exception of DWNTD, which lacks a palmitoylation site) require WLS for secretion [63], we were surprised to see that overexpression of WLS had differing effects on WNT1 and WNT3A signaling. It will be interesting to expand these studies to include other Wnt family members and to investigate the underlying mechanism.

Overexpression of WLS alone yields the highest levels of biologically active WNT1

Although overexpression of WLS caused only modest increases in the cell surface localization and secretion of WNT1, it had a dramatic effect on the ability of WNT1 to signal in target cells, particularly in paracrine assays. As compared to WLS alone, overexpression of both PORCN and WLS caused a greater increase in the cell surface localization and secretion of WNT1. However, there was no concomitant increase in biological activity. These studies highlight a disconnect between the cell surface localization and secretion of WNT1 and the activation of β-catenin dependent signaling by WNT1. Thus, we conclude that the cell surface localization and secretion of WNT1 is not fully sufficient for the activation of WNT1 signaling.

These studies are important in that they provide biochemical confirmation of key components of the current model for PORCN and WLS function during Wnt secretion. Our studies also demonstrate the functions of PORCN and WLS are likely more complex than previously anticipated. For instance, the finding that co-expression of PORCN promotes the effects of WLS with respect to trafficking and secretion, but not signaling, suggests that our understanding of these two proteins is not yet complete. Furthermore, our results have important implications for the production of recombinant Wnt proteins, as they suggest that overexpression of WLS along with WNT1 will significantly improve the yield of biologically active protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- PORCN and WLS compete for binding to WNT1.
- Overexpression of PORCN or WLS promotes the trafficking of WNT1 to the cell surface.
- Overexpression of WLS alone promotes WNT1 secretion and signaling.
- PORCN and WLS team up to promote WNT1 trafficking and secretion, but not signaling.
- Cell surface and secreted WNT1 levels are not reliable predictors of signaling.

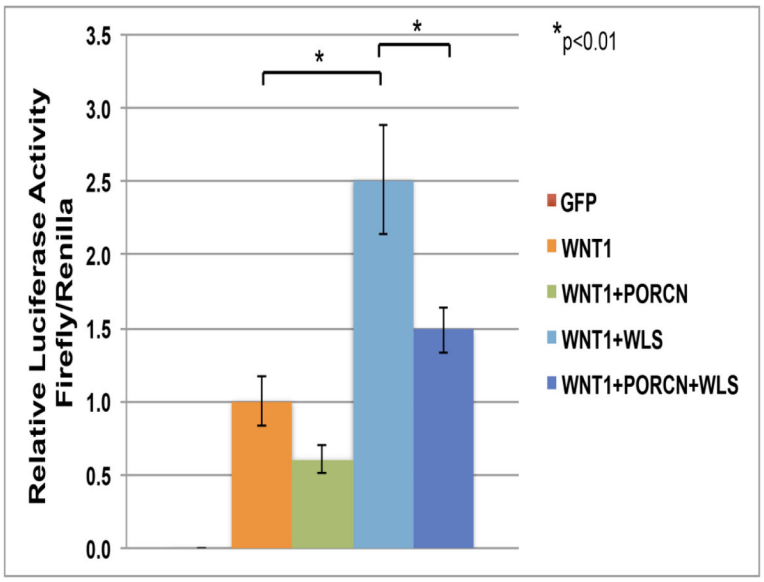

FIGURE 1.

WNT1, PORCN and WLS co-immunoprecipitate with one another. HEK293T cells were transiently transfected with constructs encoding GFP, WNT1, PORCN, and/or WLS as indicated. The amount of total DNA was held constant using pcDNA3.1.GFP as a filler. Cell lysates were immunoprecipitated (IP) with anti-HA (PORCN) in A and D or anti-H6 (WLS) in B, C, and E and subjected to SDS-PAGE. Gels were immunoblotted (WB) with A) anti-HA (PORCN), B) anti-H6 (WLS), C) anti-GA (PORCN), or D–E) anti-WNT1. Secondary antibody conjugated with Alexa Fluor® 680 was used for detection with an Odyssey CLX infrared scanner. Proteins present in total lysates and on the bead are shown. The images shown are representative of those from 4 independent experiments.

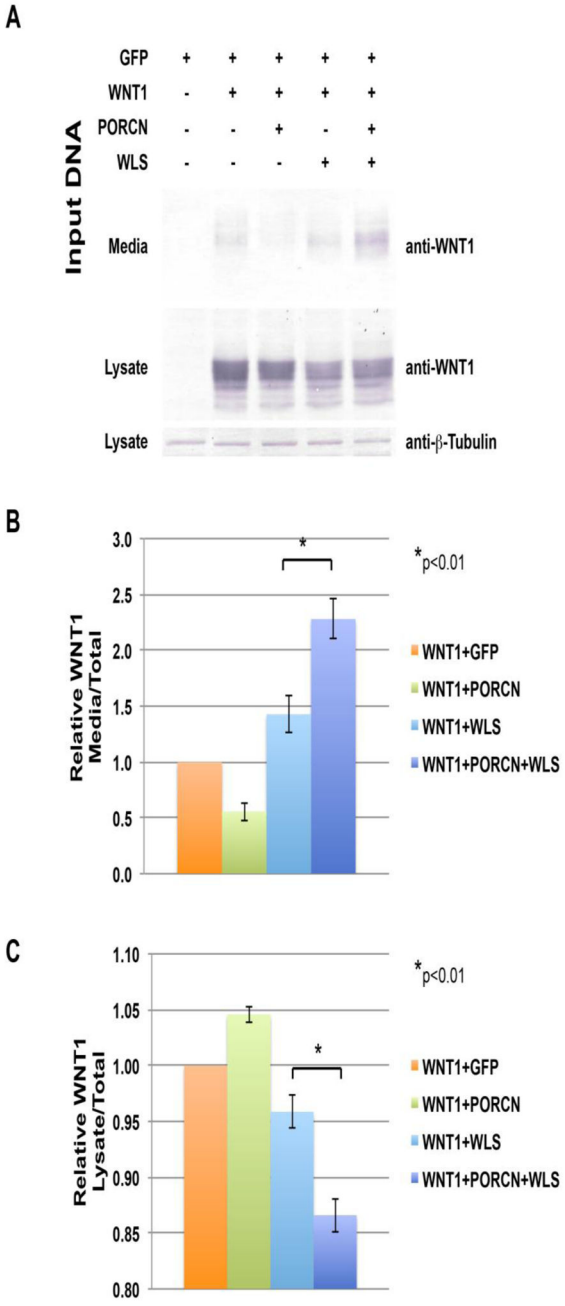

FIGURE 2.

PORCN and WLS bind to each other and compete for binding to WNT1. Quantification of the results from Figure 1 is shown. Each data point represents three independent replicates. Error bars show +/− standard error.

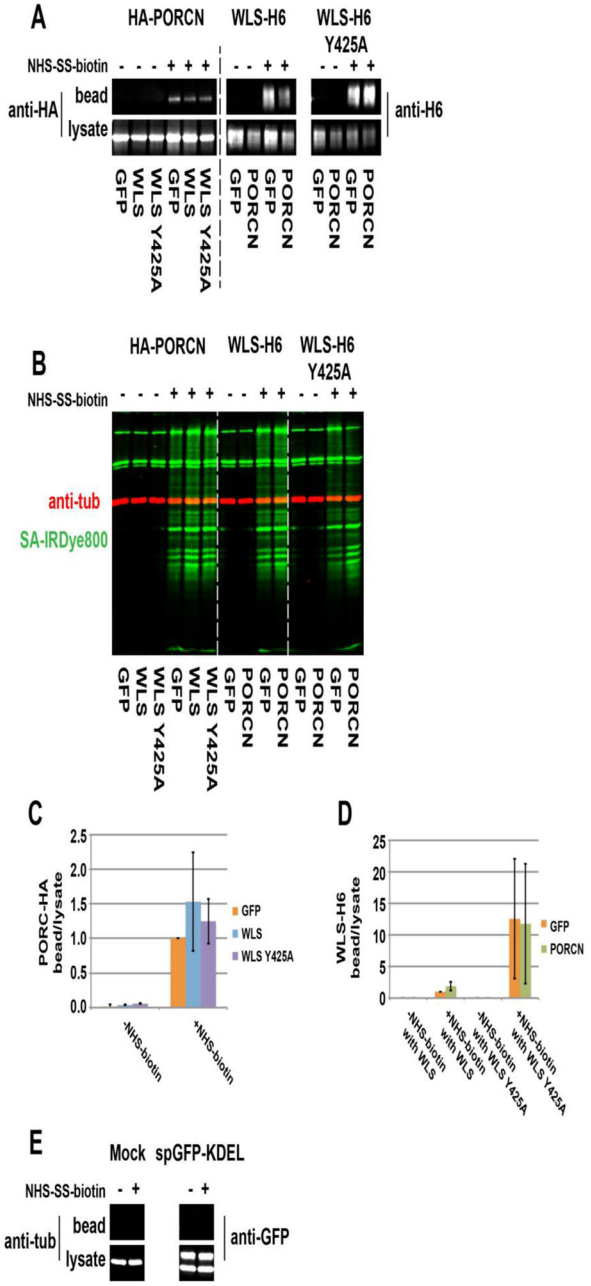

FIGURE 3.

PORCN, but not WLS, promotes the palmitoylation of WNT1. A) PORCN and WLS increase the apparent hydrophobicity of WNT1 in a TX-114 phase separation assay. HEK293T cells were transiently transfected with WNT1 along with GFP, PORCN or WLS. Cell lysates were subjected to extraction in TX-114 as described in Experimental Procedures. Aqueous (Aq) and TX-114 (TX) fractions were subjected to SDS-PAGE and immunoblotted with antibodies against WNT1. This experiment was carried out in triplicate. B) Only PORCN promotes the palmitoylation of WNT1. HEK293T cells were transiently transfected with WNT1:F_C along with GFP (control), PORCN, or WLS. Cells were

metabolically labeled with DMSO (D, control) or Alkyne-palmitate (A). Wnt1:F_c was precipitated from cell lysates using A/G Agarose beads and subjected to click chemistry to covalently attach biotin azide to the alkyne palmitate. Proteins were separated by SDS-PAGE and analyzed by probing immunoblots with anti-WNT1 followed by an Alexa Fluor 680 labeled secondary antibody and Streptavidin IR 800 dye. C) Fluorescence was quantitated using a Licor Odyssey CLX infrared scanner. Data points reflect the average of 3 independent replicates. Errors bars represent +/− standard error. The Alk-C16 data with PORCN data for each independent replicate were normalized to 1. Hence, there are no error bars for that data point.

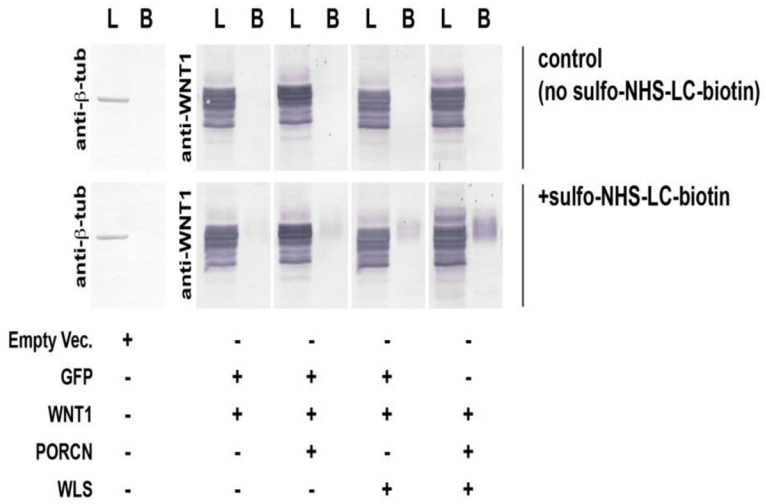

FIGURE 4.

PORCN and WLS promote the association of WNT1 with detergent resistant membranes. HEK293T cells were transiently transfected with WNT1 along with GFP, PORCN or WLS. Cell lysates were separated by density gradient centrifugation. Fractions 1–10 were collected from top to bottom and then separated by SDS-PAGE. Gels were immunoblotted and analyzed with PCNA (non-raft associated nuclear protein), Flotillin1 (lipid raft control) and anti-WNT1 antibodies. Arrows mark the presence of WNT1 in lipid raft fractions. NIH Image J was used for quantification of the results. Data points reflect the average of 3–6 independent replicates. Error bars represent +/− standard error.

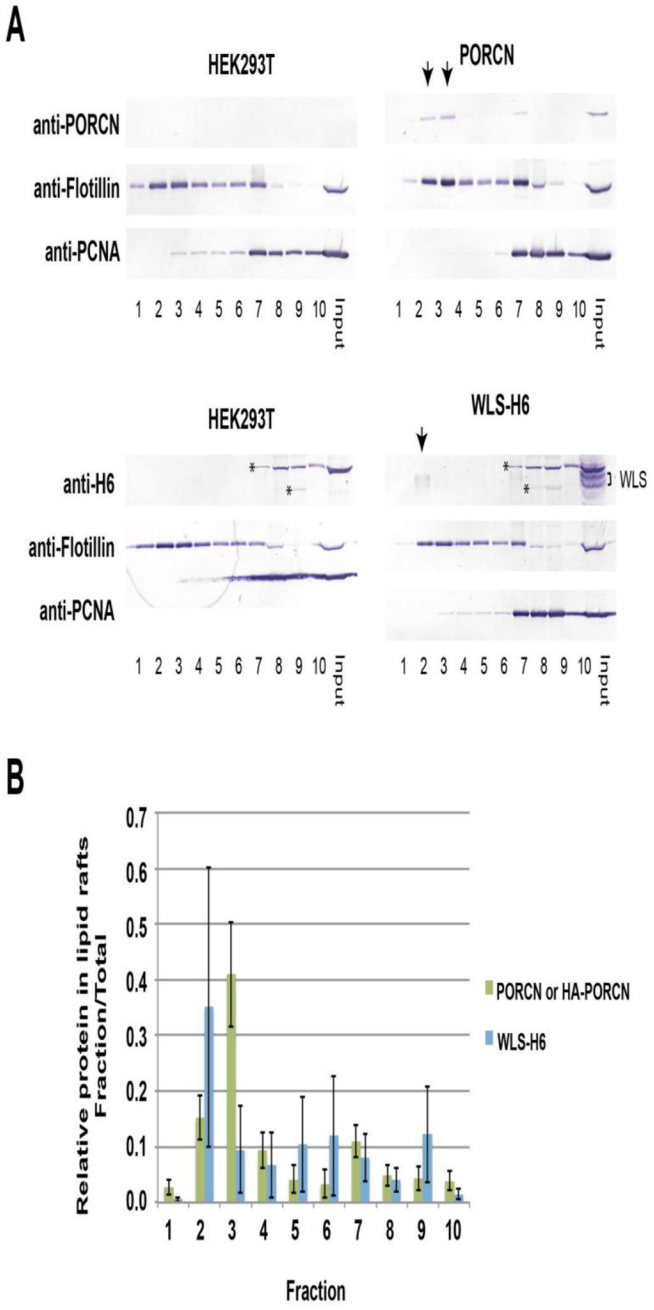

FIGURE 5.

PORCN and WLS are localized to lipid rafts. HEK293T cells were transiently transfected with PORCN (or HA-PORCN) or WLS-H6. Cell lysates were separated by density gradient centrifugation in Optiprep density gradient medium. Fractions 1–10 were collected from top to bottom and then separated by SDS-PAGE. Gels were immunoblotted and analyzed with PCNA (non-raft associated nuclear protein), anti-Flotillin1 (lipid raft control) as well as anti-PORCN, anti-HA, or anti-H6. Asterisks (*) demarcate non-specific bands. NIH Image J was used for quantification of the results. Data points reflect the average of 2–3 independent replicates. Error bars represent +/− standard error.

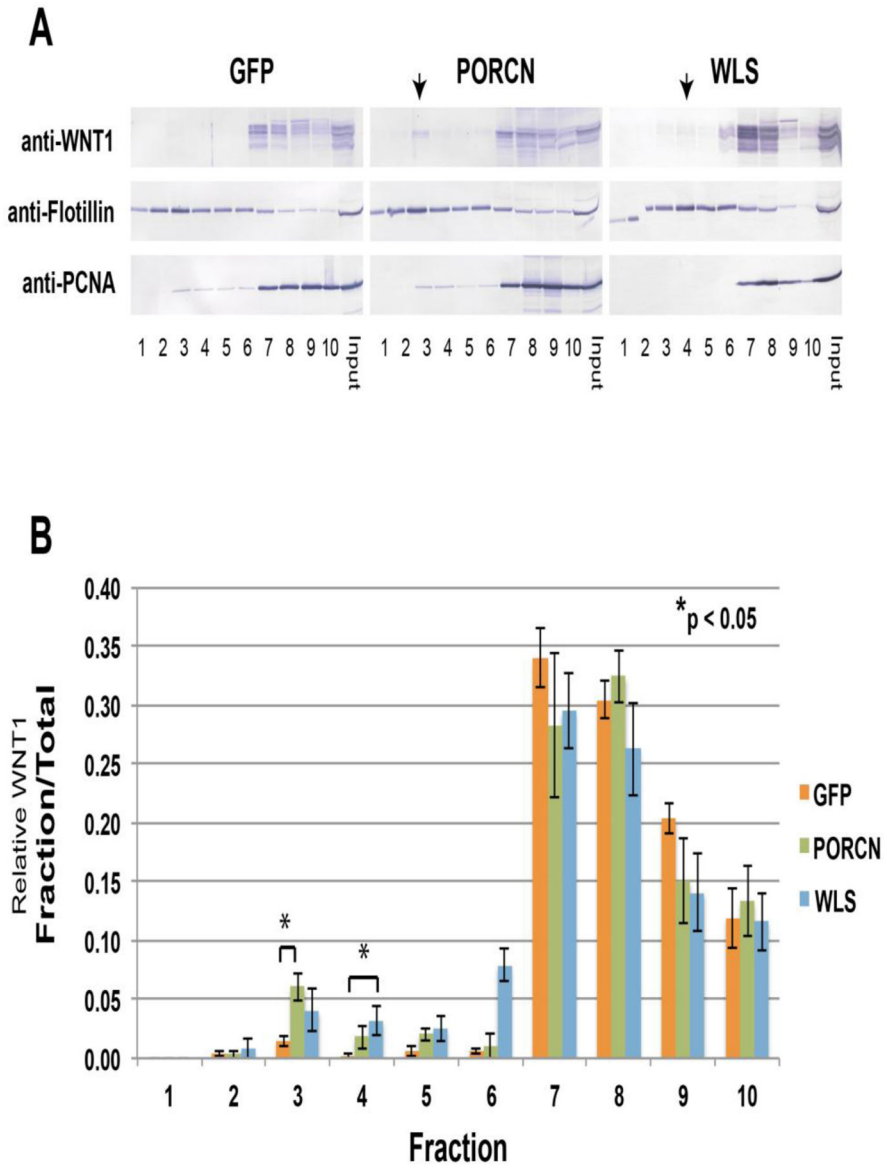

FIGURE 6.

PORCN and WLS cooperate to regulate the trafficking of WNT1 to the cell surface. HEK293T cells were transiently transfected with the indicated constructs. Cells were incubated in the presence (bottom) or absence (top) of sulfo-NHS-LC-biotin. After quenching the reaction, cells were lysed and cell surface proteins were precipitated with NeutrAvidin Agarose. Proteins were separated by SDS-PAGE and analyzed via Western blot with antibodies against β-tubulin (cytoplasmic control) and WNT1. This experiment was carried out 4 times. Representative results are shown.

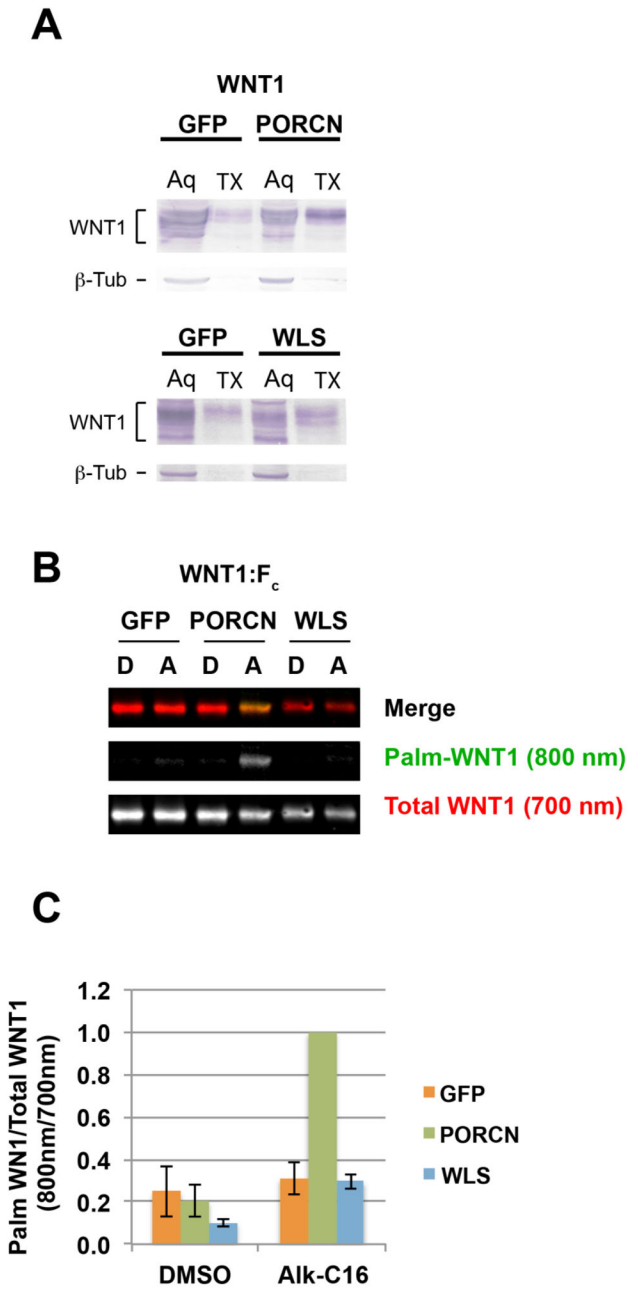

FIGURE 7.

PORCN and WLS are present on the cell surface. HEK293T cells were transiently transfected with HA-PORCN, WLS-MYC/H6 or WLS-MYC/H6 Y425A. Proteins on the cell surface were covalently labeled with Sulfo-NHS-SS-Biotin. After cell lysis, cell surface proteins were precipitated using NeutrAvidin Agarose. A) The amount of PORCN, WLS and WLS Y425A in the original lysates and on the bead was visualized by immunoblotting with anti-HA (PORCN) or anti-H6 (WLS and WLS Y425A). B) A duplicate blot with lysates was probed with anti-β-tubilin and Streptavidin-IRdye800 as loading and labeling controls, respectively. C) The levels of cell surface PORCN (bead) as compared to the total levels of

PORCN (lysate) are shown in a graphical format. The data for PORCN+GFP in the presence of Sulfo-NHS-SS-Biotin was normalized to a value of 1. D) The levels of cell surface WLS or WLS Y425A (bead) as compared to the total levels of WLS or WLS Y425A (lysate) are shown in a graphical format. The data for WLS+GFP in the presence of Sulfo-NHS-SS-biotin was set to a value of 1. Values in C–D represent the average of 2 independent replicates. Error bars reflect +/− standard error. E) Additional controls show that β-tubulin (a cytoplasmic protein) and spGFP-KDEL (an ER resident protein) are not detected on the cell surface using this method.

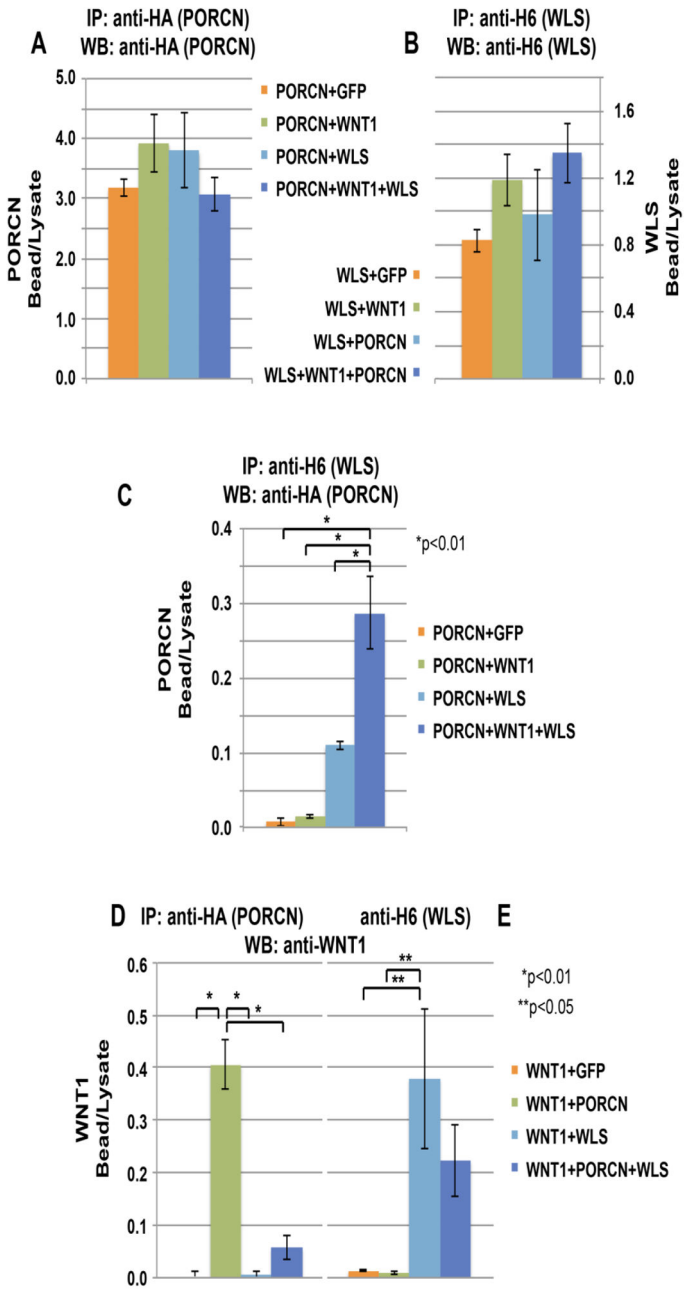

FIGURE 8.

PORCN and WLS cooperate to promote WNT1 secretion from HEK293T cells. HEK293T cells were transiently transfected with the constructs indicated. Media was collected and WNT1 was precipitated using Sepharose blue beads. A) Proteins in the (partially purified) media and cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against WNT1 and β-tubulin (as a loading control). Western blots were quantified using NIH Image J. B) This graph shows the relative levels of WNT1 protein in the media/total WNT1 protein (media + cell lysates). C) This graph shows the relative levels of WNT1 protein in cell lysates/total WNT1 protein (media + cell lysates). Samples transfected with WNT1 and

GFP were further normalized to a value of 1. Data points reflect the average of 6 independent replicates. Error bars represent +/− standard error.

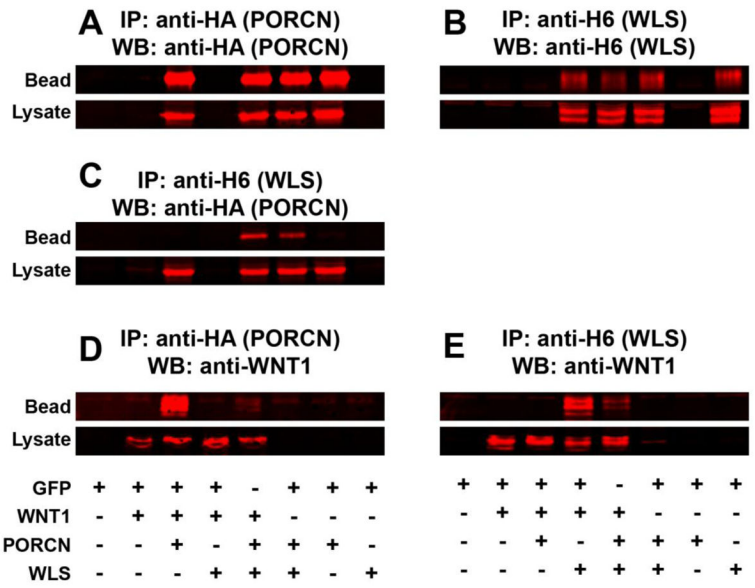

FIGURE 9.

Autocrine 8xSuperTopFlash assay reveals the effects of PORCN and WLS on WNT1 signaling. The autocrine SuperTopFlash assay was carried out as described in Experimental Procedures. HEK293T cells were transfected with GFP along with the indicated plasmid. GFP levels were varied such that total DNA levels were held constant. Data from cells transfected with GFP and WNT1 were normalized to a value of 1. Each data point represents the average of 12–15 independent replicates carried out on 3 different days. Errors bars indicate +/− standard error.

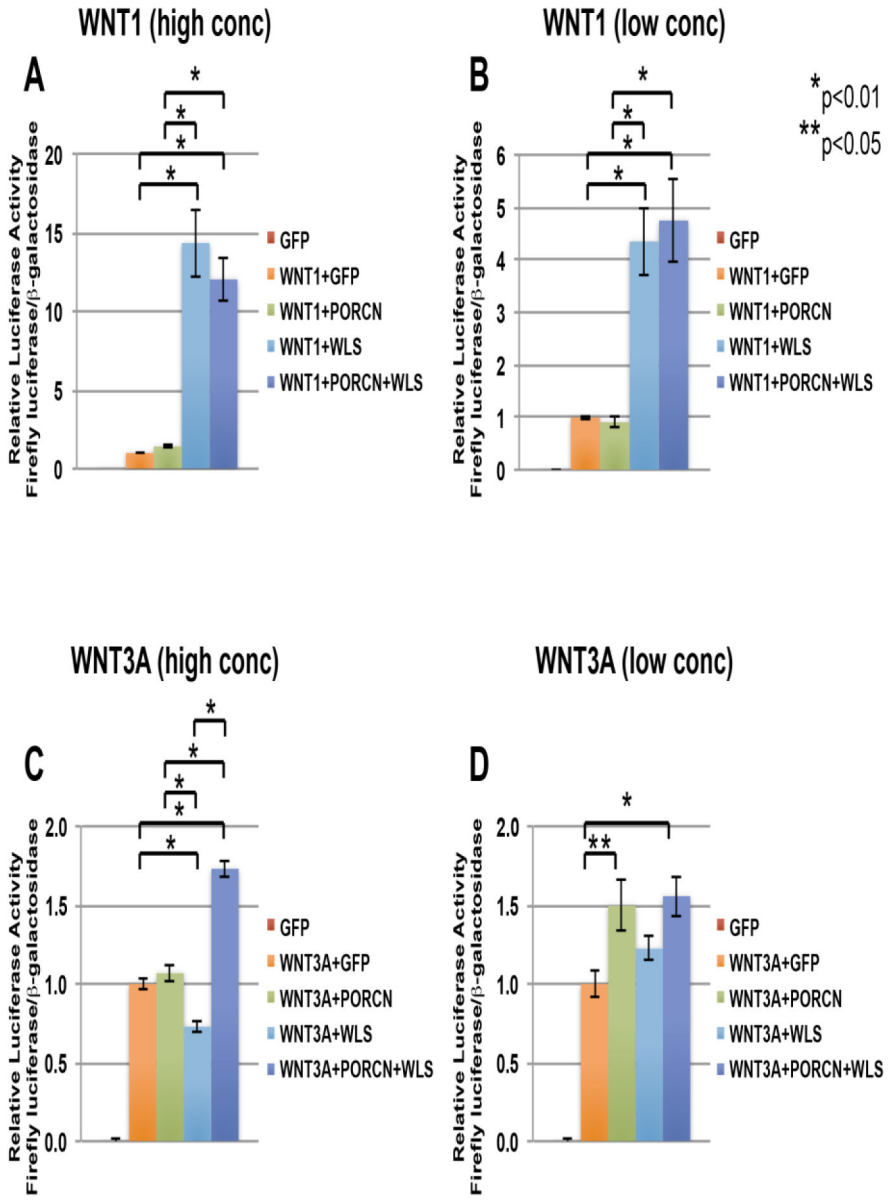

FIGURE 10.

Paracrine 8xSuperTopFlash assay reveals the effects of PORCN and WLS on WNT1 and WNT3A signaling. The paracrine SuperTopFlash assay was carried out as described in Experimental Procedures. Cells were transfected with GFP along with the indicated plasmid. GFP levels were varied such that total DNA levels were held constant. For “high concentration” transfections, 183 ng of the Wnt expression construct was transfected into cells seeded in 24 well plates. For “low concentration” transfection, 10 ng of the Wnt expression construct was used in each well. The transfected HEK293T cells were mixed with LSL reporter cells before measuring luciferase activity. Data from HEK293T cells transfected with GFP and WNT1 (or WNT3A) were normalized to a value of 1. Each data

point represents the average of 12–15 independent replicates carried out on 3 different days. Errors bars indicate +/− standard error.