FATTY ACYLATION OF WNT PROTEINS BY THE ACYLTRANSFERASE PORCUPINE

by

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DEDICATION

To my parents, Haydeé Esteves Soler and Oscar Rios Ramos, for teaching me that with effort and dedication everything is possible (H.Q.J.). Without your wisdom and life lessons, this work would have never been possible. To Ryan, for your infinite amount of support, love and patience throughout this journey; this achievement is both yours and mine.

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ABSTRACT

Wnts comprise a family of secreted signaling proteins that play major roles in coordinating proper tissue development and cell fate determination during embryogenesis as well as tissue maintenance and oncogenesis in adults**.** To signal correctly, Wnt proteins need to be processed, modified and secreted. Wnt processing involves the attachment of a unique and essential lipid modification: the mono-unsaturated fatty acid, palmitoleate ($16:1^{\Delta 9}$) is attached to a highly conserved serine residue (Ser209 on Wnt-3a). Inability to incorporate this fatty acid renders Wnt unable to initiate the intracellular signaling cascade or to be efficiently secreted. Porcupine (Porcn) is the acyltransferase responsible for the attachment of this fatty-acid adduct and represents an appealing target for the development of inhibitors that can modulate Wnt signaling activity in Wnt-related diseases. The source of this monounsaturated fatty acid (MUFA) had not been identified, and it was not known how Porcn recognizes its substrate and whether desaturation occurs before or after fatty acid transfer to Wnt. The goal of my thesis project is to unravel the mechanism by which Porcn recognizes its substrates (palmitoleate and Wnt) and catalyzes the transfer of a MUFA onto Wnt.

Our work demonstrated that Porcn transfers MUFAs but not saturated fatty acids onto Wnt proteins. We next showed that stearoyl-CoA desaturase (SCD), the rate limiting enzyme in the biosynthesis of MUFAs from saturated fatty acids, generates the 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. This establishes that conversion to an unsaturated fatty acid occurs prior to transfer by Porcn. 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.

Porcn is a multipass transmembrane protein, and its extreme hydrophobicity has hampered biochemical and functional analysis. We first examined how Porcn recognizes its fatty acid substrate by screening a panel of 10 different ^{125}I-Iodofatty acid analogues of varying chain lengths from 10 to 18 carbons. This analysis revealed that Porcn activity is sensitive to acyl-chain length, as it is unable to transfer fatty acids longer than 16 carbons. Next, we carried out a comprehensive structure-function analysis of Porcn by mutating highly conserved residues in Porcn and Wnt3a. A total of 16-point mutations in Porcn and 13 in Wnt3a were generated. Studies of these mutant proteins identified key residues required for Porcn enzymatic activity, stability and Wnt3a binding, as well as residues in Wnt3a that mediate Porcn binding, fatty acid transfer and Wnt signaling. The data generated by these biochemical analyses has enabled us to generate an initial functional map of the active site of Porcn.

BIOGRAPHICAL SKETCH

Jessica Rios-Esteves was born on October 29, 1985 in Moca, Puerto Rico and grew up in San Sebastián where she attended Academia San Sebastián Mártir Elementary/Middle School and Patria Latorre Ramírez High School. In 2003, she began undergraduate studies at the University of Puerto Rico- Mayagüez (UPRM) Campus, majoring in the field of Industrial Biotechnology. During her time at UPRM, Jessica participated in various summer research programs in the U.S. and developed a passion for biomedical research, especially in the field of biochemistry. In 2008, she obtained a BSc with high honors, and subsequently moved to New York to pursue a Ph.D. in Cancer Biology from the Gerstner Sloan-Kettering Graduate School of Biomedical Sciences. Following her passion for biochemistry, she joined Dr. Marilyn Resh's laboratory where she studied the mechanism by which Wnt proteins undergo fatty acylation.

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LIST OF ABBREVIATIONS

GPI: glycophosphatidylinositol
PAT: protein acyltransferase
ER: endoplasmic reticulum
NMT: N-myristoyltransferase
eNOS: endothelial Nitric Oxide Synthase
APT1: acyl protein thioestarase 1
GPCR: G-Protein Coupled receptors
TMD: transmembrane domains
LRP6: low density lipoprotein receptor-related protein 6
MBOAT: Membrane-bound O-acyltransferase
Erf2: effect on Ras function
Yck2: yeast casein kinase 2
CRD: cysteine rich domain
Hh: Hedgehog
Shh: Sonic Hedgehog
PM: Plasma Membrane
2BP: 2-Bromopalmitate
HPLC: High Pressure Liquid Chromatography
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PS: phosphatidylserine
PI: phosphatidylinositol
PA: phosphatidic acid
PG: phosphatidylglycerol
LPAT: lysophospholipid acyltransferases
ACAT: Acyl-coenzyme A:cholesterol acyltransferase
CE: cholesterylester
DGAT: acyl-CoA:diacylglycerol acyltransferase
VLDL: very low-density lipoproteins
TAG: triacylglycerol
HHAT: Hedgehog Acyltransferase
GOAT: Ghrelin O-Acyltransferase
GHSR: growth hormone secretagogue receptor
GH: Growth hormone
HPLC: High Pressure Liquid Chromatography
HSPG: heparan-sulfate proteoglycan
MUFA: monounsaturated fatty acid
APC: adenomatous polyposis coli
CK1: casein kinase 1
GSK3: glycogen synthase kinase 3
FAP: familial adenomatous polyposis
sFRPs : Secreted Frizzled-Related Proteins
WIF: Wnt Inhibitory Factors
Dkk: Dickkopf
NDP: Norrin
Rspo: R-spondin
MMTV: mammary tumor virus

CHAPTER ONE

INTRODUCTION

The post-translational attachment of lipids to proteins is a distinctive feature of eukaryotic cells. Proteins are covalently modified with fatty acids, isoprenoids, sterols, phospholipids and glycophosphatidylinositol (GPI) to control a dynamic range of cellular processes such as membrane association and trafficking, lipid raft targeting, stability and signaling activity (1-6). The lipophilic nature of these molecules increases target hydrophobicity, thereby altering biochemical properties, membrane affinity and the local concentration of the substrate in membrane subdomains. As with other post-translational modifications, some forms of lipid attachment are highly dynamic. Modified proteins can undergo cycles of acylation/deacylation to achieve an extra layer of regulation, complexity and versatility. This process involves the concerted action of acyltransferases (enzymes that attach lipids to substrates) and esterases or lipases (enzymes that cleave lipids from substrates), which function as regulators and points of control in this process. The three most common types of protein lipidation are: prenylation, GPI-anchorage and fatty acylation.

Prenylation. Protein prenylation involves the attachment of a 15-carbon farnesyl group ($C_{15}H_{25}$) or a 20-carbon geranyl group ($C_{20}H_{33}$) to a cysteine residue by a farnesyltransferase or a geranylgeranyltransferase, respectively (Table 1). Prenylated proteins often contain a C-terminal C*aaX* motif, where C represents the modified Cys residue, *a* is an aliphatic residue and *X* determines the identity of the isoprenoid group (X=M, S, Q, A or C for farnesyl or, L or E for geranyl). After isoprenoid attachment, the –aaX motif is cleaved, and the newly exposed terminal cysteine is carboxymethylated. Prenylated targets are typically cytosolic proteins that upon modification are directed to cytoplasmic, nuclear or endomembranes. Prenylated proteins include

the Ras superfamily of small GTPases, nuclear lamins, the γ-subunits of heterotrimeric GTPases, and several protein kinases and phosphatases (7-10). All isoforms of Ras, for example, depend on membrane targeting for signaling activity. Mutation of the farnesylated Cys residue (Cys 186) abolishes membrane localization, rendering Ras inactive (10-12). Although prenylation increases membrane affinity, a single isoprenoid moiety is not enough to establish a stable interaction with membranes, and it often co-occurs with palmitate attachment (see below *membrane tethering*).

GPI-anchorage. Modification with glycophosphatidylinositol (GPI) is one of the most studied and well-characterized forms of acylation. GPI is a complex glycolipid consisting of phosphoethanolamine (PE), an oligosaccharide core (3 mannoses and glucosamine) and phosphatidylinositol (Table 1). GPI assembly and transfer occurs in the lumen of the endoplasmic reticulum (ER), and requires the action of nearly one dozen enzymes (13). PE is attached to the C-terminus of the protein via an amide linkage whereas the phosphoinositide remains embedded in the membrane through at least two glycerol-linked acyl chains. GPI-modified substrates are packaged into COPII-coated vesicles for delivery to the outer leaflet of the PM, where they remain anchored until a phospholipase selectively cleaves and releases the protein(14). GPI-modified proteins include cell surface hydrolases (alkaline phosphatase, 5'-nucleotidase), adhesion molecules (NCAM, CD58) and neural receptors (GDNFR), among others (15,16). At the cell surface, the GPI anchor targets substrates to lipid rafts (signal transduction centers in the plasma membrane), and in polarized cells serves as a trafficking signal to target proteins to the apical surface (17,18).

Fatty acylation. Fatty acylation refers to the covalent addition of short or long, saturated or unsaturated fatty acyl chains, or a heterogeneous mixture of both, to a protein. The most common and studied forms of fatty acylation are N-myristoylation and S-palmitoylation (Table 1). N-myristoylation refers to the co-translational attachment of 14-carbon fatty acid, myristate, to an N-terminal Glycine residue. Generally, N-myristoylated proteins contain a Met-Gly-X-X-X-Ser/Thr sequence at the amino terminus. After removal of the initiating Met, N-

myristoyltransferase (NMT) attaches myristate to the amino-terminal Gly, forming an irreversible amide linkage with the protein backbone. This modification increases membrane association. One of the best-studied examples of this process is the kinase Src, which depends on myristoylation for proper localization, stability and activity (19,20). The mechanisms and functional significance of protein S-palmitoylation are extensively discussed in the following section.

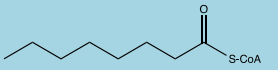
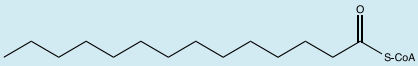
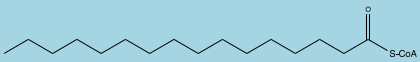
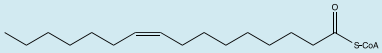
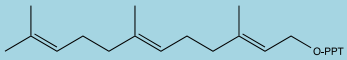
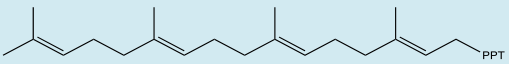
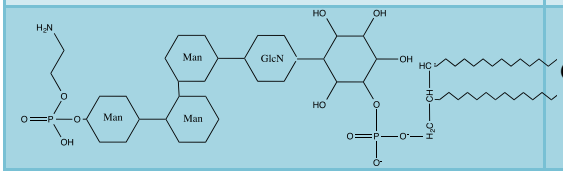
Structure	Acyl moiety	Linkage	Protein
	Octanoate	Oxyester	Ghrelin (21)
	Myristate	Amide	Src (19)
	Palmitate	Thioester or Amide	Fyn Hedgehog (22)
	Palmitoleate	Oxyester	Wnt (23)
	Farnesyl	Thioether	Nuclear Lamin A (8)
	Geranylgeranyl	Thioether	Rho (9)
	Glycophosphatidyl -inositol	Amide	NCAM (15)

Table 1. List of lipids commonly attached to proteins.

PALMITOYLATION OF PROTEINS

S-palmitoylation occurs by covalent attachment of the 16-carbon saturated fatty acid, palmitate (C16:0), to one or more cysteine residues through a thioester linkage (Table 1). Unlike prenylation, no consensus sequence, other than the presence of a cysteine acceptor residue exists for palmitoylation. Protein palmitoylation takes place on the luminal and cytoplasmic faces of membranes, typically of the ER or Golgi. Generally, a protein acyltransferase (PAT) catalyzes this reaction, utilizing intracellular palmitoyl-CoA as the fatty acid donor (1). The thioester bond between the palmitate and the protein is labile and can be readily cleaved by palmitoyl thioesterases, making palmitoylation highly dynamic and versatile. In some instances, palmitate is attached to the $-NH_2$ group of an N-terminal cysteine via an amide linkage (N-palmitoylation), as in Hedgehog (Hh) proteins and the *Drosophila* EGFR ligand, Spitz (22,24). Moreover, a unique type of palmitoylation has been identified in members of the Wnt family: the monounsaturated form of palmitate, C16:1 or palmitoleate, is attached to an internal serine residue via an oxyester linkage (O-palmitoleoylation)(23). Both, O- and N-palmitoylation are irreversible and remain attached throughout the life span of the protein.

Palmitoylation influences the structure and function of many soluble, integral and secreted proteins, including enzymes, signaling proteins, polytopic transmembrane receptors, ion channels and others (1,3,4). The functional outcomes of palmitoylation have proven more diverse and complex than that of membrane anchoring and the mechanisms are just starting to emerge.

Functions of palmitoylation

Membrane tethering: Membrane anchoring by palmitoylation is dependent upon prior lipid modification with either an isoprenyl (farnesyl or geranylgeranyl groups) or N-myristoyl group. The term kinetic trapping has been coined to describe this two-signal mechanism. The first hydrophobic modification allows the protein to indiscriminately and transiently associate with

endomembranes, whereas the second modification (generally palmitate) anchors the substrate to a specific membrane compartment (25). Thus, the subcellular localization of the PAT determines to what subcellular compartment the protein becomes permanently attached. In some cases, as in retroviral Gag proteins, Src and K-Ras4b, one of the hydrophobic signals is replaced by a stretch of basic amino acids that can mediate membrane association by electrostatic interaction with membranes (26-29). Signaling proteins such as Ras (11), Gα subunits (30,31) and Src family kinases (Fyn, Yes, Lck and Lyn) (5,32-36), enzymes such as endothelial Nitric Oxide Synthase (eNOS)(37,38), and adhesion molecules such as integrin and NCAM (39,40) require membrane localization for their function and rely on kinetic trapping to be permanently anchored.

Intracellular Trafficking: Palmitoylation can also influence the trafficking of proteins between intracellular compartments. This effect has been elegantly described for members of the Ras family of small GTPases (H, N, and K-Ras). H- and N-Ras proteins are farnesylated in the cytoplasm and homogenously distributed among cellular endomembranes (41). In the Golgi, N- and H-Ras become palmitoylated at one or two cysteine residues, respectively, and are set en route to the plasma membrane (PM) via vesicular transport. In the PM, N/H-Ras engage in signaling events and are depalmitoylated by the protein thioesterase, APT1 (acyl protein thioesterase 1). Palmitate loss sends H/N-Ras back to the Golgi via retrograde transport where it undergoes re-palmitoylation and PM targeting (42-44). Constant cycles of acylation/deacylation allow Ras to differentially bind and signal from different intracellular cell membranes.

A similar mechanism has been observed for G proteins. G proteins are heterotrimeric (G_{α}, G_{β} and G_{γ}) signal transducers at the inner leaflet of the PM that relay signals from G-Protein Coupled Receptors (GPCR) at the cell surface to intracellular effectors in different signaling cascades. G_{α} is myristoylated and palmitoylated, and G_{γ} is prenylated and caboxymethylated. The heterotrimer appears to assemble in the Golgi and then translocate to the PM (45,46). G complex localization to the PM and function depends on palmitoylation of G_{α}. It has been shown that

inhibition of palmitoylation by 2-Bromopalmitate (2-BP) treatment or site-directed mutagenesis delocalizes G_α from the PM to endomembranes and the cytoplasm, and turns off signaling (47,48). Moreover, re-expressing palmitoylated G_α or engineering a palmitoylation site in G_γ rescues this defect (45,46). Thus, dynamic palmitoylation seems to direct the transport of G proteins from the Golgi to the plasma membrane.

Lipid raft targeting: Lipid rafts are sphingolipid and cholesterol-rich subdomains of the PM that serve as platforms for signal transduction and endocytosis. The high concentration of tightly packed, highly ordered and extended lipids makes this region less fluid than the rest of the PM, thereby separating into a liquid ordered (L_o) phase that is resistant to disruption by non-ionic detergents. Lipid raft targeting by palmitoylation relies on the ability of palmitate to insert into the L_o phase of the PM, whereas modification with bulkier or polyunsaturated fatty acids excludes proteins from this region (49-51). Although palmitoylation increases protein affinity for this region, not all palmitoylated proteins localize to lipid rafts.

Palmitoylation-dependent lipid raft targeting is tightly linked to protein function. For example, eNOS is a myristoylated and palmitoylated enzyme that synthesizes nitric oxide in the cardiovascular system. Palmitoylation-defective eNOS retains enzymatic activity *in* vitro (52) and membrane association in cells (53,54), but is not targeted to rafts at the PM in cells (55,56); instead it is concentrated in a diffuse perinuclear pattern(57). Therefore, palmitoylation is required to target eNOS to a specific subdomain at the cell surface. Likewise, palmitoylation is required for raft targeting and proper function of the T-cell co-receptors, CD4 and CD8 (58,59). In neurons, palmitoylation of NCAM is required for raft targeting and for signaling during neurite outgrowth (60). Thus, lipid-raft targeting by palmitoylation influences protein function by bringing modified proteins in close proximity to their membrane-bound effectors, where they can elicit a cellular response.

Folding/Stability: Another functional consequence of palmitoylation is to protect the integrity of the modified protein. This is particularly true for polytopic membrane proteins with polar residues within transmembrane domains (TMD), causing unfavorable interactions with the lipid bilayer. These hydrophobic mismatches activate the ER quality control system and usually result in ER retention or degradation. For example, low-density lipoprotein receptor-related protein 6 (LRP6) is a single-pass TM receptor involved in mediating canonical Wnt signaling at the PM. LRP6 is palmitoylated at one or two juxtamembrane cysteine residues. When palmitoylation is inhibited, LRP6 fails to pass the last quality control checkpoint and is retained in the ER. Palmitoylation functions by creating a tilt in the TMD with respect to the plane of the plasma membrane to avoid hydrophobic mismatches, thus precluding ER retention and ensuring proper exit from the secretory pathway (61). A similar role of palmitoylation in proper folding has been observed for the yeast polytopic membrane protein, chitin synthase (Chs3) and the SNARE, Tlg1. Palmitoylation-defective Chs3 aggregates and is retained in the ER (62), whereas palmitoylation defective Tlg1 mislocalizes to the vacuole where is ubiquitinated and degraded (63).

Protein palmitoylation, a process that for years was thought to merely increase target hydrophobicity, is now known to be an essential process in the cell. Protein palmitoylation controls a myriad of cellular events, from membrane association to protein degradation, and also has an impact on development and diseases. Over 400 proteins are palmitoylated in mammalian cells, and this is probably an underestimate. Novel technologies allowing to decode the palmitoyl proteome has expanded this list and one can only anticipate this will continue to grow. This knowledge has, and will continue to expand the field in multiple directions, providing ample opportunities for future research.

ENZYMOLOGY OF PALMITOYLATION

Since the discovery of S-palmitoylation, much effort has been devoted to dissect the mechanisms by which fatty acids are attached to proteins. Two mechanisms have been described:

autoacylation or enzyme-mediated. The former refers to non-enzymatic, spontaneous attachment of palmitate to proteins in the presence of long-chain acyl-CoA. This phenomenon has been observed *in vitro* for various lipid-modified proteins, but with the caveat that it only occurs at high, non-physiological concentrations of acyl-CoA (10μM *in vitro* vs. 1μM *in vivo*) (64). Although non-enzymatic palmitoylation might not be representative of what happens in the cell, it argues that increasing the local acyl-CoA concentration could potentially drive autoacylation *in vivo*. The second mechanism requires the action of a protein acyltransferase (PAT), an enzyme that catalyzes the transfer of palmitoyl-CoA to a protein substrate. To date, two families of enzymes with PAT activity have been described: the DHHC-PAT family and the MBOAT (Membrane-bound O-acyltransferase) family.

DHHC- family of palmitoyl acyltransferases

The existence of an enzyme with acyltransferase capability was a matter of debate for many years. The lack of tools to measure enzymatic activity along with the apparent ability of proteins to autoacylate *in vitro* hampered the identification of PATs. The first evidence for the existence of palmitoyl acyltransferases originated from forward genetic yeast studies. The Deschene group conducted a synthetic lethal genetic screen using a yeast strain that depended on Ras palmitoylation for viability, and identified two candidate genes, Erf2 (effect on Ras function) and Erf 4, to be involved in Ras2 palmitoylation (65,66). In a follow-up study, Deschene and colleagues assessed Erf2/4 PAT activity by measuring ^{3}H-palmitate incorporation into purified GST-Ras. They demonstrated that partially purified, recombinant Erf2/4 complexes were sufficient to mediate Ras palmitoylation *in vitro* (67). Later, recombinant Akr1 was shown to mediate palmitoylation of yeast casein kinase 2 (Yck2)(68). Sequence analysis of Erf2 and Akr1 revealed that these enzymes shared a conserved cysteine rich domain (CRD) containing an Aspartate-Histidine-Histidine-Cysteine (DHHC) motif. Mutation of this region in Akr1 abolished enzymatic activity(68) and suggested that other proteins sharing this motif might function as

PATs, hence the name DHHC-CRD family. The identification of this signature sequence allowed for identification and characterization of other DHHC-PATs such as Pfa3, Pfa4 and Swf1 in yeast (62,63,69), as well as DHHC9 and DHHC21 in humans (70,71).

The DHHC-CRD family is the largest family of protein palmitoyl acyltransferases identified thus far, and is genetically conserved among various species, from *C. elegans* (15 genes) and *D. melanogaster* (22 genes) to mice and humans (23 genes). Most mammalian DHHC genes are expressed ubiquitously, with only 5 being tissue specific (72). Mutations and altered levels of expression have been associated with various diseases, including cancer, schizophrenia, learning disabilities and Huntington's disease (73-75).

DHHC family members are multipass transmembrane enzymes that reside and function in different cellular endomembrane compartments, including the Golgi, ER and PM (72). The intracellular distribution of DHHC proteins provides a network of acyltransferases with extensive substrate specificities, influencing *de novo* synthesized proteins and playing a role in different cellular processes. DHHC enzymes function as S-transferases, transferring primarily palmitate (C16:0) to cysteine residues on proteins at the cytoplasmic face of membranes. The mechanism of action for DHHC enzymes follows a two-step 'Ping-Pong' mechanism: 1) initial autopalmitoylation of the enzyme, where a covalent acyl-enzyme intermediate is formed, presumably with the DHHC motif's conserved cysteine residue, followed by association with the protein substrate and 2) palmitate transfer to the –SH group of an acceptor cysteine (76,77). The formation of an acyl-enzyme intermediate (step 1) is a characteristic feature of this family, and has been observed for 17 mammalian DHHC members (76,78). In the absence of substrate, DHHC enzymes retain the ability to autoacylate (76) and are competent to transfer palmitate to a protein when the substrate is added later.

Using bioluminescence resonance energy transfer (BRET) and co-immunoprecipitation experiments, recent studies revealed that DHHC2 and DHHC3 self-associate when expressed in HEK-293 cells (79). Catalytically inactive enzymes are more susceptible to oligomerization than

wild type (WT), whereas addition of palmitoyl-CoA promotes oligomer disassociation (79). Covalently linked DHHC3 dimers are less active than monomeric counterparts, suggesting that enzymatic activity might be regulated by the oligomerization status of the protein. It would be important to determine whether these findings expand to other family members and to explore the functional significance *in vivo*.

Several DHHCs are functionally redundant in terms of protein substrate specificity, as a given enzyme can palmitoylate several substrates and, conversely a given substrate can be palmitoylated by different DHHC-PATs. Several enzyme-substrate pairs have been identified and characterized *in vitro*. Metabolic labeling of cells expressing the neural post-synaptic density protein, PSD-95 and 23 different DHHCs revealed that four DHHC enzymes exhibit PAT activity towards PSD-95 (80). Similarly, eNOS can be palmitoylated by DHHC-2, 3, 7, 8 and 21 (71). DHHC17 palmitoylates Huntingtin protein but also exhibits PAT activity towards SNAP-25 and other neural proteins (81,82). DHHC18/9 palmitoylates H- and N-Ras, and DHHC21 palmitoylates eNOS and the Src family kinases Lck and Fyn (70,71). Although DHHC's generally prefer palmitoyl-CoA as the fatty acid substrate, recent findings identified different acyl-CoA specificities for DHHC 2 and 3. Using High Pressure Liquid Chromatography (HPLC) and a fluorescent peptide cell-based assay, it was demonstrated that DHHC2 can transfer C14:0 to C18:0, and to a certain extent stearoyl-CoA (C18:1) and arachidonic acid (C20:4), but C18:1 and C20:4 were highly inhibitory for DHHC3 (77). Additional studies are needed to determine whether these specificities are conserved in other family members. However, it seems the identity of an acyl moiety attached to a given substrate is dictated by the abundance and distribution of fatty acids rather than enzyme specificity.

The study of S-palmitoylation is a field in progress and many unresolved questions regarding the specific function(s) of each DHHC protein remain unanswered. The development of new sensitive and quantitative technologies to monitor S-pamitolation *in vivo* and *in vitro* promises new avenues for research and rapid progress in the field. It will be critical to identify

and validate novel palmitoylated proteins and elucidate the functional relevance of palmitoylation for protein function. DHHCs are associated with a number of human pathologies and could potentially serve as medical biomarkers or drug targets. There is a prevailing interest for structural information that will allow for rational design of inhibitors that could be used therapeutically. The high degree of redundancy is remarkable and additional studies are necessary to explore the molecular basis of PAT–substrate recognition and specificity.

MBOAT Family

The second family of acyltransferases was identified *in silico* in 2000. Using a bioinformatic approach, Hofmann detected sequence homology and functional conservancy among a group of membrane-bound enzymes that transfer long-chain fatty acids to membrane-associated hydroxyl donors (-OH) (83), and coined the term Membrane-bound O-Acyltransferase (MBOAT) to describe this superfamily. All MBOAT family members encode multipass transmembrane enzymes with 8-11 predicted TMDs, and localize to ER and Golgi membrane compartments. A region of high homology, consisting of an Asp/Asn residue within and adjacent to the membrane and an invariant Histidine residue positioned within a long hydrophobic region characterizes this family. Alterations to this region by site-directed mutagenesis abrogate enzymatic activity in all family members tested to date, and implicate the invariant His residue as the putative catalytic site.

A total 16 members, encoded by 11 genes, have been identified in mouse and human genomes. Owing to the hydrophobic and polytopic nature of this family, biochemical and enzymology studies have been limited or nonexistent. No structural data is available for MBOATs and only a few enzymes have been purified and characterized *in vitro*. According to topology mapping experiments, the active site is positioned along a TMD or in the lumen of the ER, thus restricting target specificity towards luminal and secreted molecules or proteins. Unlike DHHC-PATs, MBOAT members recognize lipids as well as protein substrates. MBOAT family

members can be classified into three subgroups based on substrate specificity: members that acylate lysophospholipids (LPLAT subfamily), members that acylate neutral lipid (ACAT/DGAT subfamily) and those with protein substrates (PAT subfamily) (Table 2).

MBOATs Involved in Phospholipid Remodeling (LPLAT subfamily)

Glycerophospholipids are major structural components of membranes, and also serve as storage units for lipid mediators and constituents of serum lipoproteins (84). Glycerophospholipids are formed by esterification of two fatty acids moieties to the sn1 and sn2 positions of a glycerol-3-phosphate. Among the most common types of glycerophospholipids are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylglycerol (PG). To establish membrane asymmetry and diversity, which is important for membrane fluidity and curvature, the fatty acid composition of a given phospholipid is constantly remodeled in a deacylation-reacylation reaction (Lands' Cycle) (85). Phospholipase A2 cleaves the fatty acids at sn2 to produce lysophospholipid (LP). LP can then be reacylated by various lysophospholipid acyltransferases (LPATs) to generate a varied population of phospholipids with different acyl groups at sn2. Four MBOAT family members, LPEAT1/MBOAT1 (lyso-PE acyltransferase 1), LPCAT3/ MBOAT5 (lyso-PC acyltransferase 3), LPCAT4/ MBOAT2 (lyso-PC acyltransferase 4) and LPIAT1/ MBOAT7 (lyso-PI acyltransferase 1) have been implicated in phospholipid remodeling (86-89).

MBOAT-LPLATs are redundant for lysophospholipid substrates but exhibit preference for fatty acid substrates (Table 2). MBOAT1 transfers 18:1-CoA to lyso-PE and lyso-PS; MBOAT5 modifies lyso-PC, lyso-PE and lyso-PS with 18:2-CoA and 20:4-CoA; and MBOAT2 modifies lyso-PE and lyso-PS with 18:1-CoA (90). Nonetheless, MBOAT7 is specific for lyso-PI utilizing 20:4-CoA as substrate. Sequence alignment of MBOAT-LPLATs, coupled with mutational analysis of conserved residues, identified 4 MBOAT motifs required for LPLAT

activity, 3 of which might be involved in mediating phospholipid recognition, as they are not present in MBOATs with PAT activity (91).

MBOAT Family Members	Lipid or Protein Substrate	Fatty Acyl-CoA Substrate
LPEAT1	Lyso-PE, PS	18:1
LPCAT3	Lyso-PC, PE, PS	18:2, 20:4
LPCAT4	Lyso-PE, PS	18:1
LPIAT	Lyso-PI	20:4
ACAT1	CE	18:1
ACAT2	CE	18:1
DGAT1	Diacylglycerol, Wax Esters, Retinol, Monoacylglycerol	18:0
HHAT	Sonic Hedgehog	16:0
GOAT	Ghrelin	8:0
Porcn	Wnt	16:1

Table 2. MBOAT Family members and preferred substrates

Summary of all mammalian MBOAT family members and preferred substrates. Abbreviations: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cholesteryl ester (CE).

MBOATs involved in Neutral Lipid Biosynthesis (ACAT and DGAT subfamily)

The ACAT subfamily consists of ACAT1 (Acyl-coenzyme A:cholesterol acyltransferase), ACAT2 and DGAT1 (acyl-CoA:diacylglycerol acyltransferase1). ACAT1/2 catalyze the conversion of excess cellular cholesterol to its storage form, cholesteryl ester (CE), using long-chain fatty acyl-CoA and cholesterol as substrates. ACAT1 is expressed ubiquitously in many cells and tissues, while ACAT2 is primarily expressed in the liver and intestine (92,93), indicating the two enzymes may play different roles *in vivo.* Since ACAT2 expression is restricted to the two main lipoprotein-secreting tissues of the body (liver and intestines), it has been suggested that ACAT2 might be directly associated with packaging of CE into the lipid core of very low-density lipoproteins (VLDL) and chylomicrons, whereas ACAT1 plays a general role in cholesterol homeostasis. At the cellular level, cholesterol esterification by ACAT1 prevents the toxic accumulation of free cholesterol in the membranes of the ER and allows storage into lipid droplets. However, chronic accumulation of CE has been linked to early stages in atherosclerosis and in Alzheimers disease, and ACAT has been considered a drug target for therapies against these diseases (94-96).

The first ACAT1 gene was identified and cloned in 1993 (97), and recombinant human protein was purified to homogeneity with full biological activity in 1998 (98). ACAT1 is homotetrameric *in vivo* and *in vitro*, harboring dimer-forming motifs at the N- and C-terminus (99,100). Deletion of the N-terminal region converts the enzyme from a tetramer to a fully functional dimer with unaltered kinetics towards cholesterol and oleoyl-CoA, indicating that multimerization is not required for activity (100). Mutational analyses of evolutionary conserved regions have uncovered a series of residues involved in substrate binding and/or catalysis. The putative active site H460 is required for enzymatic activity and has been proposed to interact with the 3- β-OH moiety of the sterol (101). Conserved residues surrounding H460 (F453, A457 and F479) may interact with the hydrophobic portion of cholesterol, whereas a highly conserved region consisting of FYXDWWN may be involved in binding fatty acyl-CoA (102). ACAT1

activity, assayed in micelles or reconstituted vesicles, responds to cholesterol in a sigmoidal manner, suggesting that cholesterol also acts as an allosteric activator (98). Further kinetic analysis revealed that ACAT1 has two sterol binding sites: one for the substrate and one for the activator. Cholesterol works better as an activator than other sterols and oxysterols, and binds the allosteric site of the enzyme in a stereospecific manner. Upon activation by cholesterol, ACAT activity is increased, and the enzyme is promiscuous towards a wide variety of sterols with 3β-OH configuration (103,104). This mechanism allows for rapid up-regulation by cholesterol that builds up in the ER.

No structural information on ACAT1 is available. However, the membrane topology of ACAT1 has been analyzed by two different groups with different results. Lin et al. first utilized epitope-tagging and indirect immunofluorescence in fixed cells and reported a topology model of ACAT1 consisting of at least 7 TMDs (105). In a follow up study, the same group published a revised topology map with 9 TMDs, which was determined using PEG_{5000}-mal combined with Cys-scanning mutagenesis. This new model positioned putative active site His460 within TMD7 (101). A second group generated successive C-terminal deletions of ACAT carrying an epitope tag and monitored the sidedness of the tag by selective permeabilization of ER-derived microsomal vesicles (106). This approach produced a 5-TMD model. Since introducing deletions might alter the folding and topology of the enzyme, the 9-TMD model is preferred over the 5-TMD model.

The other member of this subfamily is DGAT1. DGAT1 catalyzes the final step in the biosynthesis of triacylglycerol (TAG), by attaching a third fatty acyl-CoA moiety to diacylglycerol (glycerol core with two ester-linked fatty acids)(107). TAGs are the main storage molecules of fatty acids that are used for energy utilization or the synthesis of membrane lipids. TAG synthesis also serves a functional role in protecting cells from lipotoxicity, as components of the skin surface water barrier and providing insulation for organisms (108) . Excess TAG accumulation in adipocytes contributes to obesity and organ failure (109). DGAT1 is ubiquitously

expressed in mammals, with highest mRNA levels detected in the small intestine, testis, adipose tissue, mammary gland and skin. Two enzymes, DGAT1 and DGAT2, with DGAT activity have been identified in yeast and many eukaryotes. Although functionally similar, DGAT1 and DGAT2 do not share DNA or protein sequence similarity and differ in their biochemical, cellular and physiological functions. DGAT1 is a member of the MBOAT family, while DGAT2 belongs to the Diacylglycerol Acyltransferase (DAGAT) family.

DGAT1 was identified by sequence similarity to ACAT, which shares 15–25% identity, mostly in the C-terminal region (110). Since DGAT1 has yet to be purified, most biochemical studies and kinetic analyses have relied on partially purified membrane preparations from cells that overexpress this enzyme. Unlike ACAT, DGAT1 has multiple lipid substrates, and can acylate wax esters, retinol and monoacylglycerol. With regards to fatty acid substrates, DGAT exhibits preference for oleoyl-CoA (C18:1) over palmitate (C16:0). DGAT1 is a homotetramer; self-association is mediated by the non-catalytic N-terminal domain (111). This region also binds fatty acyl-CoA while the C-terminal domain binds diacylglycerol. Membrane topology mapping studies of murine DGAT1, using protease protections assays and indirect immunofluorescence in conjunction with selective permeabilization of cellular membranes, detected 8-TMDs with the C-terminus and active site His positioned in the lumen of ER (111). Mutation of the catalytic His residue reduced acyltransferase activity towards DAG and wax esterase *in vitro*. DGAT1 has also been reported to have dual membrane topology in hepatocytes: overt and latent (cytosolic and luminal)(112,113). The overt conformation catalyzes cytoplasmic synthesis of TG, while in the latent conformation, the active site faces the lumen. The existence of this dual conformation is still a matter of debate and further studies need to be performed to validate this hypothesis.

In summary, the most prominent feature of this subfamily is the dual functionality of the active site. Both ACAT and DGAT reactions occur within the membrane environment of the ER and this advantageous location has been proposed to serve a dual role: esterified substrates from both reactions can either move to cytoplasm and be stored in lipid droplets or be assembled into

lipoprotein particle in ER and be secreted. Whether this is true or is conserved for other family members is yet to be tested.

Members with PAT activity (Hhat, GOAT and Porcn)

Identification of MBOATs that possess acyltransferase activity towards proteins occurred when Porcupine (Porcn), an uncharacterized gene involved in the secretion of Wnt proteins, was shown to share sequence homology with other MBOAT family members (83). This opened the possibility that Porcn and other uncharacterized MBOAT members could modify protein substrates. To date, three MBOATs with PAT activity toward secreted proteins have been characterized: Hedgehog acyltransferase (Hhat), Ghrelin O-acyltransferase (GOAT) and the Wnt acyltransferase, Porcn.

Hedgehog Acyltransferase (Hhat)

Hedgehog (Hh) comprises a family of conserved secreted morphogens that control several stages of embryonic development such as body plan specification, tissue patterning and limb development, in invertebrates and vertebrates (114-116). Vertebrates express three Hh members, Sonic, Indian and Desert Hedgehog, of which Sonic Hedgehog (Shh) is the best studied. In humans, Shh signaling has also been implicated in tissue homeostasis, tissue repair and cancer (117). In medulloblastoma (cancer of the cerebellum) and Basal Cell Carcinoma (skin cancer), germ-line or sporadic *de novo* mutations in the cell surface receptor, Patched (PTC) have been shown to drive tumor formation. Many other Shh-driven tumors do not harbor any genetic alteration in the pathway and depend on ligand production, either by the tumor cells themselves or by the surrounding stroma, to activate Shh signaling. The reliance on ligand production for tumorigenesis represents an amenable avenue to exploit the development of drugs targeting Shh production.

The production of functional Shh protein involves a unique series of processing events, including autocleavage and post-translational modifications. Shh is synthesized as a 45kDa

precursor protein that is targeted to the secretory pathway by a signal sequence. Subsequently, Shh undergoes an autoproteolytic cleavage event to generate a 19kDa N-terminal signaling domain (Shh-N) and the C-terminal domain, which contains intrinsic protease activity that mediates the autocleavage reaction (118). Concomitantly with autocleavage, Shh-N is modified at the C-terminus with a cholesterol moiety (119). In a separate reaction, palmitate is linked to the N-terminus of Shh-N via an amide bond (22). The reaction takes place in the lumen of the secretory pathway, as mutants missing the signal sequence are not palmitoylated. Palmitoylation and cholesteroylation are independent reactions, as cholesterol-deficient Hh can still be palmitoylated, and vice versa (120,121). Dually modified or mature Hh is the most abundant form and contains all signaling capabilities *in vivo* and *in vitro.*

The attachment of both cholesterol and palmitate is required for efficient short and long range Hh signaling. It has been suggested that cholesterol attachment targets Hh to membrane microdomains and restricts diffusion range, while palmitoylation is critical for gradient formation and signaling activity(122,123) . When assayed in cell culture, Shh palmitoylation greatly potentiates signaling activity, causing a 30-fold increase in signaling compared to its unpalmitoylated form. This increase in activity correlates with the degree of hydrophobicity of the modifying molecule rather than with its identity (22,124). It has been proposed that to achieve long-range signaling, Hh proteins form multimeric protein complexs, wherein lipid modifications are sequestered from the cytosol allowing for free diffusion and gradient formation (125). This multimeric protein complex constitutes the major active component for Hh signaling and its formation requires palmitoylation (126). Furthermore, palmitoylation-deficient Hh fails to establish a protein gradient and exhibits severe patterning and limb defects in fly and mouse embryos (127,128).

The MBOAT family member, Hedgehog acyltransferase (HHAT), catalyzes attachment of palmitate to the N-terminal Cys on Hh proteins (Cys 24 in Shh after signal sequence cleavage). Three independent groups initially identified Hhat (formerly known as skinny hedgehog, central

missing or sightless; and *Rasp* in flies), as a segment polarity gene required for Hh signaling in *Drosophila* (120,121,128). The role of Hhat as a Hedgehog acyltransferase has been established both genetically and biochemically. *Rasp* null flies produce unpalmitoylated, inactive Hh protein, as evidenced by the lack of Hh target gene expression in the developing wing and a marked reduction in Hh hydrophobicity when analyzed by reversed-phase high-pressure liquid chromatography (120,128). Moreover, an Hhat knockout mouse model exhibits defective patterning of the limbs and neural tube, and phenocopies the Cys24Ser mutant and Shh-/- phenotype (127). Definitive biochemical evidence of Hhat PAT activity was provided when Buglino and Resh accomplished the purification to homogeneity of Hhat and characterized its enzymatic activity *in vitro*. Using cell-based and *in vitro* palmitoylation systems, Buglino and Resh directly monitored Hhat-dependent incorporation of radioiodinated palmitate into Shh (121). Incubation of purified Hhat with purified, recombinant Shh results in near-stoichiometric incorporation of palmitate into Shh. Palmitate is transferred to the $–NH_2$ group of the Cys24, forming a stable and irreversible amide linkage with the protein backbone (121). It has been postulated that the palmitoyl moiety is initially attached to the sulfhydryl group of the cysteine and then transferred to the free N-terminus through an intermolecular S-to-N shift (22). However, inability to detect palmitate incorporation into N-acetylated Shh *in vitro* argues against the hypothesis that a free –SH group could serve as a fatty acyl acceptor (121). Kinetic analysis determined derived V_{max} and K_m values by titrating Shh at maximal iodopalmitoyl-CoA concentration (V_{max} 0.25 +/- 0.03 pmol/min, K_m 1.25+/- 0.26 μM) and vice versa (V_{max} 0.21 +/- 0.03 pmol/min, K_m 3.0 +/- 0.26 μM), while Hhat concentration remained constant. Optimal activity was observed at pH 6.5 and low salt concentration. Although most acyltransferases are promiscuous in terms of substrate specificity, Hhat is highly specific for Hh proteins, and does not acylate other proteins such as Wnt, PSD95 or Gα subunits. Hhat recognizes fatty acyl-CoAs but not phospholipids or free fatty acids. Although palmitoyl-CoA is the authentic fatty acyl substrate for Hhat, medium-chain fatty acids such as decanoyl-CoA (10:0), lauroyl-CoA (12:0),

and myristoyl-CoA (14:0) exhibited an ability to compete with Iodopalmitoyl-CoA. This is not surprising since initial mass spectrometric analysis of purified Shh recovered peptides with a varied population of fatty acids. Furthermore, the first 6 amino acids of the mature Shh protein are sufficient for Hhat-mediated palmitoylation *in vitro* (129). Structure-function analysis of conserved regions in Hhat identified key residues required for stability and enzymatic activity, including putative catalytic sites D339 and H379, and the identification of a novel homology region required for Shh palmitoylation (130).

Owing to its importance on Shh signaling in diseases, Hhat is an attractive target for the development of inhibitors that could antagonize Shh signaling in cancer. Hhat depletion by shRNA blocks Shh signaling and anchorage dependent/independent proliferation in human pancreatic cancer cells(131). *In vivo,* Hhat knockdown reduces tumor growth in a mouse xenograft model of pancreatic cancer(131). These results fueled efforts to carry out high throughput screening for small molecules that could inhibit Hhat-mediated Shh palmitoylation *in vitro*. This work resulted in the identification and validation of RU-SKI-43, a first-in-class Hhat inhibitor with high specificity and potency for Hhat (131,132). Current studies in our lab are investigating the role of Hhat in driving pancreatic cancer, lung and breast cancer and the viability of RUSKI43 as a therapeutic agent in cancer.

Ghrelin O-acyltransferase (GOAT)

Ghrelin is a 28aa, multifunctional peptide hormone mainly known for its orexigenic properties (appetite stimulating) and its role in glucose homeostasis. Ghrelin is produced in the stomach, but exerts its effect in the hypothalamus-pituitary unit where it binds the growth hormone secretagogue receptor (GHSR) and stimulates growth hormone (GH) release (133-135). In humans, ghrelin peaks before meals, suggesting its role as a hunger signal. Intracerebroventricular administration of ghrelin to rats or peripheral administration of ghrelin in human enhances food intake and body weight (136,137), whereas deletion of Ghrelin encoding genes prevents high diet induced obesity, enhances glucose-stimulated insulin release and

increased glucose tolerance (136,138,139).

Mature Ghrelin is derived from proteolytic cleavage of an 117aa protein precursor, preproghrelin, and exists in two major forms in stomach and blood: acylated- and des-acyl-ghrelin. Acyl-ghrelin is modified with an eight-carbon saturated fatty acid, octanoate (C8:0), at Serine 3 (133). To date, Ghrelin is the only octanoylated animal hormone known. Octanoate is attached to the –OH group of Serine via formation of an oxyester linkage. About 20-30% of circulating Ghrelin is acylated and this modification is required for its hormone releasing activity (133,134). Although octanoyl-ghrelin is the most abundant form of acyl-ghrelin, mass spectrometry analysis of purified human ghrelin from stomach recovered a small population of peptides with decanoic acid (10:0), and to a lesser extent decenoic acid (10:1) (140). Furthermore, synthetically generated decanoyl-ghrelin can stimulate GH release. The N-terminal region of ghrelin is evolutionary conserved and the first 10aa are identical across species. The high degree of conservation and the universal requirement for acylation indicate this region is essential for activity. When mice are supplemented with medium-chain fatty acids, the concentration of acylated grehlin increases in the stomach, suggesting that ingested fatty acids are directly used for the acylation of ghrelin (141).

In 2008, two independent groups identified orphan acyltransferase, MBOAT4 as Ghrelin acyltransferase and renamed the gene product GOAT for Ghrelin O-acyltransferase (21,142). GOAT, but no other MBOAT, mediates Ghrelin acylation in cell culture or *in vitro* (21,143). GOAT gene knockout mice exhibit a complete absence of octanoylated ghrelin in the blood in contrast to wild-type littermate animals (142). The tissue expression of GOAT matches that of ghrelin, with highest expression levels detected in stomach and pancreas. Supplementing the medium of HEK293T cells with lipids ranging from acetate to tetradecanoic acid results in GOAT-mediated acylation of ghrelin with fatty acids up to tetradecanoic acid (C14:0), indicating the active site is not as restrictive (142). Surprisingly, *in vitro* characterization of GOAT specificity for fatty acids showed a stronger preference for hexanoyl-CoA over octanoyl-CoA

(143). A four-amino acid peptide derived from the N-terminal sequence of ghrelin can be modified by GOAT, indicating that these four amino acids constitute the core motif for substrate recognition by the enzyme (143-145). In bullfrogs, Ser 3 is substituted for a Thr, but this residue is also octanoylated (21,146), further establishing GOAT as an O-acyltransferase. Optimal reaction conditions are observed at 37–50 °C and pH 7.0-7.5 (143). Putative catalytic sites, H338 and N307 are conserved across species and required for Ghrelin acylation. While no structural information on GOAT exists, the elucidation of its topology has provided tremendous insight to the architectural organization of the enzyme. Using selective permeabilization and indirect immunofluorescence microscopy in combination with glycosylation shift immunoblotting, it was demonstrated that GOAT contains 11 transmembrane helices and one re-entrant loop (147). Catalytic site His338 is positioned in a luminal loop between TMD8 and TMD9 and Asn307 lies in a cytosolic loop. The authors propose that His338 is required for catalysis while Asn307 might be of importance in substrate interactions, transport of substrates, or protein stability. Analytical ultracentrifugation analysis of purified GOAT in detergent micelles determined that GOAT is a monomer.

Since its discovery, GOAT inhibition represents a viable target for the treatment of obesity, diabetes and metabolic diseases. To date, three classes of GOAT inhibitors have been described: acyl-peptide analogs, a small molecule inhibitor, and a bi-substrate analog. Octanoylated pentapeptides can inhibit GOAT activity with high potency (IC_{50} 1μM), presumably acting through product inhibition (143,144). However, this approach poses problems *in vivo* because these peptides also function as a GHSR agonist and possess limited ability to penetrate membranes (145). A fluorescent-based assay coupled with click chemistry was used in a high throughput screen for small molecule inhibitors of GOAT activity. Two related small molecules were discovered (148). Each molecule contains either a six- or eight-carbon alkyl chain, suggesting that they possibly compete for the octanoic acid-binding site on GOAT. These drugs represent promising leads, but their therapeutic efficacy has not been explored pharmacologically.

Lastly, a bisubstrate analog, GO-CoA-Tat, uses non-hydrolyzable amide and thioether linkages to combine octanoyl-CoA with the first 10 amino acids of ghrelin. The interaction between the substrate and the enzyme is favorable because it allows dual occupancy without the entropic loss associated with collision of individual substrate molecules. GO-CoA-Tat potently inhibits GOAT *in vitro*, in cells and in mice (149). Treatment of C57BL6 mice on medium-chain triglyceride (MCT) diets with GO-CoA-Tat at 40 mg/kg dose, but not with the control compound or vehicle, decreased plasma acyl ghrelin levels without changing the des-acyl ghrelin levels. Intraperitoneal administration of GO-CoA-Tat improves glucose tolerance and reduces weight gain in WT mice but not in ghrelin-deficient mice. Although results are promising, peptide-based derivatives with better pharmacokinetics and pharmacodynamics profiles are needed to fully exploit the therapeutic value of this inhibitor.

Porcupine (Porcn)

Porcupine is the acyltransferase for Wnt ligands. The mechanism by which Porcn catalyzes this reaction is the topic of my thesis work and it is discussed in great detail in the following sections (refer to *Porcn as a Wnt acyltransferase* section).

THE WNT FAMILY

The Wnt1 gene, originally named *Int-1*, was identified in 1982 as a common integration site for the mouse mammary tumor virus (MMTV) in virally transduced mammary carcinomas(150). Sequencing analysis later determined *Int-1* to be the human homologue of the *Drosophila melanogaster* gene, *wingless (wg);* a gene involved in many developmental processes such as embryonic segment polarity and patterning, mid-gut morphogenesis, organogenesis and limb development (151,152). The term Wnt was coined as a mnemonic for the wingless-type MMTV integration site that founded the gene family (153,154).

Since then, Wnt orthologues have been identified in many organisms, from early metazoans to humans, highlighting the conservation of Wnt function throughout evolution. The

segment polarity phenotype observed in *wg* null *Drosophila* embryos, coupled with epistasis experiments were key for the identification of genes modifying the cellular and/or developmental response to Wg, thereby defining a pathway specifically activated by Wg molecules. The observation that exogenous expression of Wnt1 in *Xenopus* embryos results in body axis duplication provided an assay to study this pathway in vertebrates (155). Similarities between the pathway components in *Drosophila* and *Xenopus*, unveiled the canonical signaling cascade that we know today. Since then, the field of Wnt signaling has extended to many aspect of human biology, from proper tissue development and cell fate determination during embryogenesis, to tissue homeostasis and oncogenesis in adults.

Wnt biogenesis and glycosylation

The human genome contains 19 different Wnt genes, all sharing the same biochemical properties but differing in functionality. Most Wnt genes encode a 35-45 KDa protein with a signal sequence, a cysteine-rich domain (CRD) and predicted glycosylation sites. During translation, Wnt proteins are directed to the ER lumen by the signal sequence, and associate with BiP chaperone proteins until a functional conformation is assumed (156). Proper three-dimensional assembly is dependent upon correct pairing of over 20-24 cysteine residues, resulting in a distinctive and extensive network of disulfide bonds. Owing to the complexity of this process, purifying recombinant, active Wnt protein has proved very challenging and eluded many attempts for two decades. To date, not all Wnt proteins have been purified.

Correctly folded Wnts are subjected to the addition of one or more N-linked high mannose glycan chains. All Wnt proteins, except *Drosophila* Wnt D, contain at least one glycosylation site. The degree of glycosylation varies among different Wnt isoforms, and ranges from Type I (containing no glycan chains) to Type IV (containing 3 glycan chains). When overexpressed in cells, differentially glycosylated Wnts appear as slow-migrating bands, forming a ladder in immunoblots (157). Blocking Wnt glycosylation either with tunicamycin

(glycosylation inhibitor) treatment or by site directed mutagenesis impairs Wnt3a and Wnt5a secretion (158,159). Paradoxically, glycosylation deficient Wnt-1 (N29, 315, 359Q) exhibits higher autocrine signaling potency compared to WT(160,161). More recently, the *in vivo* role of N-glycosylation was studied in Wg, the *Drosophila* Wnt-1. Wg mutants devoid of all the N-glycosylations exhibited no major defects in either secretion or signaling in S2 cell culture or in the developing wing imaginal discs in fly embryos, indicating this modification is dispensable for Wg activities (162). It has been suggested that N-glycan attachment mediates Wnt binding to heparan-sulfate proteoglycan (HSPG) at the cell surface and thereby increases the local concentration of Wnt in membranes (163,164). Although the functional significance of this modification is not clear, it is possible that glycosylation plays different roles in different Wnt proteins or that its function is spatiotemporally regulated. A systematic approach providing insight to the mechanism and function of these modifications will be necessary to further define this process.

Wnt palmitoylation

Based on the primary amino acid sequence, one might expect Wnt proteins to be highly soluble. Surprisingly, Wnts are extremely hydrophobic, difficult to purify and associate with membranous structures (cell surface and extracellular matrix) in tissue culture (165). The first biologically active and soluble Wnt proteins to be studied were Wg (166) and Wnt1(167). In 2003, Willert, K. et al. achieved, for the first time, the purification Wnt3a proteins from conditioned media of murine L-cells stably expressing Wnt3a. Further characterization by mass spectrometry analysis identified a thioester-linked palmitate (C16:0) attached to a highly conserved Cys residue (Cys77) (168). Functional studies using site-directed mutagenesis determined that Wnt mutants carrying an Ala substitution at Cys77 were secreted with similar efficiency to WT but were unable to bind the receptors at the PM or induce the transcription of Wnt target genes (158,168). It was therefore concluded that palmitoylation at Cys 77 was

required for signaling activity. In a subsequent study, Takada et al. uncovered a second lipid-modification site: a monounsaturated fatty acid (MUFA), palmitoleate (C16:1), is attached to Serine 209 of Wnt3a via an oxyester linkage (23). In contrast to C77, modification of Ser209 is required for Wnt3a secretion. Palmitoylation-deficient mutants, carrying a Ser to Ala mutation, are not secreted from cells in culture or in Xenopus embryos, but are retained in the ER (23).

The identification of this unprecedented set of lipid modifications raised many questions and speculation regarding the cellular advantage these modifications could confer to Wnt ligands. However, the functional duality of palmitate and palmitoleate could not be reconciled for all Wnt family members. As opposed to mammalian Wnt3a findings, acylation-deficient Wg mutants, C93A and S239A, display opposite effects. Wg C93A (homologous to C77 in Wnt) exhibits secretion defects but retains the ability to signal *in vitro*, whereas S239A is efficiently secreted but fails to signal in *Drosophila* imaginal discs (169). More recently, a report showed that both acylation sites are implicated in secretion, but S239 has a more prominent effect on signaling and engaging the receptors than C93 (162). In mammalian Wnt, Douvraska et al. showed that palmitoylation-deficient mutants Wnt1 C93A and Wnt3a C77A exhibit different signaling capabilities depending upon the testing system used (160). Strikingly, mutation of Ser209 in Wnt3a or its equivalent in Wnt1 abolishes palmitoylation, while C77A remains palmitoylated to the same extent as WT, raising the question of whether Cys77 is really acylated (160). These conflicting findings eluded explanation for years, and different mechanisms were proposed to explain these discrepancies.

Our work (Chapter II), in conjunction with the recently resolved crystal structure of *Xenopus* Wnt8 solved this scientific conundrum by providing definitive proof that Wnt proteins only carry one lipid modification. Using a Porcn-dependent cell-based palmitoylation assay, we showed that C77 is not a fatty acyl-acceptor site in Wnt3a (for a detailed description, refer to Chapter II) (170). In addition, the crystal structure of XWnt8 revealed that Cys77 is engaged in a disulfide bond with a nearby cysteine, and that the only lipid-modified residue is Ser187

(corresponding to Ser209 of Wnt3a). Since the Ser modification site is conserved across species, it is believed that all Wnt ligands are acylated; the only exception being *Drosophila* WntD, which lacks the analogous Ser209 and is not modified (171). Therefore, it seems reasonable to assume that previous signaling defects observed in Wnt C77A arise from improper folding, rather than lipid deficiency, and highlights a tight link between functional conformation and signaling activity.

Although the interplay between palmitoylation and glycosylation has been previously studied, the misconception of Wnt dual acylation may have interfered with the interpretation of results. In cells overexpressing Wnt3a, it was observed that glycosylation enhances palmitoylation, but that palmitoylation is not required for glycosylation (158,160). More recently, Gao X et al. established that glycosylation is not required for Wnt3a palmitoylation, since tunicamycin (a glycosylation inhibitor) treatment did not interfere with the palmitoylation status of the protein(172). As new tools become available, more research is needed to understand the relationship between glycosylation and palmitoylation. In summary, Wnt ligands carry one lipid modification, palmitoleate (C16:1), at a conserved Ser residue and this modification is required to produce secreted, active Wnt ligands. Therefore, fatty acylation not only confers membrane affinity but it also bestows certain advantages such as controlled diffusion range, increased potency and specificity toward receptors.

Wnt structure

The first piece of structural information on Wnt proteins became available in 2012, three decades after the first Wnt gene was discovered. Janda et al. utilized an unconventional approach to obtain soluble Wnt protein: *Xenopus* Wnt8 and the CRD-domain of the PM receptor Frizzled (Fz) were co-expressed in the same vector. This method allowed for efficient affinity purification from S2 cells in the absence of detergents, since Fz served as a shield for the lipid moiety. The crystal structure of the XWnt8-Fz-CRD complex was resolved at a resolution of 3.25Å, and

unveiled an unprecedented two-domain structure, in which Wnt contacts Fz at two opposing sites (173). The XWnt8 N-terminal Domain (NTD) is composed of a seven–α-helical bundle, containing two large inter-helical loop insertions that are stabilized by four disulfide bonds. The C-terminal Domain (CTD) is characterized by a long 38–amino acid β-strand hairpin that is stabilized by 6 disulfide bonds, and one N-glycan chain. A lipid moiety, whose electron density is consistent with that of a 16-carbon fatty acid, was captured at Ser187 (corresponding to Ser209 in Wnt3a) and is directly involved in Fz-CRD binding. A groove on the Fz –CRD surface is lined with hydrophobic amino acids, forming extensive van der Waals interaction with the lipid. Therefore, palmitoleate plays a major structural role in mediating Wnt recognition by Frizzled, which is in turn required for pathway activation. It has been proposed that in the absence of Fz the palmitate-containing loop may form a fist so that the lipid might be protected from the aqueous environment (174). This is in fact observed in the 2.1Å resolution crystal structure of *Drosophila* Wnt D fragment encompassing the N-terminal domain and linker region. In the uncomplexed WntD, this hairpin is shifted toward the direction of the α-helical core of the NTD (175).

The elucidation of Wnt structure represents one the biggest breakthroughs in the field. It is now possible to understand the physical ligand-receptor interactions governing Wnt pathway activation. This finding will also enable researchers to rationally design inhibitors aiming to disrupt Wnt-Fz interaction in the context of a disease. As new structures become available it will be possible to elucidate interactions and binding affinities between different Wnts and Fzs, or vice versa.

Wnt secretion

Wnt function as a morphogen has been extensively documented in *Drosophila* embryos, primarily in the developing wing imaginal disc. In this setting, Wnts are produced at specific sites (Wnt producing cells) and distributed throughout adjacent tissues in a gradient fashion to

influence short and long-range patterning and differentiation decisions (176,177). It has for a long time remained a paradox how a lipid-modified, hydrophobic protein can travel up to 20 cell diameters through the aqueous extracellular space. Recent findings have shed light into the cargo-receptor interaction governing Wnt trafficking from the ER to the plasma membrane. With the aid of the p24 protein family, lipid-modified Wnts are shuttled to the Golgi (178,179) where they bind Wntless (Wls; als known as Evi/Sprinter), a multipass transmembrane cargo protein whose primary function is to escort Wnt ligands through the exocytic pathway to the PM. This view has been challenged by recent findings demonstrating that the Wnt-Wls interaction takes place in the ER instead (180). Using indirect immunofluorescence microscopy to visualize native, rather than epitope-tagged Wls, it was shown that a substantial pool of Wls resides in the ER and interacts with lipidated Wnt proteins. Wnt recognition by Wls requires Ser acylation (181,182), indicating that this process occurs downstream of the palmitoylation event. However, the lipid moiety is not the sole requirement for Wls binding as other palmitoylated signaling proteins such as Hh do not interact with Wls (183). It has been proposed that Wnt lipidation may induce a conformational change enabling a functional interaction with Wls (184), but additional structure function analyses are needed to further define Wnt-Wls physical interaction. Only one Wls gene exists per genome and is necessary for the secretion of all Wnt ligands, with the exception of *Drosophila* WntD, which is not acylated and is secreted in a Wls-independent fashion (171). The release of Wnt to the PM requires vacuolar acidification, and small drug inhibition of the V-ATPase, a proton pump required for this process leads to the accumulation of Wnt-Wls complex at the cell surface and in the cell (181). After Wnt release to the extracellular space, Wls is re-internalized by clathrin-mediated endocytosis. In the endosome, Wls binds the retromer complex, a multimeric trafficking protein complex, and is retrieved to the ER via the trans-Golgi network for additional rounds of Wnt secretion. The retrograde trafficking of Wls from the Golgi to the ER requires an unusual ER-targeting sequences at the carboxyl terminus of Wls and involves ERGIC2 (a protein essential for retrograde transport of toxins) (180). Depletion of retromer

complex components Vps35 and Vps26, by siRNA treatment, results in lysosome-mediated Wls degradation, suggesting that retromer's primary function is to prevent Wls degradation (185-187).

As Wnt reaches the cell surface, it becomes tethered to the PM through its lipid moiety, increasing the local concentration for short-range signaling. However, a significant portion of active Wnt protein can be purified from the conditioned media. Thus, how is Wnt released from the plasma membrane, and how does it achieve long range signaling? Many theories have been debated over the years; most of them provide mechanisms to hide the lipid moiety from the aqueous extracellular environment. In *Drosophila* wing imaginal discs, argosomes were first described as basolateral membrane-derived particles containing Wg which serve to spread Wg throughout the epithelium to achieve long-range signaling (188). However, this mechanism of Wnt distribution has not been observed in mammals. Alternatively, Wnt proteins may become incorporated into lipoprotein complexes. In *Drosophila*, Wg co-purifies and co-localizes with lipoprotein particles in the developing wing epithelium (189). In mammalian cells, high-density lipoprotein particles (HDL) but not low-density lipoprotein particles (LDL) can mediate Wnt3a release from cultured mouse fibroblast L cells (190). This mechanism requires Wnt acylation, but it is not clear whether lipoprotein-associated Wnts are active.

Recently, Secreted Wg-interacting molecule (SWIM) proteins were identified as extracellular modulators of Wg secretion. SWIM binds Wg at the surface of secreting cells, enabling monomeric Wg to spread through wing discs and influence long-range signaling (191). SWIM-mediated secretion depends on Wnt palmitoylation, and it is believed to shield the lipid moiety from the aqueous environment, thereby maintaining the solubility of Wg in the extracellular space. A similar mechanism has been documented for Hh proteins. Scube2 (signal sequence, cubulin domain, epidermal growth factor-related protein) binds cholesteroylated-Hh proteins at the cell surface and ferries them through the extracellular space to achieve long-range signaling (192,193). It is yet to be determined whether such a co-factor exists for mammalian Wnt ligands.

A subpopulation of Wnt proteins is transported through endosomal compartments in a Wls-dependent manner and loaded onto exosomes. In the extracellular space, exosomes carry Wnts on their surface to induce Wnt signaling activity in target cells, both during *Drosophila* development and in human cells (194). Moreover, Wnt-Wls containing exosome vesicles have been observed in *Drosophila* neuromuscular junctions, further supporting the existence of this mechanism *in vivo* (195).

For years, it has been puzzling to reconcile the long range signaling of Wnt proteins with their high degree of hydrophobicity. Recent studies have challenged the current view of Wnt as a long-range signal, arguing that besides *Drosophila* developing wing, no other system exhibits long-range Wnt signaling activity. In support of this hypothesis, Alexandre et al. showed that an engineered *Drosophila* strain in which the endogenous *wg* gene has been replaced with a membrane-tethered form of Wg, is viable and forms normally patterned appendages (196). Furthermore, short range, but not long range Wnt signaling is required for stem cell maintenance *in vitro* (177). Additional *in vivo* models of Wnt secretion are necessary to fully understand the differences and requirements for long and short range signaling.

Wnt's journey through the secretory pathway is complex and highly regulated. After processing, Wnt encounters a myriad of molecules and complexes whose major function is to safely deliver Wnt to receptor cells, enabling it to engage in countless biological process. The mechanisms mediating Wnt secretion are varied, but converge in one aspect: to achieve Wnt solubility in the extracellular environment by binding Wnt's hydrophobic moiety. Evolution has apparently devised multiple ways to protect and maintain the integrity of this fatty acyl moiety, from the moment it is attached to Wnt until it reaches its final destination. Whether these mechanisms act in a context-specific manner or occur simultaneously in the cell remains to be determined. In depth analysis of the interactions and binding affinities between Wnt and its modulators will provide a better understanding of the mechanisms governing this process.

The canonical Wnt/ β-catenin signaling pathway

Wnt ligands can activate multiple signaling pathways: the canonical/ β-catenin pathway, the Wnt/Ca+ pathway and planar cell polarity (PCP). The last two pathways are often referred as non-canonical Wnt signaling. In the planar cell polarity pathway, the receptor Frizzled (Fz) activates Jun N-terminal Kinase (JNK) and directs asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets. The Wnt/Ca2+ pathway leads to release of intracellular Ca2+, possibly via G-proteins. Elevated Ca2+ can activate the phosphatase calcineurin, which leads to dephosphorylation of the transcription factor NF-AT and its accumulation in the nucleus. For detailed reviews of non-canonical Wnt signaling, refer to (197,198).

The canonical/ β-catenin-dependent pathway is best understood and is the primary subject of this review. The end result of the canonical Wnt signaling pathway is the stabilization and nuclear translocation of the transcriptional co-activator β-catenin. In the absence of Wnt, β-catenin stability is regulated by the destruction complex (Fig. 1). This complex is comprised of the scaffolding protein Axin, the tumor suppressor Adenomatous Polyposis Coli (APC) and two kinases, Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3). When the receptors are not engaged, CK1 and GSK3 phosphorylate β-catenin at a series of conserved Serine/Threonine residues, promoting recognition by the E3 ubiquitin ligase, β-Trcp. As a consequence, β-catenin is poly-ubiquitinated and degraded by the 26S proteasome. Wnt binding to the Frizzled (Fz) and low-density lipoprotein receptor-related protein 6 (LRP5/6) co- receptors at the plasma membrane triggers a series of events that culminate in the transcription of Wnt target genes (Fig. 1). First, Wnt binding induces phosphorylation of the cytoplasmic protein, Dishevelled (Dvl). Upon phosphorylation, Dvl binds the intracellular tail of Frizzled and other components of the destruction complex including Axin and CK1. The exact mechanism of action of Dvl is still not known, and Dvl phosphorylation may not be required for β-catenin stabilization (199). Next, the LRP5/6 intracellular tail is phosphorylated at a series of PPP(S/T) repeats by the

GSK/CK1 kinases. Phospho-LRP6 recruits Axin, along with the rest of the destruction complex, to the PM where they can no longer degrade free β-catenin. Subsequently, newly synthesized β-catenin accumulates in the cytoplasm and translocates to the nucleus where it binds the T cell factor (TCF) family of transcription factors and promotes transcription of Wnt target genes, such as c-myc and cyclin D1. Phospho-Dvl, phospho-LRP6 and nuclear β-catenin are common biomarkers of Wnt pathway activation in the cell, and are widely used to detect Wnt signaling activity in tissues or in cells.

The controlled activation of Wnt target genes governs many developmental programs. Most of our current knowledge comes from engineered mouse models carrying specific Wnt gene knockouts. These mice exhibit a plethora of phenotypes, ranging from embryonic lethality to severe central nervous system defects (200). In humans, disruption of Wnt machinery components has been linked to cancer, diabetes, focal dermal hypoplasia, bone density defects, and polyposis coli (201-203).

Although seemingly complete, dissecting the downstream Wnt signaling cascade is still a work in progress. Unanswered questions underlying the functional significance of Dvl, how stabilized β-catenin is ferried into the nucleus, and why there is a high degree of redundancy between Wnt and Fz isoforms, are some of the many questions to be answered in years to come.

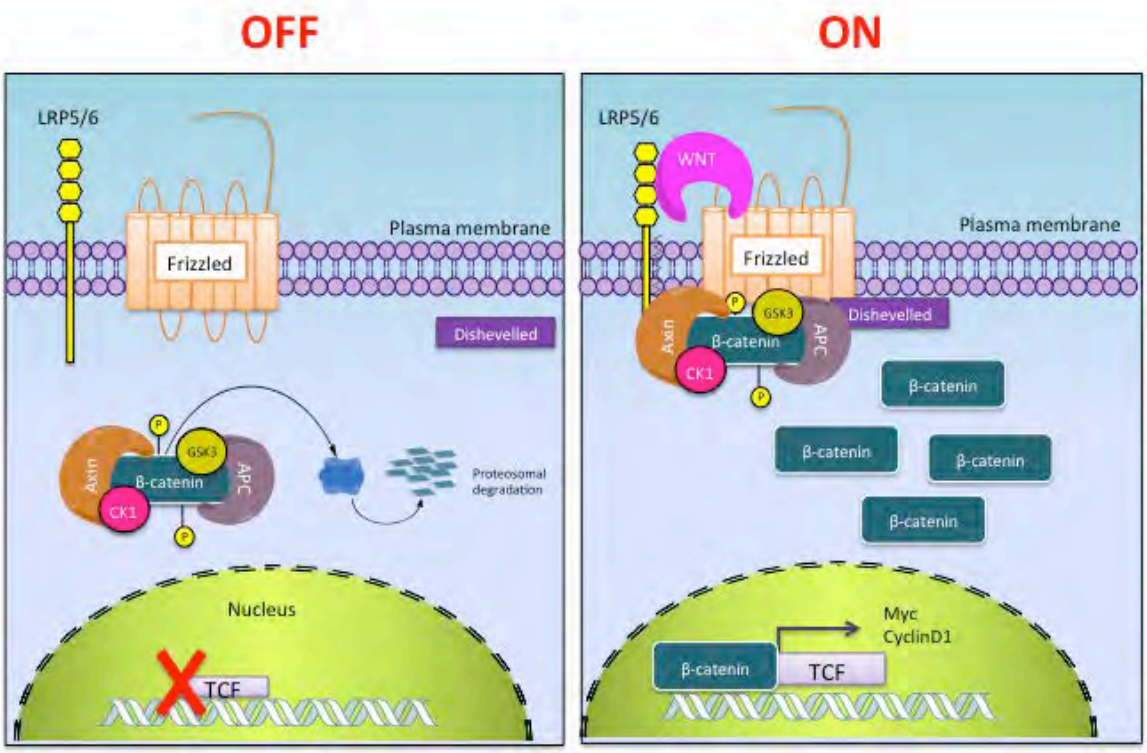


Figure 1.1 The Wnt signaling pathway

In the absence of a Wnt signal, β-catenin is recognized by the destruction complex, phosphorylated and degraded by the 26S proteasome. Upon Wnt binding to Fz and LRP5/6 at the PM, the destruction complex is sequestered to the PM and can no longer bind free β-catenin. Subsequently, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it binds the TCF family of transcription factors and induces the transcription of Wnt target genes such as c-myc and cyclin D1.

Wnt and stem cell maintenance

The role of Wnt signaling in stem cell self-renewal and tissue homeostasis has drawn particular interest in the last decade, as this aspect of Wnt is intricately connected to the development of diseases. Stem cells are defined by their capacity to differentiate into specialized lineages or to self-renew. The fate of these cells is dictated by extrinsic signals that are produced by a defined group of cells known as cell niches or signaling centers. Niche signals often comprise BMP, Hedgehog, and Delta/Notch and Wnt proteins.

Early studies showed that disruptions in mouse TCF4 result in loss of intestinal stem cells and breakdown of the tissue (204). Subsequent studies established that the Wnt pathway is required for a variety of stem cell niches, including hair follicles, bone and skin (201). In the hair follicle, Wnt signals through β-catenin and Lef1 to establish *de novo* hair follicle formation in the epidermis. Blocking Wnt signaling by overexpression of the Wnt antagonist Dikkopf (Dkk) eliminates hair follicles and other skin appendages in a tissue-specific manner. In the bone, Wnt signaling plays a key role in regulating osteoblast maturation and activity, thereby influencing bone mass and regeneration. Mice and humans carrying activating mutations in LRP5 (G171V) display increased bone density and elevated numbers of active osteoblasts (reviewed in(205)). Moreover, loss of Lrp5 in mice results in bone mass reduction and defective osteoblast proliferation and maturation (206). In the hematopoietic system, soluble or purified Wnt proteins promote proliferation and inhibit differentiation of murine hematopoietic progenitors, as measured by clonogenic assays and long-term reconstitution in irradiated mice (207). Similarly, Wnt can expand the number of clonogenic cells from the mammary gland, with retention of the developmental potential of the cells when transplanted back into animals (208). Wnt can also maintain pluripotency of mouse embryonic stem cells, and exogenous addition of Wnt protein or Wnt agonists can maintain self-renewing stem cells in culture (209).

The development of new and powerful genetic techniques has facilitated the visualization

of Wnts as stem cell modulators. Wnt target genes such as Axin2 and Lrg5 have been identified as markers of stem cell populations in intestine, colon and hair follicles (210-212). Lineage tracing of Wnt responsive stem cells has emerged as a potent tool to detect cell fate *in vivo*, and shows how these cells contribute to the different cell lineages of the mammary epithelium, colon and intestine(210,211). To dissect the mechanism by which Wnt promotes self-renewal *in vitro*, Wnt proteins were immobilized on beads and introduced to embryonic stem cells in culture. An interesting pattern was observed: the proximal cell retains stem cell markers whereas distal cell acquire hallmarks of differentiation, suggestive of a mechanism whereby a spatially restricted Wnt signal induces an oriented cell division that generates distinct cell fates at predictable positions relative to the Wnt source (213). Whether or not this mechanism exists *in vivo* is yet to be determined.

Wnt signaling and cancer

Owing to the importance of Wnt signaling in maintaining tissue homeostasis and stem cell control, it is not surprising that mutations tipping this balance lead to malignant cell growth. The first connection between Wnt and human cancer was evidenced in patients with familial adenomatous polyposis (FAP), a hereditary cancer syndrome, and spontaneous forms of colon cancer. FAP patients carry germline, inactivating mutations in the APC gene (214). Loss of APC function leads to constitutive activation of Wnt signaling and the formation of intestinal β-catenin/TCF4 complexes, resulting in activated transcription TCF target genes. Additional mutations in genes such as p53, kras or smad4 induce the formation of colon adenomas or polyps that can then progress towards malignancy. In rare cases of colorectal cancers wild-type for APC, Axin2 is mutated (215) or activating point mutations in β-catenin remove the regulatory N-terminal Ser/Thr residues (216) thus leading to constitutive Wnt activation. The connection between stem cell biology and colon cancer is evident in several recent reports establishing a correlation between Wnt signal strength, stem cell signature, and cancer stem cell behavior

(217,218). Alternative mechanisms of Wnt activation have also been described. Activating mutations in β-catenin, loss-of-function mutations in Axin or decreased production of Wnt antagonist contribute to the progression of several types of human cancer including breast, melanoma, hepatocellular carcinoma, and head and neck (201,219,220), while inactivating mutations in the TCF family member Lef1 have been detected in sebaceous skin tumors (221).

Modulators of Wnt signaling

Natural Wnt antagonists. Secreted Frizzled-Related Proteins (sFRPs) and Wnt Inhibitory Factors (WIF) are secreted molecules that bind Wnts, thereby inhibiting the interaction between Wnt and Wnt receptors (222,223). Members of the Dickkopf (DKK) and WISE/SOS families antagonize Wnt signaling by binding LRP5/6 and disrupting the Wnt-induced Fz-LRP6 complex formation (223,224).

Wnt Agonists. To date, two Wnt agonists, Norrin and R-spondins (Rspo), have been identified. Both Norrin and Rspo are small, secreted proteins that act through the Fz/LRP6 complex. Norrin binds Fz4 with high affinity, and co-expression of Norrin, Fz and Lrp potently activates Wnt signaling. Norrin knockout mice exhibit a severe retinal hypovascularization phenotype, which resembles a loss-of-function mutation in LRP5 (225,226). Rspo are ligands for Lrg5, a family of 7-TM receptors that physically reside within Fz/LRP complexes. Lrg5 mediates Rspo input into the Wnt pathway by potentiating signaling enhancement of low-dose Wnt. In HEK293 cells, RSPO1 enhances canonical WNT signals initiated by WNT3A. Removal of LGR4 does not affect WNT3A signaling, but abrogates the RSPO1-mediated signal enhancement, a phenomenon rescued by re-expression of LGR4, -5 or -6 (227).

Small molecules. Due to the extensive involvement of Wnt in diseases**,** many targeted therapies have been directed against multiple components of the Wnt signaling pathway including Axin,

GSK3, CK1 and β-catenin/TCF. The most effective drugs would be those aimed at disrupting the interaction between β-catenin and TCF, as they would inhibit the final step of signaling cascade. However, this interface has proven an elusive target and the binding interface is not easily accessible to chemical compounds. Small molecules PKF115-854, CGP049090 and iCRTs, have been suggested to target Wnt signaling at this level, but their specificity and efficacy remains to be established (228,229). Other molecules target more upstream events. Tankyrase inhibitors, IWR(230) and XAV939(231) stabilize and increase Axin levels, which, in turn, destabilize β-catenin to inhibit Wnt signaling. The CK1 agonist Pyrvinium modulates Wnt signaling output by activating CKI activity and promoting β-catenin phosphorylation and degradation(232). GSK3 inhibitors (LiCl, Bio, CHIR and SB-216763) are commonly used in the laboratory to activate Wnt signaling and induce the transcription of Wnt target genes (233,234). However, this approach should be used with caution as GSK3 is involved in many other cellular processes and its inactivation has pleiotropic effects. Surprisingly, there are no effective small-molecule inhibitors of the receptors for Wnt, Fz or LRP5/6.

PORCN AS A WNT ACYLTRANSFERASE

Early genetic studies identified Porcupine *(porc)* as a segment polarity gene involved in Wingless (Wg) signaling during *Drosophila melanogaster* embryonic development (235). Embryos devoid of both maternal and zygotic *porc* genes display an embryonic mutant phenotype similar to *wg* null mutants, including defective patterning of the embryonic epidermis and the imaginal discs (236). Mutations in the *porc* gene lead to retention of Wg proteins within producing cells, severely affecting Wg distribution and diffusion range (237,238). Conversely, exogenous co-expression of Porcupine and Wg in S2 cells enhances Wg secretion compared to cells transfected with Wg alone (239,240). In this scenario, it was also observed that the ratio between highly glycosylated Wg (type IV) to the other forms (type I, II, III, containing none, one or two glycan chains, respectively) increased in a Porcn concentration-dependent fashion

(236,239). In *C. elegans*, the porcupine homolog *mom-1* has a similar function in promoting secretion of the Wnt protein, Mom-2 (241). In Porcn-deficient chick embryos, the range of activity of Wnt1 and Wnt3a is drastically reduced, suggesting that Porcn-mediated Wnt gradients promote proper tissue patterning in the chick neural tube (242). Porcn deficient cells exhibit a cell-autonomous defect in Wnt secretion but remain responsive to exogenous Wnt. Early genetic approaches, therefore, placed Porcupine as a mediator of Wg processing, secretion and proper Wg distribution during development.

Porcn orthologues have been identified in nematodes (mom-1), frogs (XPorc), zebrafish, mouse (mPorcn), and humans (MG61/PPN), all sharing a high degree of conservancy (240,243,244). Porcupine function is evolutionarily conserved across species, as injecting murine Porcn mRNA into *porc* null fly embryos completely rescues the mutant phenotype (240). In eukaryotes, four isoforms of Porcn mRNA (A-D) are generated from a single gene by alternative splicing, the main difference being the presence of a polybasic fragment in Porcn D but not in Porcn A-C. All four isoforms are sufficient to allow for secretion of functional Wnt3a (245), but are expressed in a tissue specific fashion (240,243). Isoform D expression is more prominent in brain, heart and muscle, whereas isoform A is more abundant in kidney, live, lung, spleen and testis (240). Kyte-Doolitle hydrophobicity plot data show that the *Drosophila* and mouse Porcupine genes encode a 52KDa polytopic transmembrane protein with eight predicted trans-membrane domains at conserved positions (239,240,245). Exogenously expressed Porcn localizes to the ER and co-stains with calnexin, an ER marker (240). Biochemical studies in S2 cells have shown that Porcupine and the N-terminal portion of Wingless can be co-immunoprecipitated in the same complex, suggesting that Porcupine may directly interact with Wingless (240,246).

Although Porcn's biochemical function remained elusive for a long time, its similarity to other MBOATs suggested a role as an acyltransferase (83). This hypothesis gained more prominence when Wnt proteins were shown to be lipid-modified. However, detecting Porcn-

dependent Wnt acylation *in vitro* or in cells presented a very challenging task. Standard approaches such as metabolic labeling of cells expressing Wnt and Porcupine with ^{3}H-palmitate were extremely inefficient (158,159,247). Alternatively, several groups utilized TX-114 phase separation assays to test the effect of Porcn expression on Wnt hydrophobicity. In this scenario, Porcn overexpression targets Wnt to lipid rafts and promotes Wnt partitioning into the detergent phase of TX-114 phase separation assay, probably due to the attachment of a fatty acyl chain (246,247). Others utilized luciferase-based reporters of Wnt signaling activity as a readout for Porcn activity (242,245). These methodologies are indirect and fail to provide direct readout of the initial biochemical event, Porcn-mediated acylation of Wnt. In 2006, Takada et al. successfully achieved metabolic labeling of Wnt3a using ^{14}C-palmitate. Moreover, when Porcn expression was blocked by siRNA treatment, Wnt palmitoylation was abolished (23), providing for the first time compelling biochemical evidence of Porcn-mediated Wnt acylation. With new technologies, Wnt palmitoylation can now be visualized at the single cell level. A new imaging technique employs clickable bioorthogonal fatty acids and *in situ* proximity ligation to visualize palmitoylated Wnt proteins with subcellular resolution as it travels throughout the secretory pathway (172).

At the time this thesis work was initiated, there was no biochemical evidence that Porcupine functions directly as a Wnt acyltransferase, and it was not known how, when, or where the enzyme recognizes its substrate. The mechanism used by the enzyme to transfer palmitate onto Wnt and/or other substrates, and the residues required for protein stability and enzymatic activity were not known. There was also prevailing interest in defining the possible role of this protein during the development of Wnt-related diseases, such as cancer, focal dermal hypoplasia and schizophrenia. Unfortunately, none of these problems had been fully addressed. The long-term objective of this project is to unravel the mechanism by which Porcupine recognizes its substrates (palmitate and Wnt) and catalyzes the transfer of fatty acids onto Wnt, which is the focus of Chapters 2 and 3.

PORCN IN DEVELOPMENT AND DISEASES

Porcn is required for Wnt activity during development, as mutations in the PORCN gene cause focal dermal hypoplasia (FDH) in humans, also known as Goltz syndrome. FDH is an X-linked multisystem disorder characterized by developmental malformations, primarily involving the skin, skeletal system, eyes, and face, tissues where Wnt signaling is active (248-250). FDH patients are mainly female heterozygotes, and the syndrome is never transmitted to male offspring, suggestive of male-specific embryonic lethality. Conditional (Cre-mediated) deletion of a Porcn allele in mouse revealed patterning abnormalities, such as dermal atrophy and ectodermal appendages defects (nail, hair and teeth), resembling those observed in human patients with FDH (251). Embryos generated by aggregation of Porcn mutant ES cells with wild-type embryos fail to complete gastrulation, as evidenced by the lack of *HoxB1and Lhx1* expression, and embryos appear to remain in an epiblast-like state (252). Moreover, *in vitro* studies show that Porcn mutant ES cells fail to form endodermal and mesodermal germ layers, consistent with the requirement for Wnt signaling and Porcn activity during gastrulation. However, Porcn-dependent Wnt signaling is not required prior to mouse gastrulation, such as in pre-implantation or blastocyst lineage specification(253). Thus, gastrulation is the first Porcn/Wnt-dependent event in embryonic development and disruption of early development underlies the male lethality of human PORCN mutants.

Since Porcn is required for the acylation of all Wnt ligands, it is often referred as a bottleneck for all Wnt signaling and represents a therapeutic target for blockade of Wnt pathway activation in Wnt-driven disorders. Currently, two small-molecule Porcn inhibitors are commercially available, IWP (Inhibitor of Wnt Production) and WntC59. IWP was identified in a cell-based screen for small molecule antagonists of Wnt/β-catenin signaling pathway using a 200,000 compound synthetic chemical library (247). IWP compounds interfere with both β-catenin dependent and independent pathways (254), and have been extensively used in various *in*

vitro settings ranging from tissue engineering and stem cell biology. However, the high hydrophobicity of this compound poses a challenge for *in vivo* delivery and clinical use. A screen for compound derivatives with diverse chemical scaffolding revealed that Porcn exhibits an ability to accommodate diverse chemical inhibitors (254). This led to the identification of potent, sub-nanomolar inhibitors with effects on Wnt-dependent developmental processes, including zebrafish posterior axis formation and kidney tubule formation (255).

Wnt C59 is a small-molecule (2-(4-(2-methylpyridin-4-yl)phenyl)-*N*-(4-(pyridin-3-yl)phenyl) acetamide), developed and patented by Novartis as a Wnt signaling modulator. It is commercially available as a highly potent Porcn inhibitor (IC50 75pmol/L), but no peer-reviewed published information on its efficacy and molecular target was released by the company. A recent study biochemically validated C59 as a bona fide PORCN inhibitor, showing that C59 inhibits Wnt palmitoylation, Wnt interaction with Wntless, Wnt secretion and Wnt activation of β-catenin reporter activity (256). Unlike IWP, C59 is readily bioavailable *in vivo* with a half-life in blood of 1.94 hrs, and one daily administration maintains the concentration well above the IC50. WntC59 reduces tumor growth in Wnt-driven mammary cancers in mice and downregulates the expression of Wnt/ β-catenin target genes, *Axin2*, *Ccnd1*, *c-Myc*, and *Tcf7* (256). A Novartis PORCN inhibitor, LGK974, which is structurally similar to Wnt C59, is currently in Phase I clinical trials for Wnt ligand-dependent malignancies (i.e melanoma, breast neoplasms, triple-negative breast cancer, pancreatic adenocarcinoma, head and neck cancer). In a Wnt1-driven breast cancer mouse model, LGK974 treatment led to tumor regression at a well-tolerated dose with no apparent toxicity (257). The effectiveness of this drug has also been corroborated in head and neck cancer cell lines and *in vivo* mouse models. Surprisingly, mice exhibit no apparent toxicity, as measured by no pathologic changes in the gut or other tissues. Taken together, these results offer preclinical proof-of-concept that inhibiting mammalian Wnts can be achieved by targeting PORCN, and suggest that this is a safe and feasible strategy *in vivo*.

STEAROYL-COA DESATURASE

A poorly investigated aspect of Wnt acylation by Porcn is the origin of the fatty acid substrate, palmitoleate. To date, Wnts are the only secreted signaling molecules known to be modified with palmitoleate (C16:1), a rare and highly regulated fatty acid in the cell. Palmitoleate (or cis-delta-9-hexadecenoic acid) is a constituent of glycerides and phospholipids in human adipose tissues and liver, and was recently described as a lipokine (having hormone-like effects). It was shown that fatty-acyl binding-Protein (FABP)-deficient mice are resistant to high fat diet induced insulin resistance and exhibit a marked enrichment in the amount of palmitoleate in the plasma, leading the authors to propose that plasma-derived palmitoleate stimulates insulin action in muscle and suppress liver steatosis (258). Since then, there have been a number of articles focusing on the relationship between the amount of palmitoleate in tissue and blood lipids and human metabolic diseases such as obesity (259), insulin resistance (260,261) and diabetes (262).

Palmitoleate is can be derived from dietary sources such as animal, vegetable or marine oils. Alternatively, at low plasma level concentrations palmitoleic acid is synthesized by *de novo* lipogenesis and fatty acid desaturation. This process involves the action of Stearoyl-CoA Desaturase (SCD), the rate-limiting enzyme in the biosynthesis of MUFAs from saturated fatty acid precursors. SCD 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 (263,264). MUFAs synthesized by SCD are then used as major substrates for the synthesis of phospholipids, triglycerides and cholesteryl esters.

As a result of gene duplication during evolution, different species contain different numbers of SCD genes and express different numbers of SCD isoforms (265). In mice, all four SCD isoforms share >80% amino acid sequence identity and exhibit desaturase activity (SCD1, SCD2 and SCD4 desaturate palmitoyl-CoA and stearoyl-CoA; SCD3 activity is restricted to palmitoyl-CoA (263)). Human SCD1 shares 85% homology with mouse Scd1-Scd4, whereas SCD5 shares limited sequence identity (65%); both isoforms exhibit desaturase activity.

Since Wnt ligands are modified with palmitoleate, our work investigates the possible role of SCD in mediating Wnt acylation by Porcn. Using a set of biochemical tools, we identified SCD as a key molecular player during Wnt biogenesis and processing and demonstrated that SCD activity is required to generate an appropriate substrate for Porcn (Refer to Chapter 2).

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Antibodies and reagents were purchased as follows: rabbit polyclonal anti-Wnt3a, rabbit polyclonal anti β-catenin, rabbit polyclonal anti-Dvl2 (Cell Signaling Technology); rat polyclonal anti-Wnt3a, goat polyclonal anti-Shh (N-19), rabbit polyclonal anti-Shh (H-160), protein A/G plus agarose (Santa Cruz Biotechnology); mouse monoclonal anti-FLAG M2, palmitoleic acid (Sigma-Aldrich); mouse monoclonal anti-Myc 9E10 (Monoclonal Antibody Core Facility, MSKCC), HRP-conjugated secondary antibodies (Thermo Scientific), Goat anti-Rabbit Alexa Fluor594 and Goat anti-Mouse Alexa Fluor 488 (Invitrogen). Anti-Fyn serum was generated as reported (49). Small molecule compounds were purchased as follows: CAY10566 (Cayman Chemical), A939572 (BioVision), and Wnt C59 (Cellagen Technology). Small interfering RNA duplexes (siRNA) 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 in (121). N-terminal FLAG-tagged Porcn cDNA was generated from a murine Porcupine clone in pcDNA3.1 (a gift from Dr. Joseph Goldstein (UT Southwestern)). WT Porcn corresponds to NCBI Accession NP_076127.1. PCR fragments encoding C-terminally FLAG-tagged Porcn constructs Δ1–55, Δ1–94, and N-terminally FLAG-tagged Porcn Δ416–613, and Δ371–461 were amplified by PCR and ligated into the NheI/KpnI restriction sites of pcDNA3.1. All Wnt3a and Porcn point mutants were generated by site-directed mutagenesis using the Quikchange mutagenesis kit (Agilent Technologies). COS-1, HEK 293T, L, L-Wnt3a cells were

obtained from ATCC. COS-1, HEK 293T and L-cells were grown in DMEM/10% FBS. L-Wnt3a cells were grown in DMEM/10% FBS and 0.5g/L Geneticin. Super 8X TOP/FOP FLASH and pRL-TK plasmids were a gift from Dr. Anthony MC Brown (Weill Cornell Medical College). Super TOP Flash (STF) has 8x TCF binding sites upstream of a Thymidine Kinase promoter and firefly luciferase ORF. Super FOP Flash contains 8x mutated TCF- binding sites. Cells were transfected with Lipofectamine 2000 (Invitrogen) in 100mm-plates. When using siRNA, L-Wnt3a cells in 60mm plates were co-transfected with 1µg FLAG-Porcn plasmid and 20nM 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 125I-iodo fatty acids was performed using ^{125}I-NaI (Perkin Elmer) (266,267).

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 6µg FLAG-tagged Porcn plasmid +/- 20nM of siSCD1 and/or siSCD2, were incubated in DMEM containing 2% dialyzed FBS for 3hr. 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 37ºC. Cell lysates were immunoprecipitated with 5µL of the indicated antibody and 60µL of protein A/G-agarose for 16hrs at 4ºC, eluted in sample buffer containing 60mM DTT and electrophoresed on 12.5% SDS-PAGE gels. When indicated, IPs were treated with 40µL of 1M Tris or 1M NH2OH pH 7.0 for 1hr at RT and eluted with 5x Sample Buffer. Radio-label 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

WB using enhanced chemiluminescence (Thermo Scientific) and quantified with Quantity One software (BioRad) on a GS-800 Calibrated Densitometer.

Secretion Assays

L-Wnt3a cells were transfected with 6µg of FLAG-Porcn plasmid. 24 hrs post-transfection, cells were switched to DMEM/2% dialyzed FCS and either DMSO, Wnt C59 or CAY10566 and grown for 48hrs. Conditioned media was cleared by centrifugation at 500xg for 5min and concentrated from 5mL to a final volume of 500µL using 10,000 MWCO 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 100mm plates were transfected with 3µg of STF or Super FOP and 0.3µg pRL-TK. 24 hrs post-transfection, cells were co-cultured in 6-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 the 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 100mm plates were co-transfected with plasmids encoding Wnt3a-myc (WT or mutant), STF or Super FOP Flash and RL. 24 hrs post-transfection, cells were seeded in 6-well plates at a density of 0.5x106 cells/well in DMEM/2% dialyzed 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 100mm plates were transfected with 1µg Wnt3a-myc plasmid. 24 hrs post-transfection, cells were switched to DMEM containing 2% dialyzed FCS and either DMSO, 10nM Wnt C59 or 25nM CAY10566 and cultured for 24hrs. Cells were lysed in 1X RIPA containing protease and phosphatase inhibitors for 10min at 4ºC. Lysates were cleared by centrifugation at 14,000rpm for 30 min and analyzed

by SDS-PAGE and WB with the indicated antibodies. A parallel set of plates was subjected to cell fractionation to obtain the cytosolic pool of β-catenin, as described in (268).

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Forty-eight hours after transfection, COS-1 cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% (v/v) paraformaldehyde in PBS for 20min at room temperature. Cells were washed with PBS and permeabilized with 0. 2% Triton X-100 in PBS for 5min at room temperature, then washed twice with PBS. Cells were stained with anti-FLAG antibody at 1:250 dilution in 3% BSA and incubated for 1hr. Cells were placed in PBS for 20 min, followed by 45-min incubation with secondary antibodies and 1:5000 dilution of Hoechst dye in PBS. Coverslips were mounted on slides using ProLong Gold mounting solution (Invitrogen). Images were acquired on a Leica inverted SP5 confocal microscope using a 63x oil immersion objective.

PROTEIN STABILITY ASSAYS

Porcn-transfected COS-1 cells were split into 60mm dishes and cultured for 24hr at 37 °C. Cells were placed in DMEM media containing 10% FBS, 100µg/mL of cycloheximide and 40µg/mL of chloramphenicol and incubated for 0, 5, 10 and 24hr. At each time point, cells were lysed in 500µL of 1X RIPA buffer and Porcn was immunoprecipitated from the total cell lysate with anti-FLAG antibody and 60µL of protein A/G-agarose beads for 16hrs at 4ºC. Samples were eluted in sample buffer containing 60mM DTT, electrophoresed on 12.5% SDS-PAGE gels, transferred onto PVDF membranes and probed with anti-FLAG antibody**.** Protein expression was quantified as described above.

CO-IMMUNOPRECIPITATION ASSAYS

COS-1 cells were co-transfected with 3µg of Wnt3a-Myc and 4µg of FLAG-Porcn cDNAs. Forty-eight hours after transfection cells were rinsed with STE and lysed with 500µL of modified RIPA for 15min (50mM Tris pH 7.4, 1% NP-40, 0.25% Na-Deoxycholate, 50mM NaCl, 1mM

EDTA). Total cell lysates were immunoprecipitated with 5µL of the indicated antibody and 60µL of protein A/G-agarose beads for 16hrs at 4ºC and eluted in sample buffer containing 60mM DTT. Samples were electrophoresed on 12.5% SDS-PAGE gels, transferred onto PVDF membranes and probed with anti-Wnt3a or anti-Myc or anti-FLAG antibodies, as indicated.

BIOINFORMATICS

MBOAT family sequences (*Mus musculus*) were identified by querying the NCBI-conserved domain database against pfam03062. Multiple sequence alignment of murine MBOAT and Wnt family members was carried out using the ClustalW2 Multiple Sequence Alignment server. Porcn membrane topology prediction was performed using the MEMSAT-SVM server (http://bioinf.cs.ucl.ac.uk/psipred) and a graphical model was generated by Protter Server (http://wlab.ethz.ch/protter/#)

CHAPTER TWO

STEAROYL-COA DESATUARASE IS REQUIRED TO PRODUCE ACTIVE, LIPID-MODIFIED WNT PROTEIN[1]

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 (200,202). Activation of the canonical Wnt signaling pathway stabilizes the transcriptional co-activator β-catenin, which translocates to the nucleus, where it binds the T-Cell Factor (TCF) family of transcription factors and activates expression of Wnt target genes. 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-mono-unsaturated fatty acid, palmitoleate ($C16:1^{\Delta 9}$) is attached to a highly conserved serine residue, corresponding to Ser 209 on Wnt3a (23). Lipid modification is required for Wnt secretion, as mutants lacking the Ser modification site are retained in the endoplasmic reticulum (ER) (23) and are unable to interact with Wntless (Wls) (181,182), 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 (159,173). Thus, Wnt fatty acylation is necessary to produce secreted, fully active Wnt protein.

Genetic (237,239,240) and biochemical (23,247) studies have identified Porcupine

[1] This research was originally published in Cell Reports. Rios-Esteves, J and Resh MD. Stearoyl CoA desaturase is required to produce active, lipid-modified Wnt proteins. Cell Rep (2013), 4(6): 1072-1081

(Porcn) as the acyltransferase responsible for lipid modification of Wnts. Porcn is a member of the membrane-bound O-acyltransferase (MBOAT) family (83) and is predicted to modify all Wnt family members containing the conserved Ser 209 equivalent (23). Wnt signaling is tightly linked to and fine tuned by Porcn expression (245), placing Porcn as an attractive target for the development of drugs that modulate Wnt pathway activity in Wnt-driven diseases (247,254,256). 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 mono-unsaturated fatty acid is attached to Wnt proteins. All studies of Wnt acylation to date have relied on labeling cells with the saturated fatty acid palmitate (23,158,160,168,247), but mass spectrometric analysis indicates that under these conditions, palmitoleate is the major fatty acid attached to Wnt (23). Thus, a mechanism must exist to convert the saturated fatty acid (SFA) to a mono-unsaturated fatty acid (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 saturated fatty acid 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 (263,264). 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 saturated fatty acids, to Wnt3a. Furthermore, SCD inhibition blocks Wnt3a secretion and renders Wnt inactive. These findings establish a novel role for SCD as an essential intermediate in Wnt protein biogenesis and processing.

RESULTS

^{125}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 co-transfected with cDNAs encoding Myc-tagged Wnt3a and either empty vector (pcDNA3.1) or FLAG-tagged Porcn, and labeled with ^{125}I-IC15:0 for 6hrs, 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 co-expressed (Fig. 2.1a). A similar result was obtained in mouse L cells stably expressing Wnt3a (L-Wnt3a) (Fig. 2.1b). 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 (Fig. 2.1b). Radiolabel incorporation into Wnt3a was detectable in cells transfected with 200 ng of Porcn plasmid, but was maximized when higher amounts of Porcn were co-expressed. 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 (Fig. 2.1c), consistent with the model that Wnt3a is lipidated only at Ser209 and not at Cys77 (168,173). Moreover, treatment of cells with the Porcn inhibitor WntC59 resulted in a 90% decrease in label incorporation into Wnt3a (Fig. 2.1d), 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 (Fig. 2.1d), 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 (121,267). Surprisingly, we failed to detect ^{125}I-IC16:0 incorporation into Wnt3a when Porcn was co-expressed (Fig. 2.1e). 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 (Fig. 2.1e). 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 (Fig 2.2).

Although IC16:0 was not incorporated into Wnt3a, low levels of IC16:1 were detected in the presence of Porcn (Fig. 2.1e). 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 (Fig 2.1e). 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.

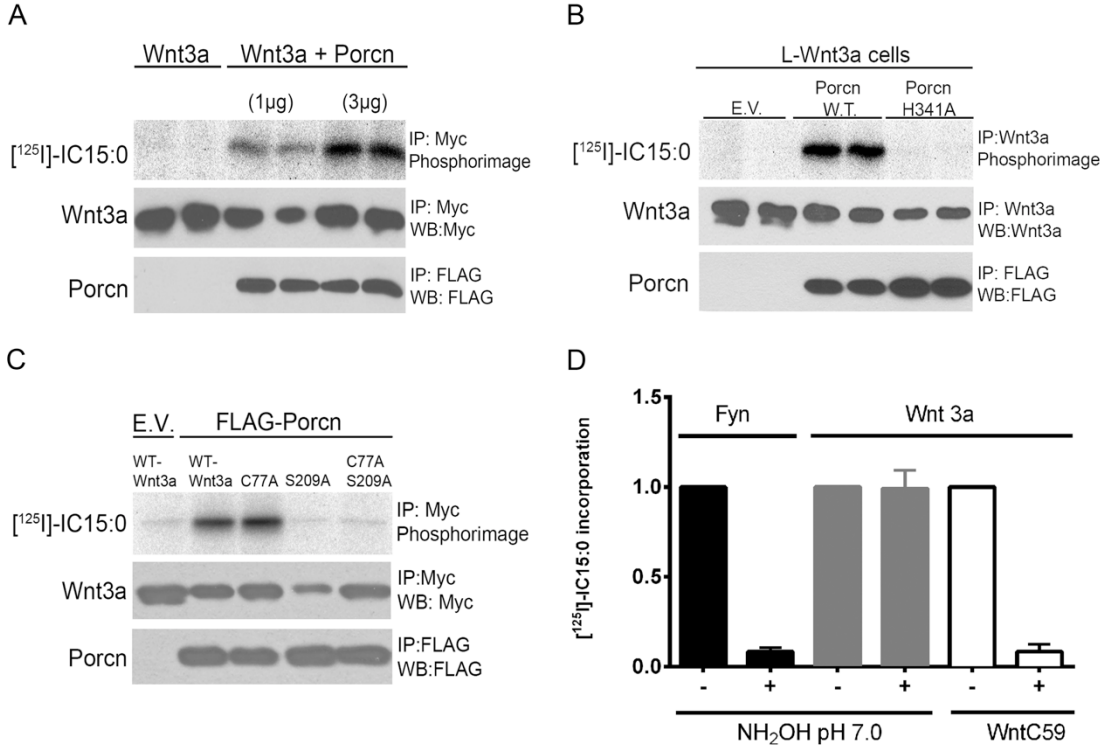
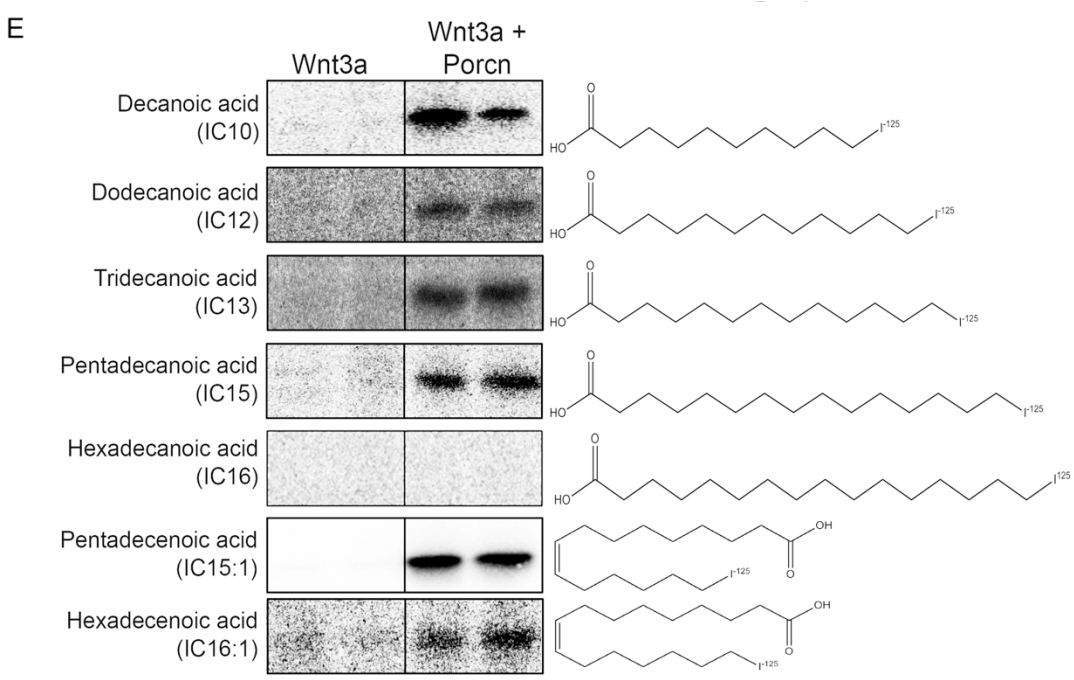


Figure 2.1 Reconstitution of Wnt3a palmitoylation by Porcn in cells.

(A, C) COS-1 cells were co-transfected with plasmids encoding Myc-tagged Wnt3a constructs and either empty vector (pcDNA3.1) or FLAG-tagged Porcn and labeled with 40μCi of 125I-IC15:0 for 6 h. 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 (1μg or 3μg) 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 125I -IC15:0 were treated with 1M Tris or NH_2OH pH 7.0 for 1hr at RT, and analyzed as in A. For WntC59 experiments, cells were treated with DMSO or 10nM of drug 3hrs prior to label addition. Phosphorimaging signals were normalized to protein levels; NH_2OH and WntC59 sensitivity are expressed as % of Tris- or DMSO-treated controls, +/-SEM. The experiment was performed two times in duplicate. (E) COS-1 were co-transfected with the indicated plasmids, labeled with 40μCi of the indicated radio-iodinated fatty acids for 6hrs and analyzed as in A. Gel images represent duplicate samples.

Figure 2.1 (Continued)



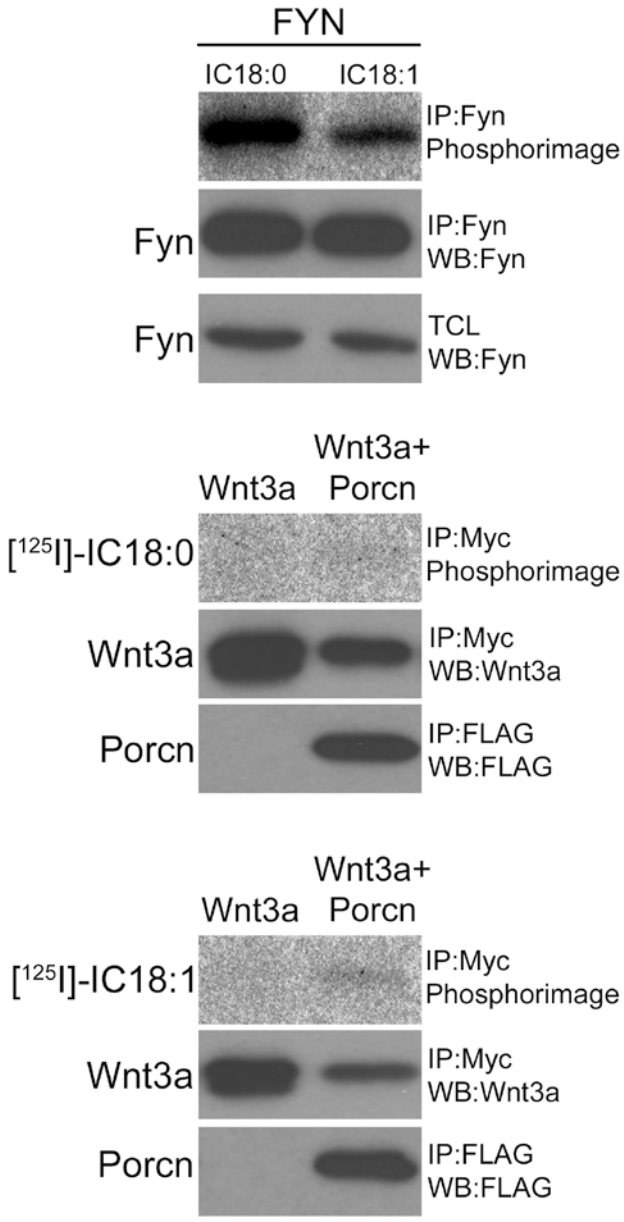


Figure 2.2 Porcn Does Not Transfer IC18:0 or IC18:1 to Wnt3a

COS-1 cells expressing the indicated plasmids were labeled with 40μCi of ^{125}I-IC18:0 or ^{125}I-IC18:1 for 6hr. Total cell lysates (TCL) were collected and immunoprecipitated with anti-Fyn, anti-Myc or anti-FLAG antibodies. Immunoprecipitates were analyzed by SDS-PAGE, phosphorimaging (top panel) and Western Blotting (WB) with the indicated antibodies (lower panels).

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 (Fig. 2.3a,b). A similar result was obtained in COS-1 cells co-expressing Wnt3a and Porcn (Fig. 2.3c). Treatment with CAY10566 also blocked Porcn-mediated acylation of Wnt5a, a non-canonical Wnt ligand (Fig. 2.3d), 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 (Fig 2.3a-d). Moreover, CAY10566 did not alter fatty acylation of Sonic hedgehog (Shh), which is palmitoylated by the MBOAT family member Hhat, or Fyn (Fig. 2.3e,f), 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 (269,270). qPCR analysis revealed that mouse L-Wnt3a cells primarily express SCD1 and SCD2 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 (Fig 2.4). 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, and suggest that Porcn transfers MUFAs, but not saturated fatty acids, to Wnt family members.

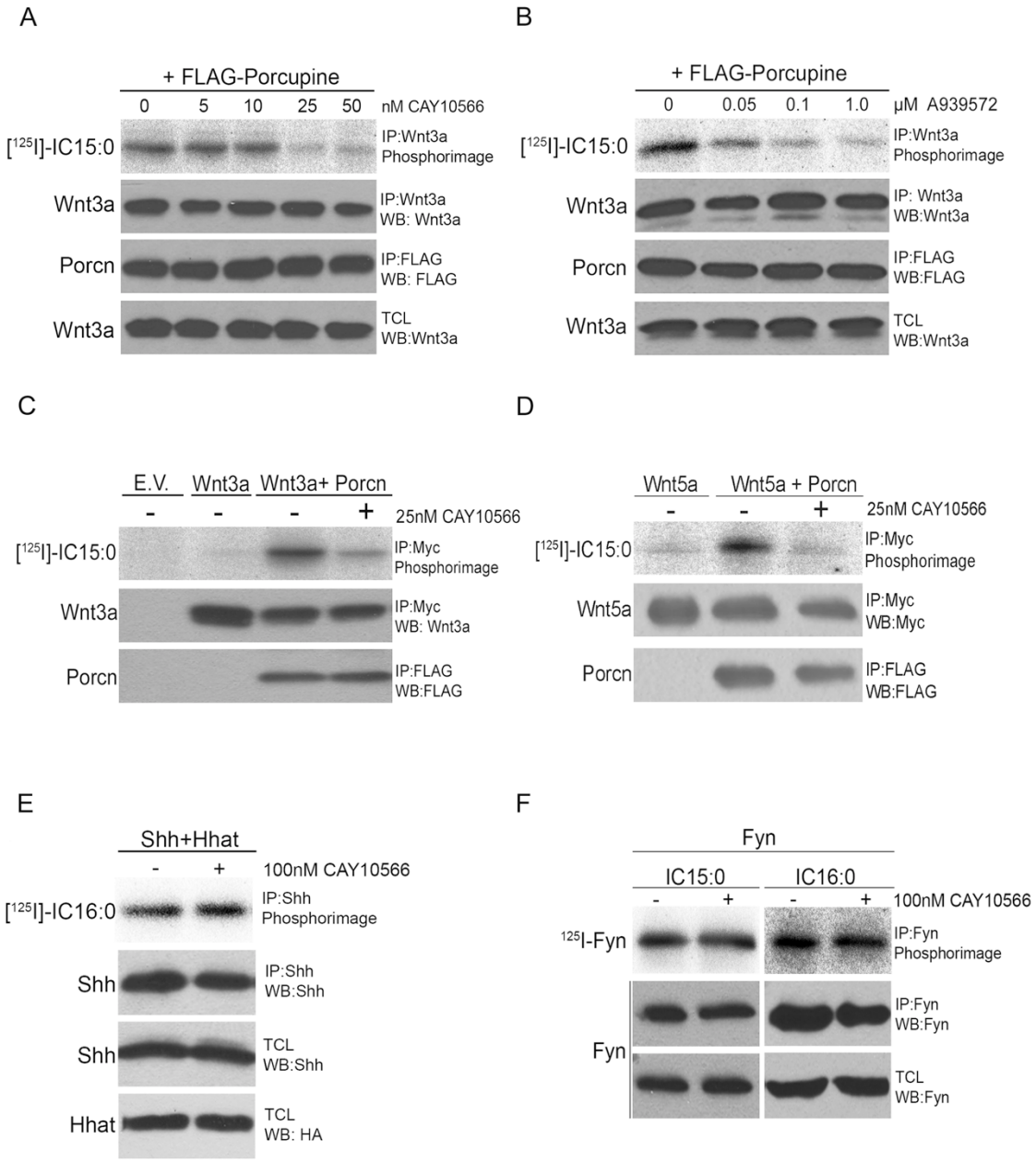


Figure 2.3 SCD activity is required for Wnt fatty acylation

L-Wnt3a cells **(A, B)** or COS-1 cells **(C-F)** expressing the indicated constructs were incubated for 3hrs in media containing CAY10566 or A939572, then labeled with 40μCi of 125I -IC15:0 (**A-D,F**) or 125I -IC16:0 (**E,F**) for 6hrs. Cell lysates were analyzed as in Fig 2.1. Lower panels (**A,B,E,F**), total cell lysates (TCL) were analyzed directly by WB using the indicated antibodies.

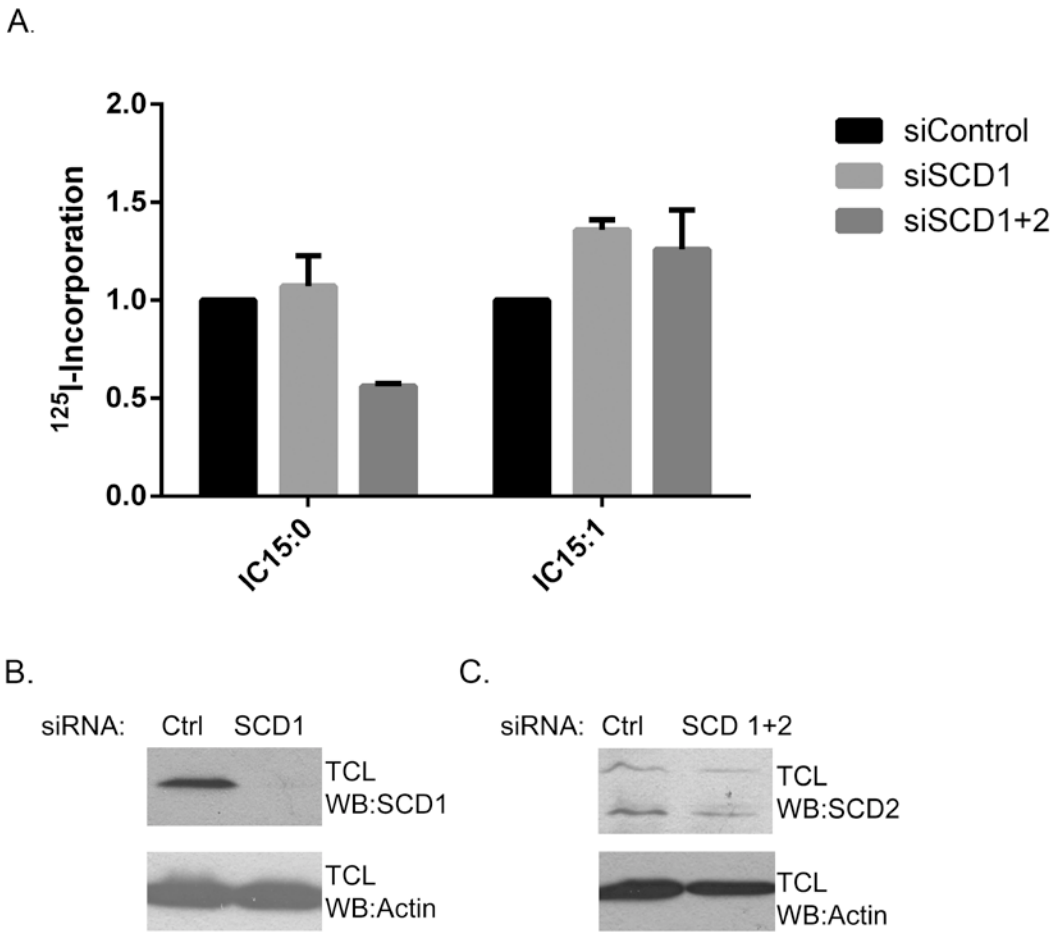


Figure 2.4 Knockdown of Both SCD1 and SCD2 Reduces Label Incorporation into Wnt3a

L-Wnt3a cells were transfected with 1 μg FLAG-Porcn +/− 10nM SCD1 and/or SCD2 siRNA. Thirty-six hours after transfection, cells were labeled with 40 μCi of ^{125}I-IC15:0 or ^{125}I-IC15:1 for 6hr and processed as in Figure 2.2
(A) Levels of ^{125}Iodine incorporation were quantified and normalized to control; bars represent mean ± SEM (n = 2).
(B and C) Levels of SCD1 (B) and SCD2 (C) were monitored by WB. SCD2 migrates as two bands of ~40 kDa.

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-mono-unsaturated fatty acid, iodo-pentadecenoate (IC15:1). ^{125}I-IC15:1 was incorporated into wild-type but not S209A Wnt3a (Fig 2.5a). No radiolabeling of Wnt3a occurred in cells expressing H341A Porcn, or Hhat, the Shh acyltransferase (Fig 2.5b). 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 or with SCD-1 and SCD-2 directed siRNAs had no effect on incorporation of either ^{125}I-IC15:1 (Fig. 2.5c,d; Fig 2.4) or ^{125}I-IC16:1 (Fig. 2.6) 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 (Fig. 2.7a). Treatment with CAY10566 had no effect on secretion of Shh (Fig 2.8), 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 10nM or 25nM CAY10566, or 10nM WntC59. The conditioned medium (CM) was used to stimulate HEK293T cells co-expressing 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 (Fig. 2.7b).

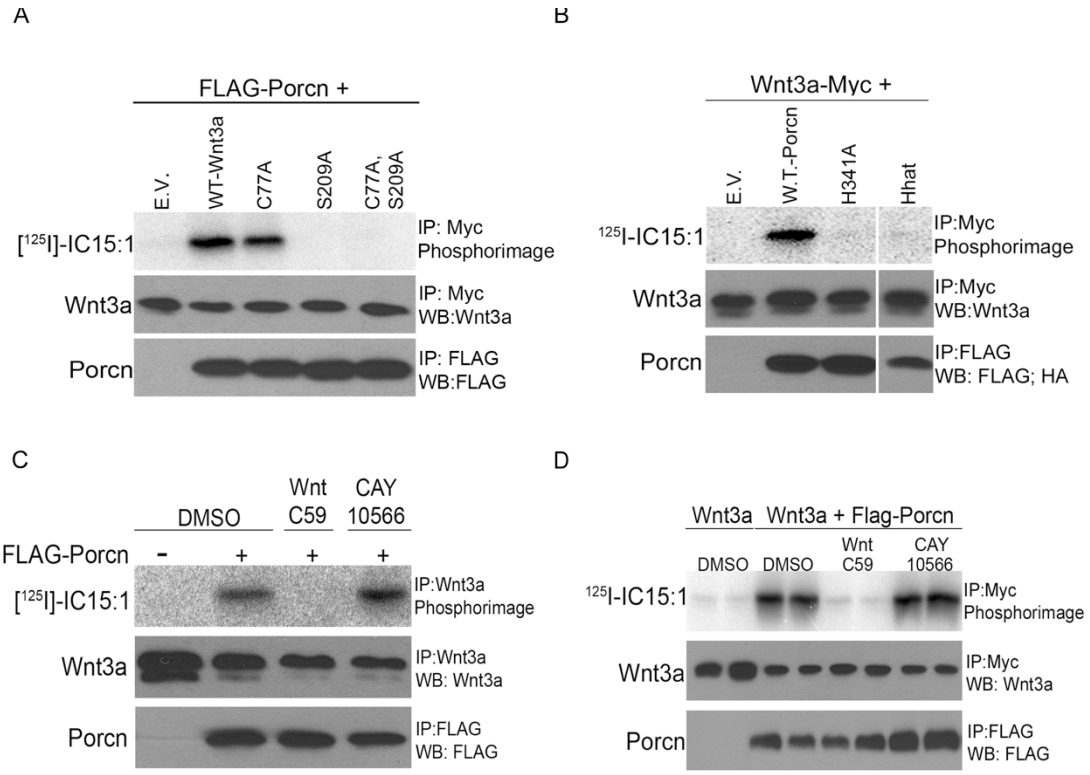


Figure 2.5 The SCD requirement is bypassed by exogenous addition of MUFAs

COS-1 cells expressing Myc-tagged Wnt3a and/or FLAG-tagged Porcn or HA-tagged Hhat constructs (A, B, D) or L-Wnt3a cells expressing FLAG-tagged Porcn constructs (C), were labeled with 125I-IC15:1 -/+ 100nM CAY10566 or WntC59. Samples were analyzed as in Fig 2.1. (D) Gel images represent duplicate samples. (E) A schematic model illustrating successive Wnt processing by SCD and Porcn.

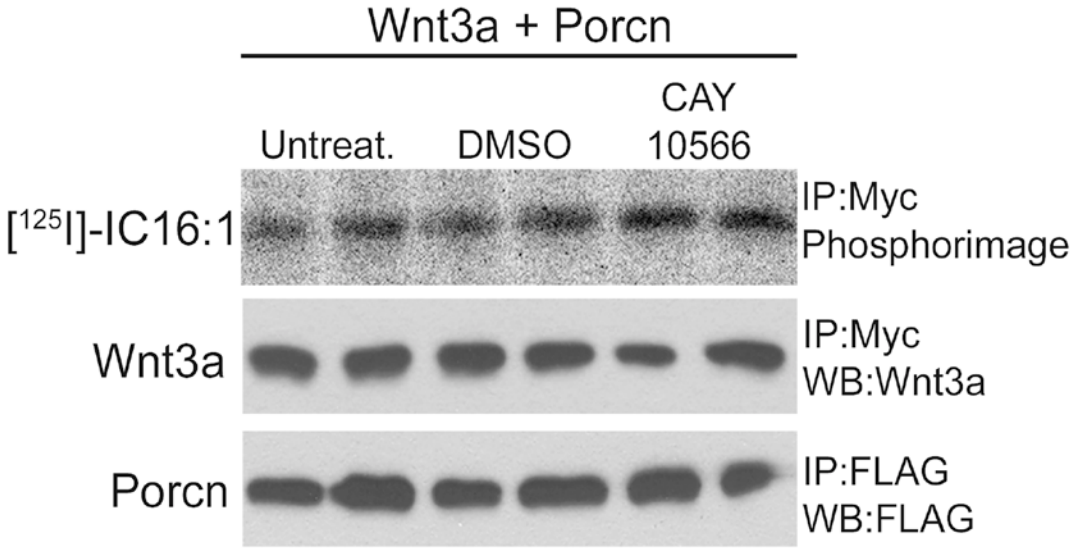


Figure2.6 IC16:1 Incorporation into Wnt3a Is Not Affected by SCD Inhibition

COS-1 cells co-expressing Wnt3a-Myc and FLAG-Porcn were cultured in media containing DMSO or 1 μM CAY10566 for 3hrs and labeled with 40μCi of ^{125}I-IC16:1 for 6hrs. TCL were immunoprecipitated and analyzed by SDS-PAGE and phosphorimaging (top panel) or WB (lower panels) with the indicated antibodies.

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 co-cultured with HEK293T cells co-expressing STF and RL. Treatment with CAY10566 resulted in ~50% decrease in Wnt signaling activity compared to DMSO-treated controls (Fig. 2.7c). Of note, this signaling defect was rescued by supplying exogenous palmitoleic acid to the co-cultures, whereas the inhibitory effect of Wnt C59 could not be rescued (Fig. 2.7c). 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 (Fig. 2.7d). 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, 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 (Fig. 2.7e). 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 (Fig 2.7f). Taken together, these data indicate that SCD inhibition blocks Wnt secretion as well as autocrine and paracrine signaling activity.

Figure 2.7 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 medium was concentrated and analyzed along with the TCL by WB using anti-Wnt3a antibody.

(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.

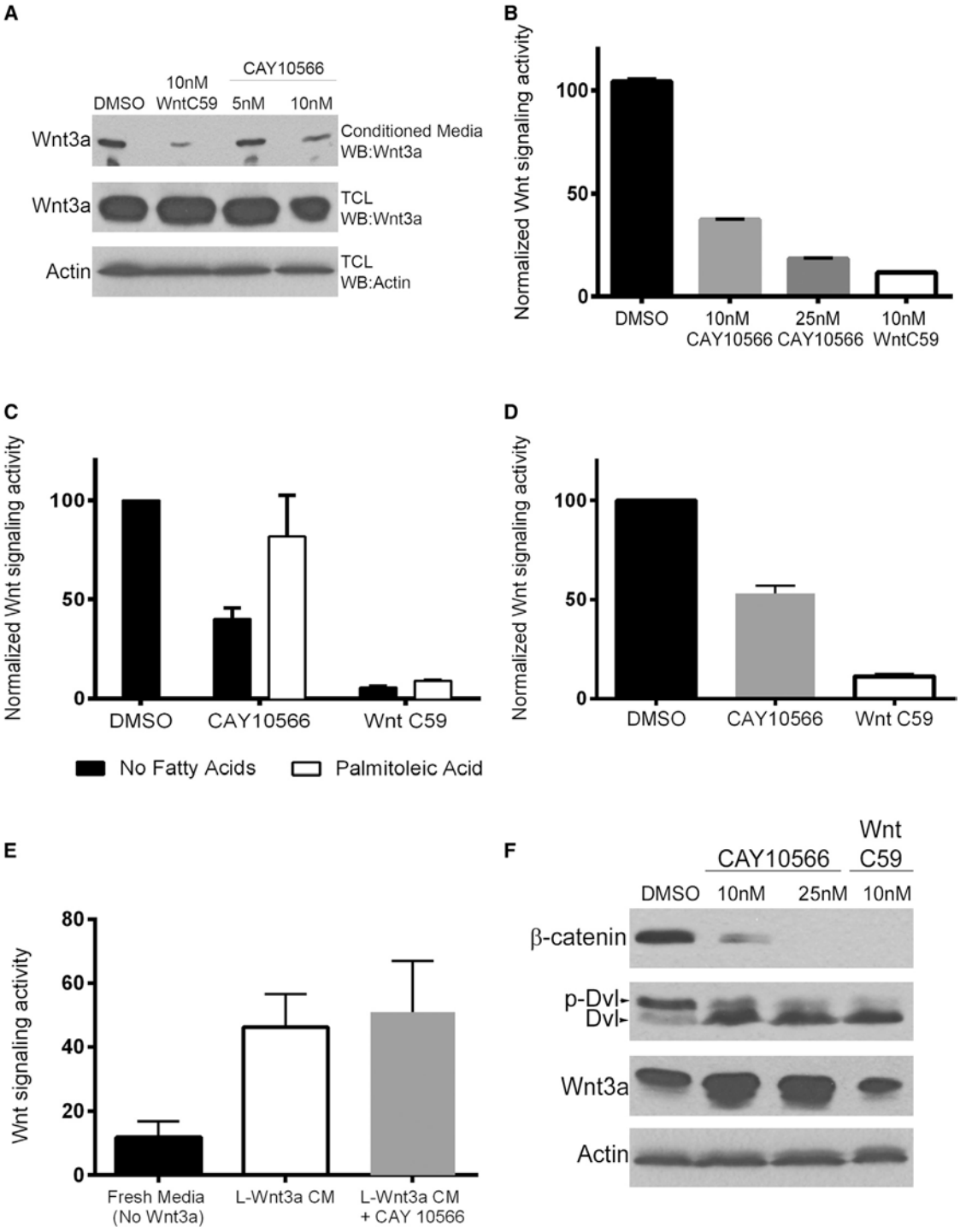
(C) L-Wnt3a cells were cocultured with HEK293T reporter cells and grown for 24 hr in the presence of DMSO, CAY10566, or WntC59. A total of 100 mMpalmitoleic acid was added to the culture medium where indicated. Luciferase activity was measured 24 hr after stimulation, normalized to RL and FOP-Flash activity,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

(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.

Figure 2.7 (Continued)



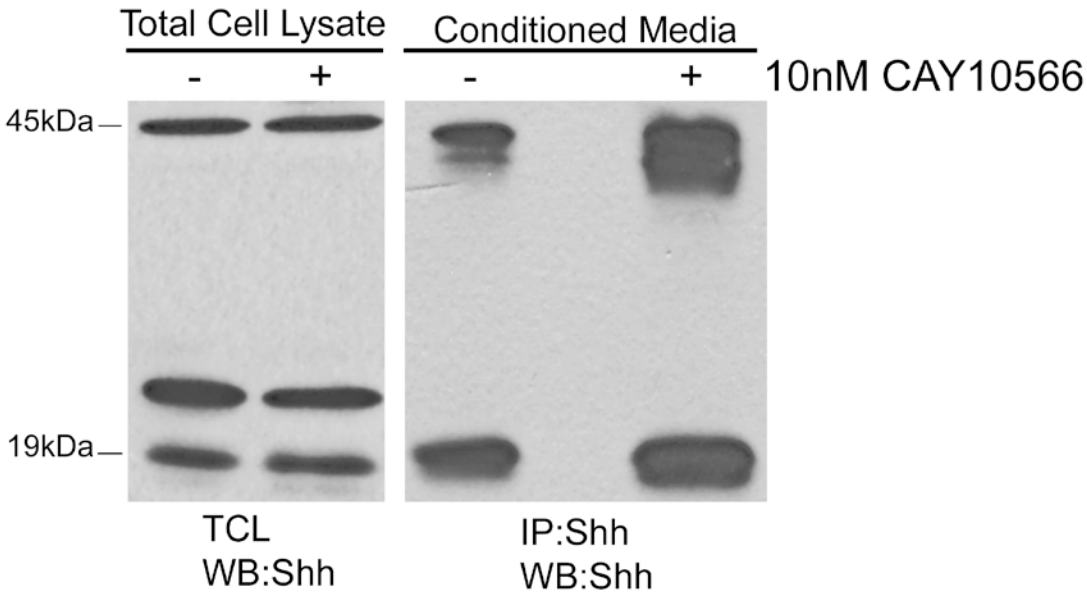


Figure 2.8 SCD Inhibition Does Not Affect Shh Secretion

L-Wnt3a cells transfected with a plasmid encoding Shh were grown in media containing DMSO or 10nM CAY10566 and cultured for 48hrs. The conditioned media from 3 plates was pooled, concentrated and analyzed along with the TCL by WB using anti-Shh antibody. The 45 kDa protein band represents the Shh precursor, the 19 kDa protein band represents the mature, processed Shh protein; the band that migrates above the 19 kDa band is a non-specific band.

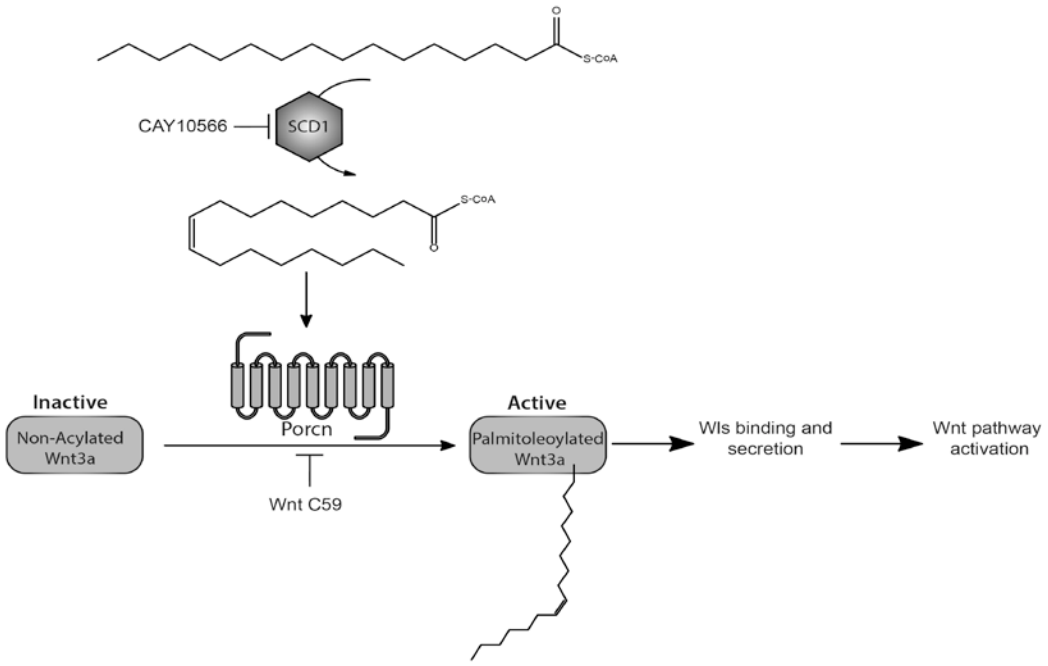


Figure 2.9 New model of Wnt processing

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

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 2 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; 3) Porcn uses palmitoleoyl CoA as a substrate and transfers the unsaturated fatty acid to Wnt (Fig 2.9).

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 (32,121,267). A notable feature of these reagents is their limited metabolic interconversion to longer or shorter fatty acid species (266). 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 2.5 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 (258). 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 (271). The bent conformation of palmitoleate may provide an appropriate three-dimensional conformation for interaction of Wnt with Wls (5), 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 (173). 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, dictates 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 (265). 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 (263)). 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 (272), 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 (273,274). 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 (275). SCD1 expression levels are upregulated in colon, breast, prostate and lung cancers and inhibition of SCD blocks cell proliferation and tumor growth (276,277). Potential mechanisms to explain how SCD1 inhibition blocks cancer cell proliferation include attenuation of AKT signaling, and induction of senescence and apoptosis (276). 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 □-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 (278). In another study, constitutive over-expression of SCD5 in neuronal cells increased MUFA production, Wnt5a secretion into the media, and non-canonical Wnt5a signaling (279).However, opposite effects on Wnt7a were observed: SCD5 over-expression 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 is the first to provide a novel 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.

Acknowledgments

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CHAPTER THREE

IDENTIFICATION OF KEY RESIDUES AND REGIONS IMPORTANT FOR PORCUPINE-MEDIATED WNT ACYLATION [2]

INTRODUCTION

Post-translational modification of proteins with one or more fatty acids controls a dynamic range of cellular processes such as membrane trafficking and targeting, lipid raft targeting, and signaling activity (1,5,23,42,280). The most common form of fatty acylation is protein S-palmitoylation, which occurs by covalent attachment of the 16-carbon saturated fatty acid, palmitate (C16:0), to one or more cysteine residues through a thioester linkage. The mechanism of action of S-palmitoyl acyltransferases has been well documented and extensively reviewed (1). Recently, secreted signaling proteins (Hedgehog, Wnt) and hormones (Ghrelin) have been reported to contain fatty acids attached via amide (N-) linkage or oxyester (O-) linkage (22,23,133). The diverse mechanisms governing these reactions are currently being uncovered.

Wnts are secreted, lipid-modified signaling proteins that control embryonic development and self-renewal in adult tissue (203,207,281-283). Wnt proteins activate signaling cascades that result in the transcription of genes involved in proliferation, differentiation and migration. Aberrant activation of the Wnt signaling pathway has been linked to a variety of human cancers, including melanoma, breast, colon, and head and neck (282,284,285). Owing to its importance in diseases, there is significant interest in understanding the biochemistry of Wnt processing and secretion.

Production of an active Wnt signal is initiated by the acyltransferase Porcupine (Porcn),

[2] This research was originally published in The Journal of Biological Chemistry. Rios-Esteves, J; Haugen, B and Resh, MD. Identification of key residues and regions important for Porcupine-mediated Wnt acylation J. Biol. Chem (2014) 289, 17009-19 © The American Society for Biochemistry and Molecular Biology

which catalyzes transfer of palmitoleate (C16:1), a 16-carbon monounsaturated fatty acid (MUFA), to a highly conserved serine residue (Ser 209 in Wnt3a) (23,170,173,246). Attachment of palmitoleate is required for Wnt function. Mutation of the conserved Ser to Ala in Wnt, or pharmacological inhibition of Porcn, blocks Wnt fatty acylation, intracellular trafficking and secretion, binding to its cell surface receptor, Frizzled, and signaling activity (23,170,173,181,182). Porcn is required for Wnt activity during development, as mutations in the *PORCN* gene cause focal dermal hypoplasia (FDH), also known as Goltz syndrome, an X-linked multisystem disorder characterized by developmental malformations(248,249,251). In adults, Porcn is a therapeutic target for blockade of Wnt pathway activation in Wnt-driven disorders (247,254). Porcn inhibitors reduce tumor growth in Wnt-driven mammary cancers in mice and in head and neck cancer cell lines (256,257).

Porcn is an endoplasmic reticulum (ER)-resident integral membrane protein. It has 11 predicted transmembrane domains (TMDs) and belongs to the MBOAT (membrane-bound O-acyltransferase) family (83). In mammals, 16 MBOAT members encoded by 11 genes have been identified. Biochemical analysis of this family has been hampered by the extreme hydrophobicity of the proteins due to the presence of multiple TMDs. Most MBOAT family members are involved in neutral lipid biosynthesis (ACAT1/2, DGAT1/2) and phospholipid remodeling (LPEAT1-4, GUP1). Only three members, Porcn, Hhat (Hedgehog acyltransferase) and GOAT (Ghrelin O-Acyltransferase), have been shown to have protein substrates. Hhat catalyzes attachment of palmitate to the N-terminal Cys of Hedgehog proteins (121) and GOAT mediates transfer of octanoate to Ser3 of proghrelin, an appetite-stimulating hormone (21). All family members share a conserved MBOAT homology domain that is thought to be necessary for enzymatic activity. This region harbors an invariant His residue positioned within a stretch of highly conserved hydrophobic amino acids and a well-conserved Asp/Asn surrounded by hydrophilic residues (83). Mutation of the conserved His reduces the catalytic activity of ACAT1/2, DGAT1, GOAT, Hhat and Porcn, placing this residue as part of the putative active site

(21,111,130,170,286). Mutational analyses of other conserved residues in ACAT, DGAT and Hhat have identified several residues within the MBOAT homology domain that are required for catalysis. However, no unifying consensus model for the MBOAT active site has been described.

In this study, we present a structure-function analysis of Porcn acyltransferase activity. Rather than relying on reporters of Wnt signaling activity as an indirect readout for Porcn activity (245), we directly monitored MUFA transfer to Wnt by Porcn. We exploited a cell-based fatty acylation assay that uses 125I-Iodo-pentadecenoic acid (^{125}I-IC15:1), a radioiodinated palmitoleate analogue, as a substrate for Porcn. ^{125}I-IC15:1 is sterically similar to palmitoleate (C16:1) and provides a robust, accurate, and direct readout of Porcn acyltransferase activity in the cell (170). To determine the functional significance of highly conserved residues, we generated a series of truncation and point mutants within Porcn that targeted the MBOAT homology domain as well as residues that are mutated in FDH. We identified residues that are important for Wnt binding, acyltransferase activity and stability of Porcn. In addition, the positional requirement of highly conserved residues surrounding the Wnt3a palmitoleoylation site was analyzed. Taken together, this structure-function analysis has enabled us to generate an initial working map of the active site of Porcn and to define a consensus sequence for Wnt palmitoleoylation.

RESULTS

N- and C-terminal mutants of Porcn lack acyltransferase activity and aggregate in the ER

To identify a minimal region of Porcn retaining acyltransferase activity, we generated N- and C-terminal truncations. A web-based topology prediction program, MEMSAT-SVM (287), was utilized to aid in the design and placement of truncation points to avoid membrane-spanning regions. We used the MEMSAT-SVM topology prediction server for its consistency in predicting the topology of Porcn across species, and its accuracy in predicting GOAT topology (147). MEMSAT-SVM predicted 11 TMDs with invariant His341 embedded in TMD 9 (Fig. 3.1a).

Based on this model, we engineered N- and C-terminal truncations, missing one or more predicted transmembrane helices, each carrying a FLAG tag at the opposite end of the truncation site. The enzymatic activity of these mutants was tested in a cell-based Wnt palmitoylation assay (170). COS-1 cells were co-transfected with cDNAs encoding Myc-tagged Wnt3a and either wild type (WT) or truncated Porcn, and labeled with 125I-Iodo-pentadecenoic acid (^{125}I-IC15:1). Wnt3a was immunoprecipitated from cell lysates, and the amount of ^{125}I-IC15:1 incorporated into Wnt3a was determined by phosphorimaging analysis after SDS-PAGE. Two C-terminal truncations (Δ415-461 and Δ374-461) expressed truncated Porcn proteins, but these mutants failed to promote Wnt3a acylation (Fig.3.1b). We tested whether the enzymatic defects observed were due to altered intracellular localization. Analysis by indirect immunofluorescence and confocal imaging revealed punctate immunostaining, a pattern characteristic of aggregated and misfolded protein (Fig. 3.1c). Expression of the N-terminal deletion mutants was not detected by Western blot, but immunofluorescent confocal imaging analysis again revealed aggregated protein (data not shown). Thus, these N- and C-terminal deletion mutants are misfolded and devoid of protein acyltransferase (PAT) activity.

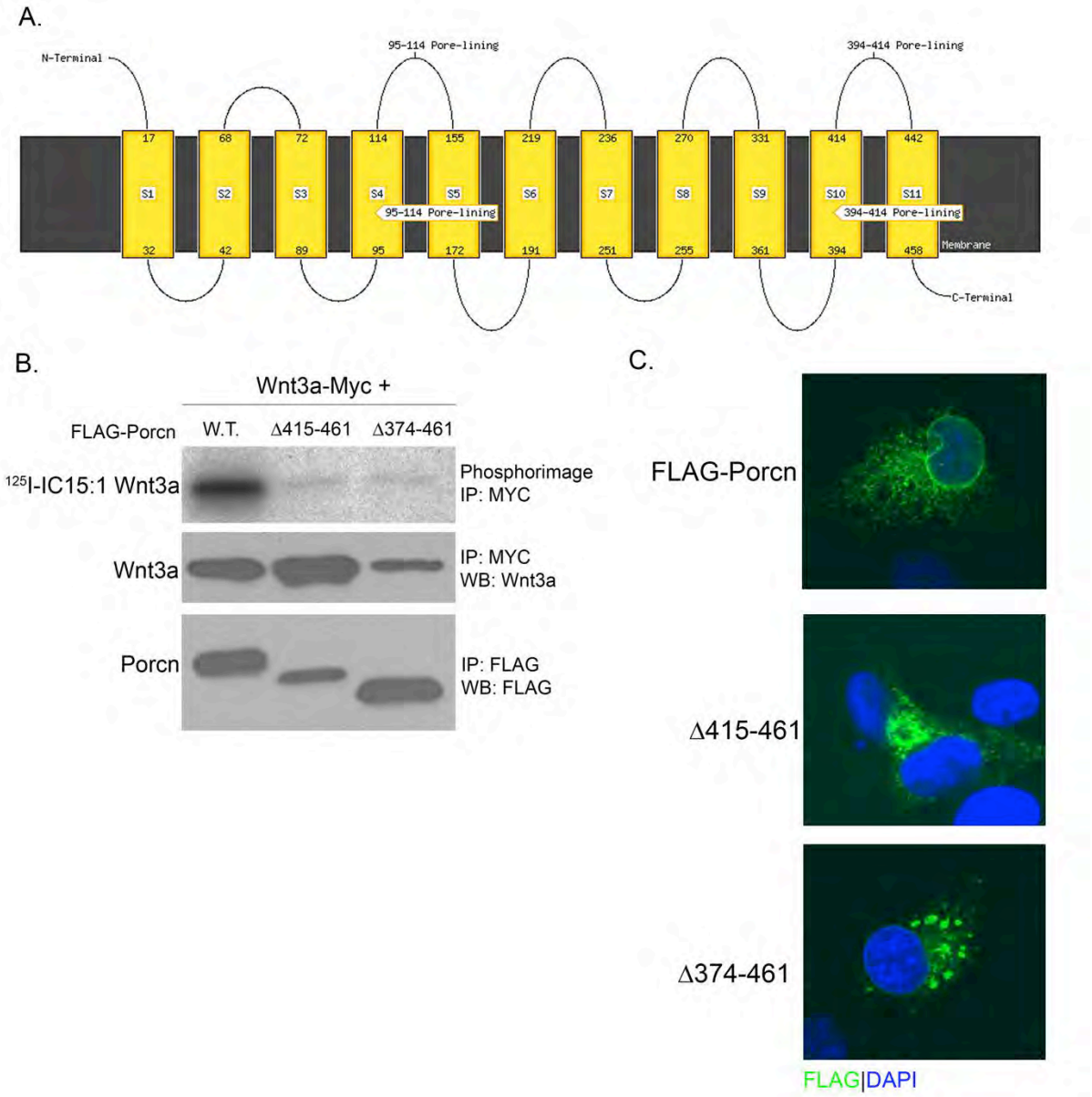


Figure 3.1 Porcn truncation mutants are inactive and aggregate in the ER.

A, Predicted transmembrane topology map of murine Porcn protein (NP_076127.1) generated using the MEMSAT-SVM server. Rectangles represent predicted transmembrane helices and numbers indicate boundaries. **B,** COS-1 cells co-expressing Wnt3a-Myc and FLAG-Porcn, either WT or truncated, were labeled with ^{125}I-IC15:1 for 5h. Cell lysates were immunoprecipitated (IP) with anti-MYC or anti-FLAG antibodies and analyzed by SDS-PAGE and phosphorimaging (top panel) or Western blotting (middle, lower panels) with anti-Wnt3a or anti-FLAG antibodies. **C,** COS-1 cells expressing WT or truncated FLAG-tagged Porcn were fixed, stained with DAPI (blue) and analyzed by indirect immunofluorescence with anti-FLAG antibody (green) and confocal microscopy.

Identification of key residues in the MBOAT homology domain of Porcn

The MBOAT homology domain is the most conserved region of the MBOAT family, with residues sharing 50% or more conservation (Fig. 3.2a). Mutational analysis identified several residues in this region in ACAT1/2, DGAT and Hhat that are critical for catalytic activity (111,130,286), but the requirement of these amino acids for Porcn PAT activity has not been explored. In murine Porcn, the MBOAT homology domain spans residues 305-342 and harbors putative catalytic sites Asn 306 and His 341. To determine the functional significance of this region in Porcn, 9 conserved residues within the MBOAT homology domain were individually mutated to Ala. Based on their effect on Porcn PAT activity, these mutants could be classified into three categories. Two mutations did not significantly alter Porcn activity (N306A and W312A) indicating these residues are not required for acyltransferase activity (Fig 3.2c). Three other mutants (W305A, Y316A and Y334) exhibited moderate defects in activity (30-50% of WT) (Fig 3.2c). Three mutants (S337A, L340A and H341A) had little to no activity (<20% of WT), indicating that these residues are critical for acyltransferase activity (Fig. 3.2b,c). S172A was used as a control, as it does not localize to the MBOAT homology domain and mutating this residue had no effect on Porcn activity. T333A was the only point mutant whose expression was not detected by Western blotting. Immunofluorescent confocal imaging analysis detected low expression levels, altered intracellular localization and protein aggregation in the ER. Thus a complete assessment of the effect of this mutant on Porcn activity and/or biochemical properties could not be established and was not included in further characterization (Fig. 3.2d). Taken together, this mutational analysis identified 6 residues within the MBOAT homology domain of Porcn that are essential for enzymatic activity.

We next tested the effect of the MBOAT homology domain mutations on protein stability. Porcn transfected COS-1 cells were treated with cycloheximide (CHX), to block new protein synthesis, and levels of WT and mutant proteins were monitored as a function of time. WT Porcn was very stable, with 60% still present after 24hr of CHX blockade. All mutants,

except Y316A, exhibited stability comparable to WT, ranging from 48-98% remaining at 24hrs (Table 3.1). Y316A was much less stable than WT, with 24% remaining after 24hrs (Table 3.1). With the exception of T333A, proper intracellular localization of all point mutants to the ER was confirmed by indirect immunofluorescence and confocal microscopy, indicating that defects in enzymatic activity were not due to mislocalization or misfolding (Fig 3.2d).

The ability of the Porcn mutants to bind to the Wnt3a protein substrate was assessed in a co-immunoprecipitation assay using COS-1 cells co-expressing Wnt3a and Porcn. Three Porcn mutants with severe enzymatic activity defects (Y334A, S337A and H341A) exhibited impaired ability to co-immunoprecipitate with Wnt3a; all other mutants associated with Wnt3a to the same extent as WT (Fig 3.2e). Interestingly, the catalytically dead mutant L340A was still able to bind Wnt3a, suggesting that this residue might be involved in facilitating fatty acid recognition rather than Wnt protein substrate binding.

Porcn	% Remaining at 24h	SEM
MBOAT Homology Domain Mutants		
Wild Type	60.78	3.27
S172A	61.47	10.76
W305A	68.89	2.46
N306A	76.33	3.64
W312A	48.35	4.32
Y316A	24.74	10.03
Y334A	70.53	4.11
S337A	58.47	8.56
L340A	59.00	0.14
H341A	98.99	1.01
FDH-Associated Mutants		
S136F	21.93	13.95
R228C	82.86	5.84
L331R	34.06	8.18
R365Q	30.28	19.31

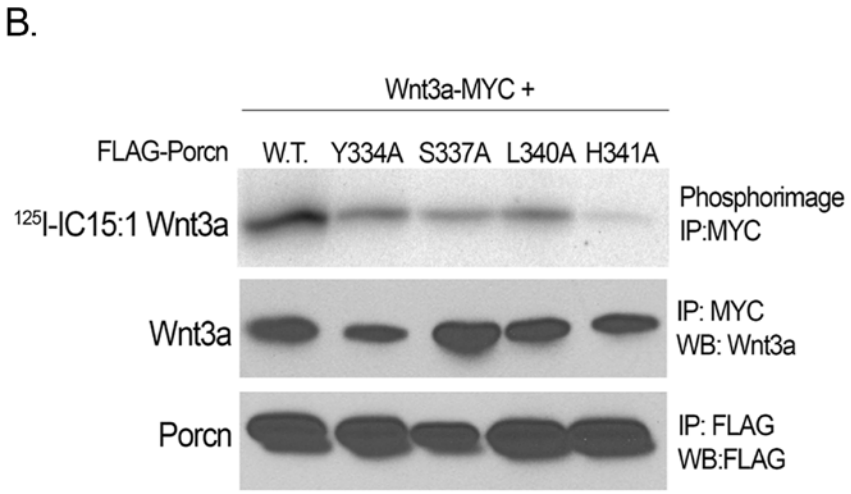
Table 3.1 Relative stability of Porcn point mutants.

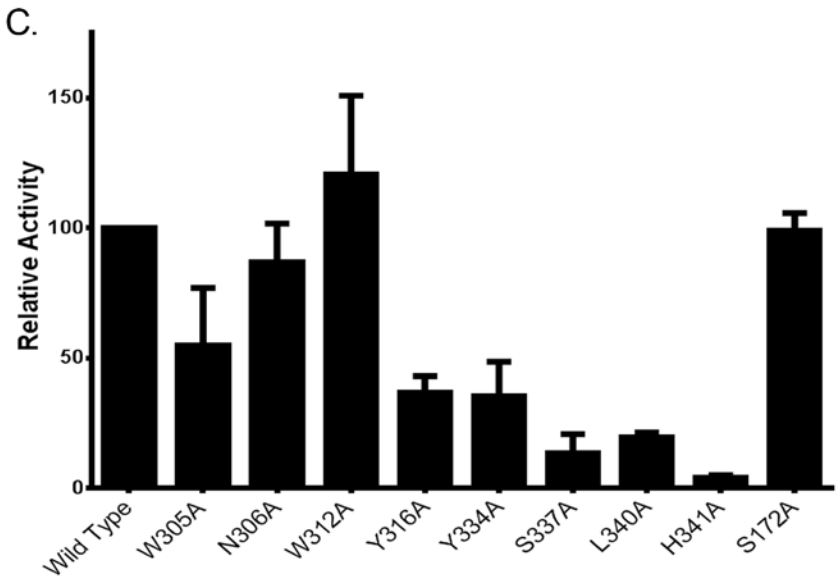
Relative stability of Porcn point mutants after 24 h of incubation with cycloheximide and chloramphenicol, as determined by western blotting with anti-FLAG antibodies and quantified by densitometry analysis. The data are expressed as percentages of 0 h control(n = 2).

Figure 3.2 Mutational analysis of conserved MBOAT homology domain residues

A, Residues that are conserved (black boxes) or similar (gray boxes) in the MBOAT homology domain in 50% or more of the16 MBOAT family members in the mouse (*Mus musculus*) are highlighted. **B,** COS-1 cells co-expressing Wnt3a-Myc and either WT or mutant FLAG-Porcn were labeled and analyzed as in Figure 1b. The experiment was performed three times in duplicate; a representative image is shown. **C,** Quantification of experiments in (b). Relative acyltransferase activity was determined by calculating the level of ^{125}I-IC15:1 incorporation per amount of immunoprecipitated Wnt3a for each mutant Porcn construct, then normalizing to WT Porcn, which was set to 100%. Each bar represents the average of three experiments and is expressed as the percentage of WT Porcn activity; error bars indicate SD. **D,** The intracellular localization of WT and mutant Porcn was analyzed as in Figure 1c. **E,** Lysates from COS-1 cells co-expressing Wnt3a and the indicated Porcn constructs were immunoprecipitated with anti-FLAG antibody followed by Western blotting with anti-Wnt3a or anti-FLAG antibody (top 2 panels). Total cell lysates (TCL) were analyzed directly by Western blotting with anti-Wnt3a and anti-actin antibodies.

Figure 3.2 (Continued)





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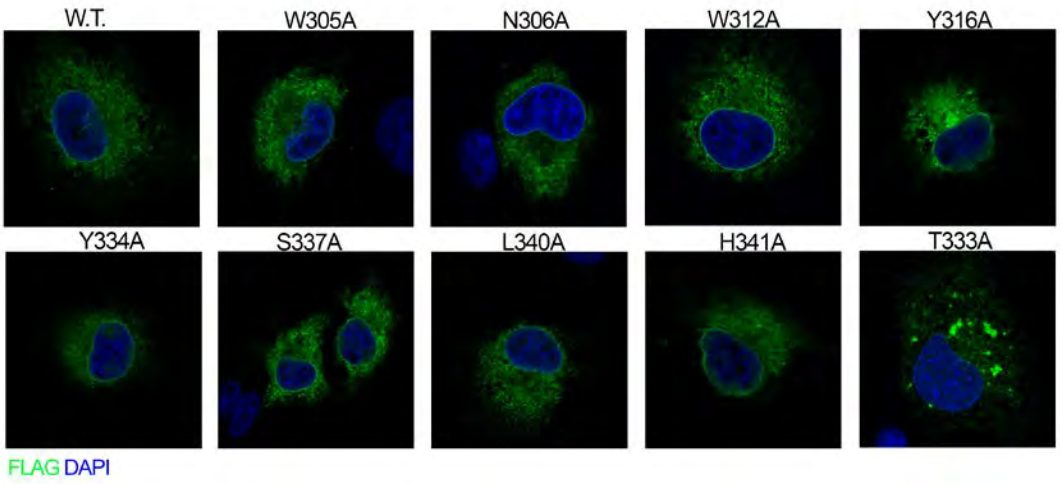
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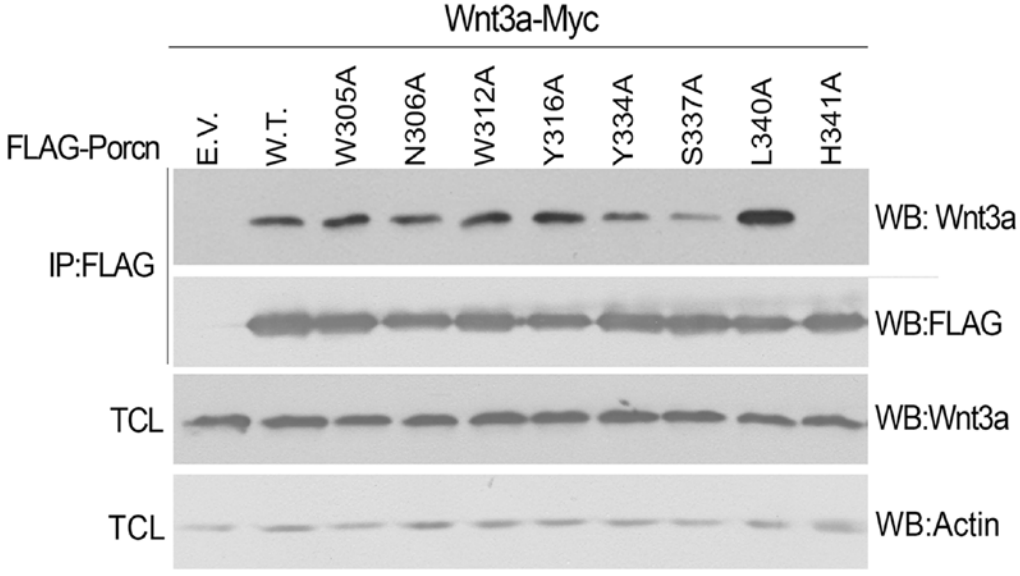
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FLAG-Porcn
E.V.
W.T.
W305A
N306A
W312A
Y316A
Y334A
S337A
L340A
H341A
IP:FLAG
WB: Wnt3a
WB:FLAG
TCL
WB:Wnt3a
TCL
WB:Actin

FDH-associated mutations are detrimental to Porcn stability

Over 70 mutations in the PORCN gene have been identified in FDH patients, including single nucleotide changes, micro-deletions, insertions and others (288-290). Approximately 26% of FDH-causing mutations are missense mutations in the PORCN coding region, but how these mutants affect Porcn function is not clear. In order to understand the biochemical mechanism by which missense mutations alter Porcn activity, the FDH-associated Porcn mutants L331R, R365Q, R228C and S136F were generated and tested for PAT activity, stability and intracellular localization. In contrast to MBOAT homology domain mutants, all FDH-associated mutations, except R228C, exhibited moderate to severe enzymatic defects (Fig. 3.3a) and compromised protein stability (Fig. 3.3b). The R228C mutation was described in an FDH patient with two heterozygous mutations (291). Since Arg228 is not conserved across species, and the patient's mother, who is clinically unaffected, has the same heterozygous mutation, the pathological significance of the R228C mutation was uncertain. Our finding that R228 Porcn is essentially indistinguishable from WT Porcn suggests that this mutation does not contribute to the disease state. In addition, indirect immunofluorescence and confocal imaging analysis of S136F revealed protein aggregation, probably due to misfolding (Fig. 3.3c). These findings suggest that altered stability and/or folding could be the underlying cause for enzymatic dysfunction in at least some of the FDH mutants described to date.

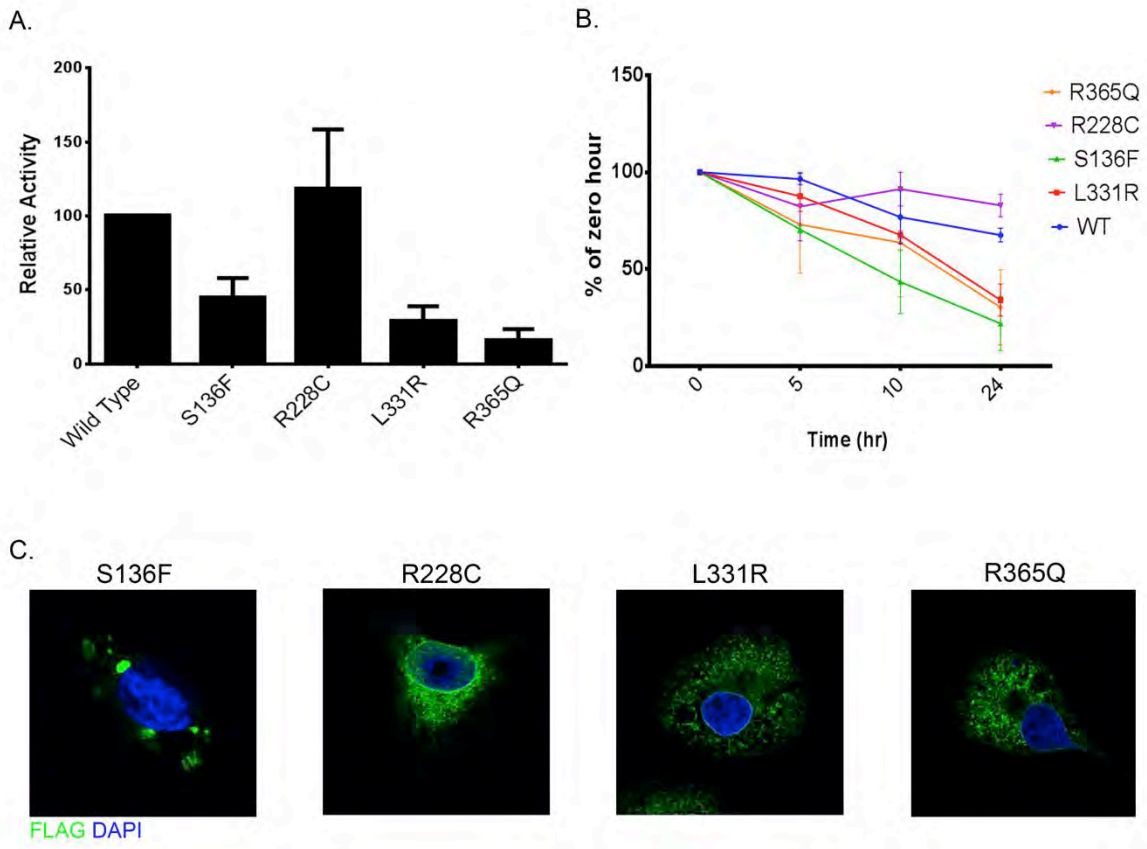


Figure 3.3 FDH mutations alter stability and intracellular localization of Porcn

A, COS-1 cells co-expressing Wnt3a-myc and the indicated Porcn constructs were labeled and analyzed as in Figure 1b. Each bar represents the average of three experiments and is expressed as the percentage of WT Porcn activity (mean ± SD). **B,** COS-1 cells expressing the indicated Porcn constructs were treated with 100µg/mL of cycloheximide and 40µg/mL of chloramphenicol for 0, 5, 10 or 24 hrs. At each time point lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by WB after SDS-PAGE. The amount of FLAG signal at each time point was determined by densitometry; each point indicates mean ± SD (n=2). Values for the percentage of Porcn protein remaining at 24h were: 60.78% for WT, 21.93% for S136F, 82.86% for R228C, 34.06% for L331R and 30% for R365Q. **C,** The intracellular localization of WT and mutant Porcn constructs was analyzed as in Figure 1c.

Identification of conserved residues in Wnt required for Porcn-dependent fatty acylation

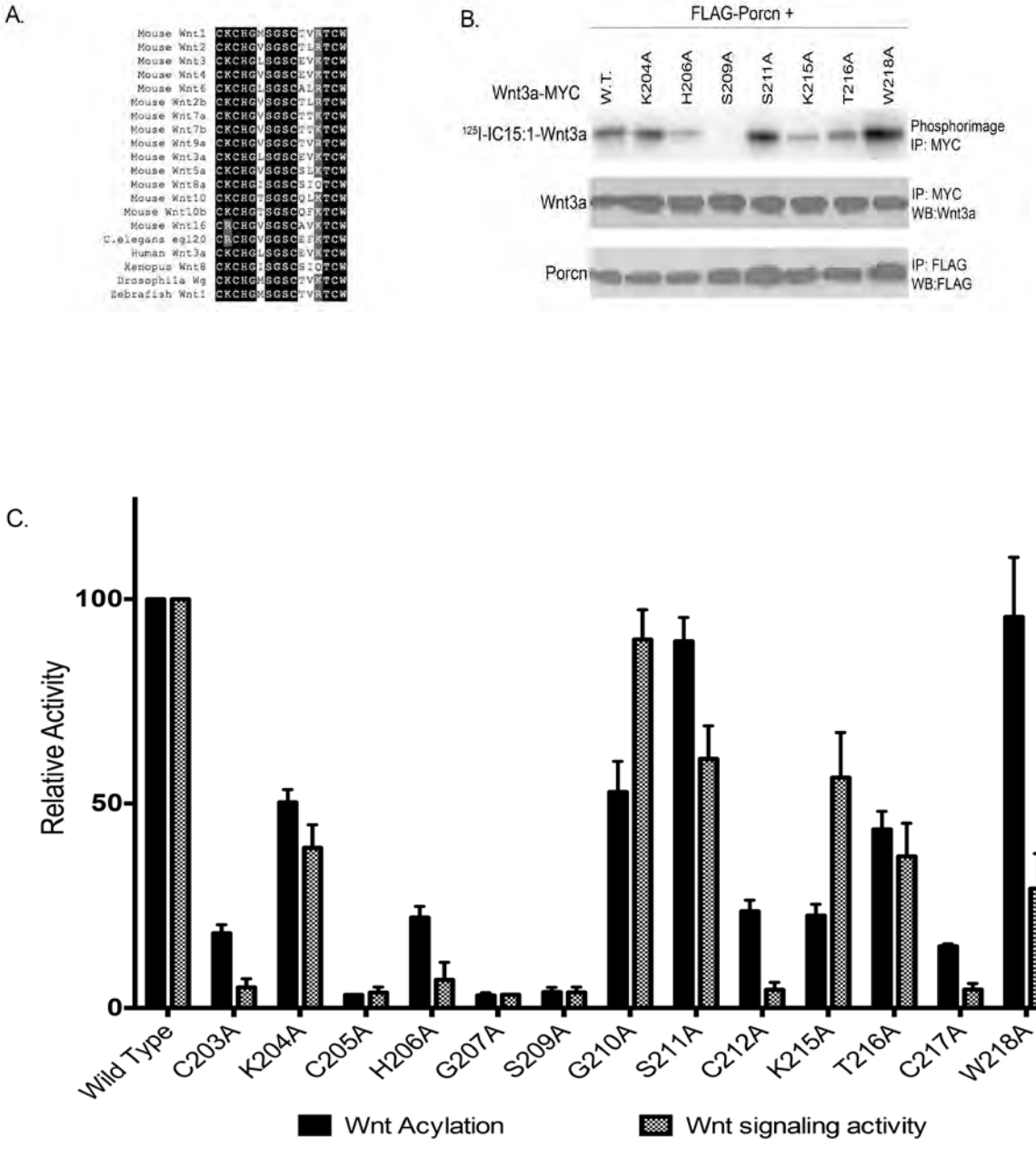
Although Wnt3a was originally reported to be palmitoylated on Cys77 (168), further studies revealed that this residue is instead engaged in a disulfide bond, and that the authentic fatty acylation site is Ser209 (23,170,173). The residues surrounding Ser209 in Wnt3a are highly conserved across species, from hydra to human Wnt proteins (Fig. 3.4a), but the importance of these amino acids for Porcn-mediated fatty acylation has not been explored. Alanine-scanning mutagenesis was used to alter conserved residues surrounding Ser209 in Wnt3a. The ability of each mutant protein to incorporate ^{125}I-IC15:1 was tested. Five of the Wnt3a mutants were acylated to the same extent (S211A, W218A) or to levels 40-50% (K204A, G210A, T216A) of WT Wnt3a (Fig. 3.4b,c). The other mutants exhibited severe acylation defects (<20% of WT) (Fig. 3.4c). Of note, the presence of each of the four Cys in this region that form disulfide bonds in Wnt proteins (173,175) was required for fatty acylation. We then tested the ability of the Wnt3a mutants to bind Porcn in a co-immunoprecipitation assay (Fig 3.4d). S211A and W218A, which are acylated to WT levels, associated with Porcn, as did K204A, which has moderately decreased fatty acylation. None of the acylation-deficient mutants were able to co-immunoprecipitate with Porcn.

The S209A mutant of Wnt3a has been shown to have defective signaling activity, and this has led to the conclusion that Wnt palmiteoylation is required for signal transduction (23,170,245). To examine the correlation between protein fatty acylation levels and Wnt signaling, we utilized a luciferase-based reporter of Wnt activity, Super Top-Flash (STF). HEK293T cells were transfected with STF, Renilla Luciferase (RL) and Wnt3a and luciferase activity was measured. All of the Wnt3a point mutants exhibited reduced signaling activity compared to WT Wnt3a (Fig. 3.4c). These reductions in signaling output, in general, correlated with reduced levels of fatty acylation. However, the W218A construct exhibited near WT levels of ^{125}I-IC15:1 incorporation but displayed signaling defects, arguing that this residue might be involved in mediating events downstream of Porcn activity. In summary, these results indicate

that the highly conserved region surrounding Ser209 plays a critical role in mediating the interaction between Porcupine and Wnt and that disruption of this region results in acylation defects, thus affecting downstream signaling activity.

Porcupine can acylate Ser and Thr but not Cys residues

With the exception of Hhat, all MBOAT family members with known substrates are O-acyltransferases, transferring fatty acids to hydroxyl groups (-OH) of proteins or lipids. For Porcn and GOAT, the modified residue on the protein substrate is Ser. We tested whether Thr or Cys in position 209 of Wnt3a would substitute for Ser as fatty acid acceptor for Porcn. As depicted in Figure 3.5a, ^{125}I-IC15:1 was transferred to Wnt3a containing S209T but not to S209C. The S209T construct exhibited signaling activity even greater than that of WT Wnt3a, whereas S209A and S209C mutants were inactive (Fig 3.5b). Thus, Porcn strictly functions as an O-acyltransferase and can form oxyester linkages to either Ser or Thr.


A.
Mouse Wnt1
Mouse Wnt2
Mouse Wnt3
Mouse Wnt4
Mouse Wnt6
Mouse Wnt2b
Mouse Wnt7a
Mouse Wnt7b
Mouse Wnt9a
Mouse Wnt3a
Mouse Wnt5a
Mouse Wnt8a
Mouse Wnt10
Mouse Wnt10b
Mouse Wnt16
C.elegans egl20
Human Wnt3a
Xenopus Wnt8
Drosophila Wg
Zebrafish Wnt1
B.
FLAG-Porcn +
Wnt3a-MYC
W.T.
K204A
H206A
S209A
S211A
K215A
T216A
W218A
125I-IC15:1-Wnt3a
Phosphorimage
IP: MYC
Wnt3a
IP: MYC
WB:Wnt3a
Porcn
IP: FLAG
WB:FLAG
C.
Relative Activity
100
50
0
Wild Type
C203A
K204A
C205A
H206A
G207A
S209A
G210A
S211A
C212A
K215A
T216A
C217A
W218A
Wnt Acylation
Wnt signaling activity

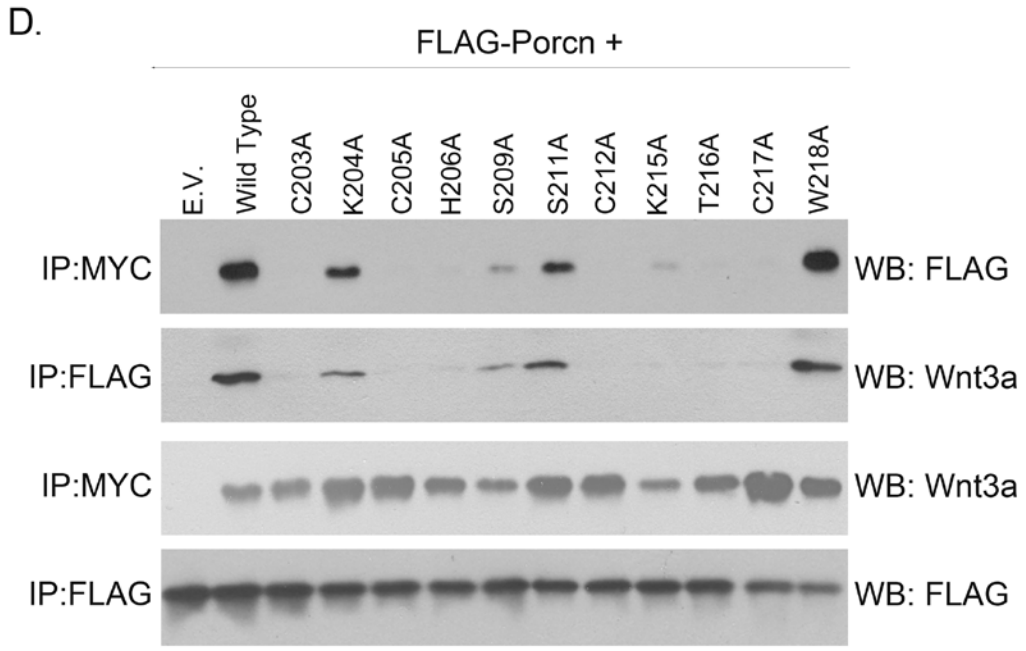


Figure 3.4 Mutational analysis of conserved residues surrounding Ser209 in Wnt3a

A, Multiple sequence alignment of a highly conserved region of Wnt family members from diverse species. Black boxes denote residues that are conserved in 80% or more sequences. **B,** COS-1 cells transfected with FLAG-Porcn and the indicated Wnt3a constructs were labeled with ^{125}I-IC15:1 for 5h. Cell lysates were immunoprecipitated (IP) with anti-MYC or anti-FLAG antibodies and analyzed by SDS-PAGE and phosphorimaging (top panel) or Western blotting (middle, lower panels) with anti-Wnt3a or anti-FLAG antibodies. The experiment was repeated three times in duplicate; a representative image is shown. **C,** Black bars: Quantification of experiments in B. Phosphorimaging signals were normalized to Wnt3a protein levels, then expressed as a percentage of WT Wnt3a (set at 100%). Each bar represents mean ± SD (n=3-6). Grey patterned bars: HEK293T cells were transfected with STF or FOP, RL and either WT or mutant Wnt3a. 48hr after transfection, STF luciferase activity was measured, normalized to RL and FOP-Flash activity, and expressed as a percentage of WT; bars represent mean ± SEM (n=3). **D,** Lysates from COS-1 cells co-expressing WT Porcn and the indicated Wnt3a constructs were immunoprecipitated with anti-MYC or anti-FLAG antibody followed by Western blotting with anti-Wnt3a or anti-FLAG antibody.

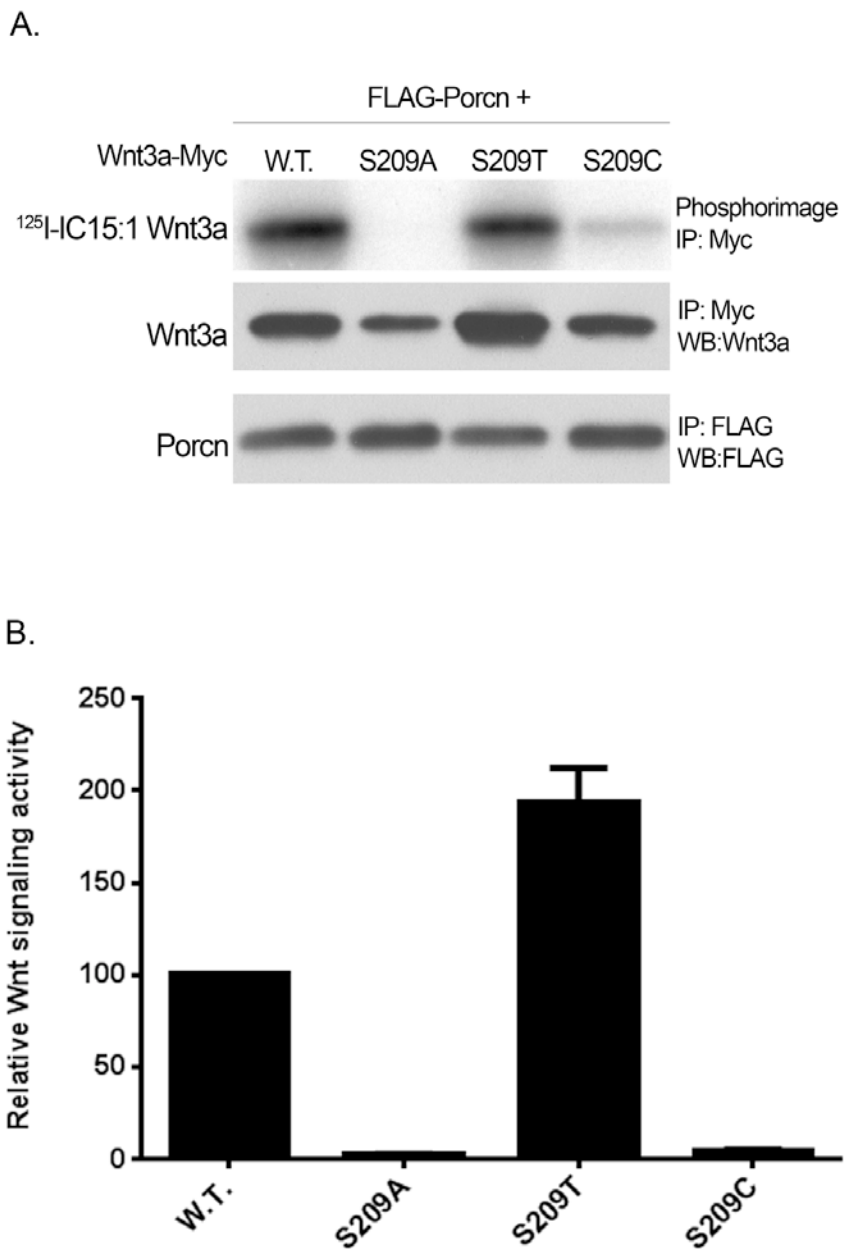


Figure 3.5 Porcn can transfer a fatty acid to either Ser or Thr at position 209 in Wnt3a

A, COS-1 cells expressing WT Porcn and the indicated Wnt3a constructs were labeled and analyzed as in Figure 1b. The experiment was repeated two times in duplicate; a representative image is shown. **B,** Relative luciferase activity for the Wnt3a constructs in panel A was determined as in Fig 4c.

DISCUSSION

A map of the active site of Porcn

Porcn-mediated attachment of palmitoleate to Wnt proteins ensures Wnt passage through the secretory pathway and proper signaling activity. At the biochemical level, this reaction has been difficult to study in a quantitative manner, partly due to the hydrophobic nature of Porcn and the lack of tools to accurately detect acylated Wnt. In this report, we undertook a systematic approach to dissect the complexities of the Porcn-mediated fatty acylation reaction. A cell-based fatty acylation assay that provides an accurate and direct measurement of Porcn acyltransferase activity (170) was combined with alanine-scanning mutagenesis of highly conserved residues and regions within Porcn and Wnt3a. Here we identify key residues that are crucial for the intracellular localization, stability, and activity of these proteins and provide molecular-level information regarding interactions between Wnt and Porcn. In total, 16 Porcn mutants (2 truncation mutants, 9 MBOAT homology domain mutants, 1 random site mutant, and 4 FDH-associated mutants) and 13 Wnt point mutants were analyzed. When mapped within a predicted topology model of Porcn, a striking pattern was observed for the Porcn mutants. Nearly all of the inactive Porcn mutants localize to TMD 9, whereas all of the constructs retaining catalytic activity localize to predicted cytoplasmic or luminal loops (Fig 3.6). This analysis defines a critical active site region within predicted TMD9 essential for catalysis by Porcn.

A recent study examined the effect of multiple Porcn mutations that occur in FDH on Wnt signaling activity (245). Several of the mutants overlap with those tested in this study. Three of the four FDH mutants we analyzed (S136F, L331R, R365Q) showed decreased stability and mislocalization, which is likely the cause of the decreased fatty acyltransferase activity that we observed (Fig 3.3) and could potentially explain the disease phenotype. Proffit and Virshup also noted decreased expression levels for R365Q Porcn, but obtained the opposite result for S136F and L331R Porcn, which exhibited nearly normal activity when assayed in a -catenin signaling

assay (245). The reason for this difference may be related to the use of an assay that is distal and not a direct readout of the initial biochemical event, Porcn-mediated acylation of Wnt.

We observed that N- and C-terminal truncation mutants of Porcn were unstable and inactive. These residues are likely needed to maintain enzyme integrity, and when deleted cause the protein to become misfolded and more susceptible to degradation. However, this was not the case for the MBOAT homology domain mutants which, except for Y316A, had stabilities close to that of WT Porcn. Despite its lower stability, Y316A retained a moderate level of acyltransferase activity. With the exception of T333A, none of the MBOAT homology domain mutants that we examined exhibited observable alterations in subcellular localization. Thus, residues in the inactive mutants are likely involved in mediating the acyltransferase reaction, by either binding to the protein or the fatty acid substrate, or directly catalyzing fatty acid transfer.

Invariant His and Asn/Asp residues are highly conserved and regarded as putative catalytic sites for all MBOATs. However, several differences regarding the requirement for these residues have been observed. Mutation of the invariant Histidine residues abrogates activity in all family members tested, but only reduces activity by 50% in Hhat. The residue immediately adjacent to the conserved His is required for catalytic activity in Porcn and Hhat, but not in ACAT1 (130,286). Of note, the L340A Porcn mutant was catalytically inactive but could still bind to Wnt3a. This suggests that L340 might be involved in binding to palmitoleolate, consistent with its localization within a TMD (Fig 3.6). Conversely, the conserved Asn/Asp is not required for Porcn acyltransferase activity (N306) but it is essential for Hhat and GOAT activity (21,130). Mutational analyses of other conserved residues within the MBOAT homology domain have been performed for ACAT1, ACAT2, DGAT1 and DGAT2, based on sequence conservation across species, and in Hhat, based on sequence homology among MBOATs with protein acyltransferase activity. Similarities and differences among the overlapping mutants are summarized in Table 3.2. Mutation of the S337 equivalent results in complete inactivity or severe enzymatic defects in ACAT1 and ACAT2 (292). We observed a similar result for Porcn, whereas another group

reported that the S337A Porcn mutation was able to fully restore Wnt signaling activity to Porcn null cells (245). In Porcn, Y334A exhibited moderate defects in activity whereas mutation of this residue in Hhat has no detectable effect (130). Mutations of W305 in Porcn or F338 in Hhat results in partial loss of acyltransferase activity (130). Considering that Porcn, Hhat and GOAT catalyze distinct biochemical reactions with different fatty acid and protein substrates, it is perhaps not surprising that the active sites of these enzymes are not identical.

Residue in Porcn	PORCN	HHAT	GOAT	ACAT1	ACAT2
W305	50%	32%	-	-	-
N306	80%	10%	0%	-	-
Y334	40%	100%	-	-	-
S337	13%	-		0%	27%
L340	20%	30%	-	70% [a]	-
H341A	4%	50%	0%	0%	0%
References		(130)	(21)	(292)	(293)

Table 4. Relative acyltransferase activity of other MBOAT family member mutants

Percentages indicate relative enzymatic activity compared with wild type controls.

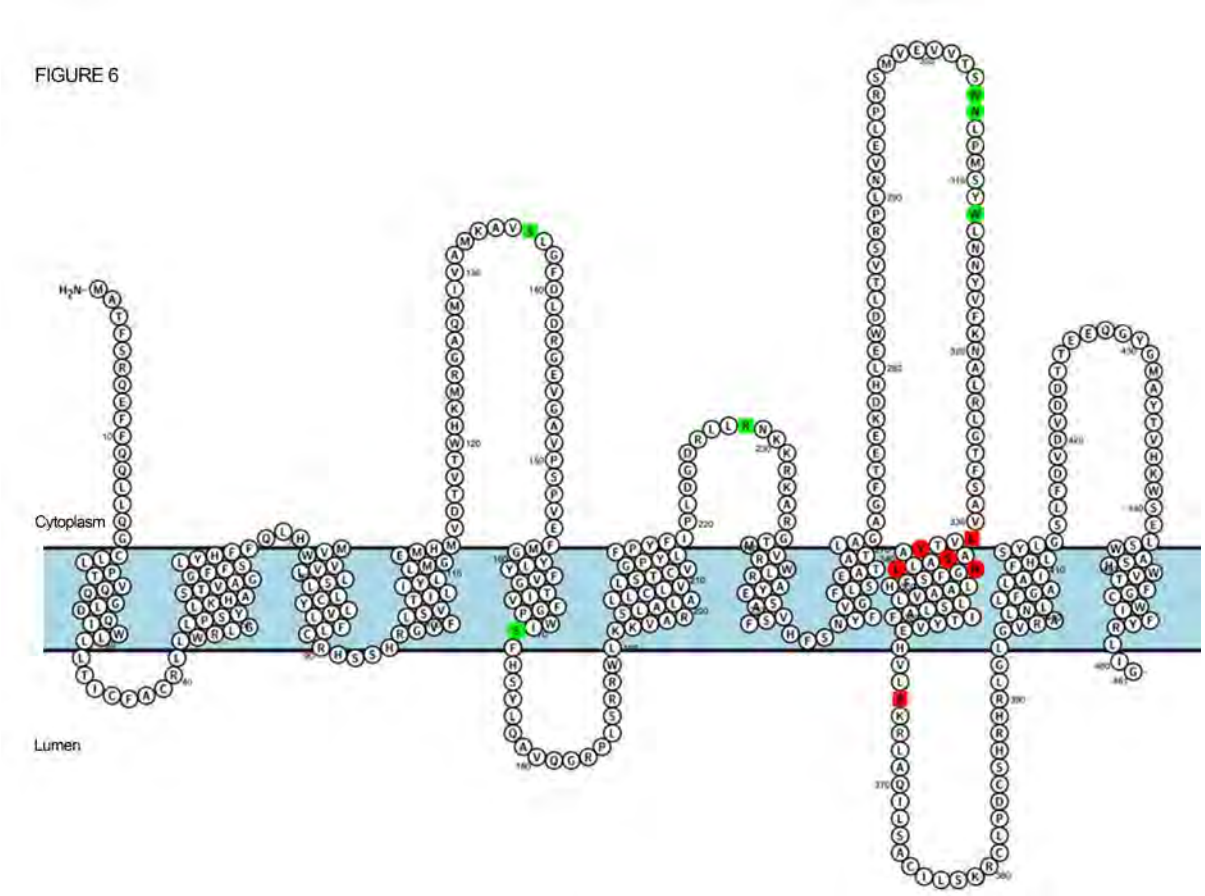


Figure 3.6 Predicted transmembrane topology map of the active site of Porcn

Graphical representation of the predicted topology of Porcn as generated by Protter Server (41). MBOAT homology domain (circles) and FDH-associated mutants (squares) are highlighted according to their effect on Porcn PAT activity. Red boxes represent residues that are essential for enzymatic activity (inactive mutants) and green boxes indicate residues that are not required for PAT activity (active mutants).

Identification of a consensus sequence within Wnt necessary for Porcn-mediated fatty acylation

Site directed mutagenesis of the sequences immediately upstream and downstream of the conserved Ser acylation site led to identification of residues required for fatty acylation of Wnt3a. Based on this analysis, we propose the following consensus sequence for Porcn-mediated acylation: **C**X**C**H**G**X**S**XX**C**XX**KXC.** This sequence overlaps significantly with the Wnt family sequence conservation surrounding Ser209 (Fig 3.4a), but identifies multiple conserved residues (K204, G210, S211, T216, W218) that are not required for Wnt acylation. The products of a blastp interrogation of the human proteome are nearly all Wnt proteins. However, we found two proteins that retain 6 of the residues surrounding "Ser209": β-defensin and netrin G1, both of which are secreted proteins that could be potential Porcn substrates. Further experiments will be required to explore this possibility.

A close correlation was observed between ^{125}I-IC15:1 incorporation and signaling activity of the Wnt mutants (Fig 3.4c). However, W218A Wnt3a was acylated to levels near WT but displayed signaling defects. This suggests that this residue might be involved in mediating distal events downstream of Porcn, such as the interaction between Wnt and its cell surface receptor, Frizzled. In fact, the equivalent W218 residue in Xenopus Wnt8 (Trp 196) is engaged in van der Waals interactions with an Asn residue in the cysteine-rich domain of Frizzled (173). The K215A construct was a poor substrate for Porcn-mediated acylation, but exhibited nearly 50% of the signaling activity of WT Wnt3a. One could speculate that the Lys to Ala mutation induces a conformational change that is conducive to Wntless binding and Wnt pathway activation, thereby bypassing the acylation step.

Both GOAT and Porcn transfer a fatty acid to a Ser residue via oxyester linkage. Although hedgehog proteins, which are the only known substrates for mammalian Hhat, are palmitoylated on an N-terminal Cys, we have shown that Hhat can also attach palmitate to

Ser(129). This could potentially occur through initial attachment via an oxyester link, followed by intramolecular rearrangement to an amide bond. Here we show that Porcn can also recognize a Thr residue placed within the consensus sequence for Wnt3a palmitoleoylation, a property shared by GOAT (21). This finding supports the designation of Porcn as an O-acyltransferase that attaches fatty acids via oxyester linkage. Since all Wnt proteins described to date contain Ser and not Thr at this site, it is likely that Ser is required for downstream functions of Wnt that occur after acylation.

Acknowledgments

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FOOTNOTES

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CHAPTER FOUR

CONCLUSIONS

Over the years, our lab has developed and optimized *in vitro* and *in vivo* technologies to study protein palmitoylation in cells, and has accomplished groundbreaking research on the mechanisms underlying the palmitoylation and/or myristoylation of Src, Fyn, Shh and HIV Gag proteins, among others. Five years ago, Dr. Resh and I embarked on a journey to elucidate the mechanism by which Wnt proteins undergo fatty acylation by the enzyme Porcupine (Porcn); a seemingly effortless project for The Resh Lab. To our surprise, the study of Wnt acylation was more challenging that we anticipated. Wnt proteins did not respond to standard methodologies used in the lab, requiring us to pioneer an assay that would allow detection of acylated Wnt *in vitro*. We established a cell-based palmitoylation assay using a radio-iodinated palmitoleate analogue (IC15:1). Although puzzling at first, this new system unveiled an unprecedented mechanism: a) Porcn transfers MUFAs, preferably palmitoleate, but not saturated fatty acids to Wnt substrates; b) Stearoyl-CoA Desaturase (SCD) generates the appropriate MUFA substrate for Porcn and c) blocking SCD activity inhibits Wnt acylation, reduces Wnt secretion as well as autocrine and paracrine Wnt signaling activity. The SCD inhibitor effects were rescued by exogenous addition of monounsaturated fatty acids. This also establishes that conversion to an unsaturated fatty acid occurs prior to transfer by Porcn. Based on these results, 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. With an assay in hand, we then analyzed the structure-function relationship between Wnt and Porcn. Over 20 point mutants in Wnt and Porcn were generated and tested for activity, stability and intracellular localization. We identified key residues and regions required for Wnt acylation by Porcn,

enabling us to produce an initial map of Porcn's active site and to define a consensus sequence for Wnt palmitoleoylation.

This work contributes to the advancement of the field by providing compelling biochemical evidence of the mechanism governing Wnt acylation by Porcn, a process that precluded the scientific mind for years. This knowledge sets the stage for the development of an *in vitro* acylation system that will aid in the purification of recombinant Porcn protein and kinetic analysis of the enzyme.

The requirement of a MUFA substrate

The requirement for fatty acid desaturation prior to Porcn-binding and transfer to Wnt is one of the most important findings of this work, not only because of its unique nature but also due to its implication in signaling. This modification might confer certain advantages that enable Wnt to perform diverse biological functions, but the exact mechanism is yet to be determined. It is known that modification with unsaturated fatty acids displaces proteins from lipid rafts, as these moieties do not fit within the liquid ordered phase that characterizes this region. Although Wnts have been shown to associate with rafts, it is possible that palmitoleate precludes Wnt from permanently binding to these membranous surfaces, thus facilitating travel across tissues. A bent conformation may also assist in insertion of palmitoleate into the hydrophobic groove of Frizzled that binds Wnt. For the enzyme Porcn, this requirement implies that the active site of the enzyme has evolved to discriminate between MUFA and SFA, further underlying the significance of the fatty acid identity.

Despite major setbacks in the field of Wnt acylation in recent years, the future seems to hold more promise. Emerging technologies allowing visualization of acyl-Wnt at the single-cell level, along with increasing number of Wnt crystal structures and the development of new Wnt pathway inhibitors, are just but a few recent advancements in the field. This level of detail and sophistication will continue to provide insight into this fundamental biological process.

FUTURE DIRECTIONS

Define the relationship between lipid metabolism and Wnt signaling

We identified SCD as a key molecular player during Wnt biogenesis and processing. Inhibiting SCD activity, chemically or by siRNA treatment, blocks Wnt palmitoylation and renders Wnt inactive. This finding suggests that there is a direct correlation between the intracellular pool of fatty acyl-CoAs and Wnt signaling. It would be interesting to investigate how changes in lipid composition influence Wnt signaling in normal tissue or in the context of a disease. To further characterize the relationship between SCD and Wnt signaling *in vivo*, one could measure Wnt pathway activation in SCD-/- (whole body or tissue specific knockout) mice, particularly in the stem cell population of the skin, hair follicle and gut where Wnt is activated.

It is possible that when palmitoleate levels are limiting, Porcn utilizes shorter fatty acid substrates. This would be suggestive of a model in which the identity of an acyl moiety attached to a given substrate is dictated by the abundance and distribution of fatty acids rather than enzyme specificity. It would be interesting to characterize the pool of acylated Wnt during starvation and measure how Wnt signaling responds to metabolic stress. 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*. This could indicate that Wnt signaling might respond to changes in the metabolic state of the cell.

Porcn purification and enzymology studies

Enzymology and kinetic studies on Porcupine have been hindered by the lack of tools to measure Porcn activity *in vitro* and the inability to obtain purified, recombinant Porcn. Our findings uncovering the authentic fatty acid substrate for Porcn may facilitate the establishment of an *in vitro* palmitoylation system. The source of Wnt protein is problematic, as investigators have not been able to purify soluble, recombinant Wnt proteins expressed in E. coli, yeast or Baculovirus expression. Alternatively, it could be possible to synthesize a cyclical, 16-residue

peptide containing the conserved sequence surrounding Ser-209 acylation site, mimicking the natural conformation of the loop region. Our lab has successfully used a Shh peptide to recapitulate Hhat-mediated Shh palmitoylation *in vitro(121)*. Experimentally, membranes from Porcn-transfected cells could be incubated with ^{125}I-IC15:1-CoA and WT Wnt peptide or a peptide carrying a Ser-to-Ala mutation. Radiolabel incorporation into the peptide would be quantified in a gamma counter.

Recently, our lab successfully achieved the purification to homogeneity of recombinant HHAT from transiently transfected HEK293T cells. One could employ a similar approach to achieve the purification of recombinant Porcn. Having established an *in vitro* assay, we could then validate the purification of functional Porcn enzyme at various steps during the process. Once Porcn is purified to homogeneity, we could measure kinetic parameters such as V_{max} and K_m values, determine optimal pH and temperature, among others. This information will be instrumental for drug development and structural studies.

Determine Porcn Topology

Porcn is localized to the ER. To date there have been no studies of Porcn membrane topology. It would be of great interest to determine where catalytic residues (identified in Chapter 3) lie relative to the plane of the surrounding lipid bilayer. The Protter Server program predicts 11 potential TMDs and 8 loops between TMDs (Figure 3.6). Topology-mapping experiments would allow us to determine whether the loops reside on the luminal or cytosolic side of the ER membrane. This could be accomplished using 2 alternative approaches: 1) Protease Protection Assays or 2) Indirect immunofluorescence and selective permeabilization. In the absence of structural data, this approach would allow us to map transmembrane domains, and to obtain useful information for the rational design and analysis of targeted inhibitors.

Does Porcupine form oligomers?

Other MBOAT family members such as ACAT and DGAT exist in higher oligomeric forms (homodimer/tetramer) and oligomerization is involved in regulating enzyme activity (99). Currently, no information about the oligomeric state of Porcupine is available. To address whether Porcn forms higher order oligomers, one could take two approaches: Co-immunoprecipitation experiments of differentially epitope-tagged Porcn constructs or cross-linking of membrane-bound Porcn-Flag. Monomeric Porcn migrates at ~ 45kDa and higher oligomeric forms are expected to appear ~ 90kDa and 180kDa for a Porcn dimer and tetramer, respectively. If Porcn dimerizes, the region mediating the interaction can be mapped, and the effect of mutations in this region on Porcn function, stability or intracellular localization can be determined. To map the protein-protein interface, Flag-Porcn constructs carrying various N-or C-terminal truncations could be co-transfected with WT–myc Porcn. The ability of WT Porcn to bind the truncated form would be assessed by co-immunoprecipitation.

Functional significance of Porcn Palmitoylation

While investigating Wnt acylation by Porcupine, we serendipitously discovered that in the absence of Wnt protein, Porcn incorporates both IC16 and IC16:1 (Figure 4.1). Recently, another group reported a similar finding using ω-alkynyl palmitic acid (Alk-C16) and Click-Chemistry (172). However, the functional significance of this modification is yet to be determined. We propose two possible models to explain this novel finding: (1) Palmitate incorporation represents Porcn autoacylation; (2) Porcn is palmitoylated by a different PAT. These two models can be distinguished by testing the ability of catalytically inactive (FLAG- or Myc-tagged) Porcn mutant, H341A to incorporate Iodo-palmitate. Experimentally, COS-1 or HEK293FT cells would be transfected with empty vector (pcDNA3.1), or cDNA encoding H341A, labeled with ^{125}I-IC16 or ^{125}I-IC16:1, and lysed. Cell lysates would be IP'ed with anti-Flag (or myc), and analyzed by SDS-PAGE and phosphorimaging. ^{125}I-IC16 incorporation would be quantified using Image Gauge software and normalized to expression levels of each Porcn

construct (as determined by Western blotting). If palmitate incorporation is abolished or greatly reduced in the mutant Porcn, it would be highly suggestive of the autoacylation model. We will then consider two additional possibilities: i) autoacylation represents formation of an acyl-enzyme intermediate, which then transfers palmitate to the substrate; ii) or autoacylation occurs at a non-catalytic site. We favor the first explanation, as formation of an acyl-enzyme intermediate has been observed in members of the DHCC family of PAT's, which are responsible for the palmitoylation of many intracellular proteins, including Ras, eNOS, PSD-95. An alternative outcome is that the mutant Porcn constructs still incorporate IC16, suggesting that Porcn does not autoacylate, but rather is palmitoylated by a different PAT. This modification could potentially regulate Porcupine's acyltransferase activity, subcellular localization, trafficking or stability.

Regardless of whether Porcn is palmitoylated by itself or another PAT, we would next map the site(s) of Porcn acylation. In human Porcn, the site of palmitoylation has been identified as Cys187 (3), but this residue is substituted with an Arg in mouse. Since we can still detect IC16 and IC16:1 incorporation in murine Porcn, we hypothesize that an alternative, or multiple residues serve as a fatty acyl acceptor sites. IC16 incorporation is sensitive to hydroxylamine treatment, indicating that the fatty acid is likely attached to a Cys residue via a thioester linkage (Figure 4.2). There are 11 Cys residues in the mPorcn primary sequence. A series of Porcn mutants carrying N- or C-terminal truncations, internal deletions, as well as point mutations of potential acceptor sites could be generated by PCR-directed and/or site directed mutagenesis. The ability to incorporate palmitate could be tested, and mutants lacking the ability to incorporate palmitate could be further analyzed.

Palmitoylation of multipass transmembrane proteins has been shown to regulate their sorting, trafficking, localization and/or stability. Once the site(s) of mPorcn palmitoylation are identified, it will be possible to investigate the functional significance of this modification. In human Porcn, it was observed that when Porcn "autoacylation" was blocked, Wnt signaling was slightly increased. We would compare mutants vs. WT Porcn in terms of :

A) Porcn stability: Porcn-transfected COS-1 cells would be treated with cycloheximide and expression levels of WT and mutant proteins, determined by WB, would be monitored as a function of time.

B) Palmitate turnover: The half life of the ^{125}I-IC16-palmitate moiety on Porcn would be determined by performing pulse-chase experiments.

C) Porcn Trafficking and localization would be examined by indirect immunofluorescence analysis.

D) Wnt binding would be tested by co-immunoprecipitation experiments.

E) Wnt acylation: We would monitor ^{125}I-palmitate incorporation into Wnt, in the presence or absence of WT or mutant Porcn.

F) Wnt secretion and signaling: Cells co-expressing Wnt3a and mutant Porcn would be assessed for their ability to secrete Wnt3a into the media. Wnt signaling activity would be evaluated by analyzing markers of pathway activation, which include cytoplasmic and nuclear β-catenin levels, Dishevelled phosphorylation and/or using a TOP FLASH Luciferase reporter system.

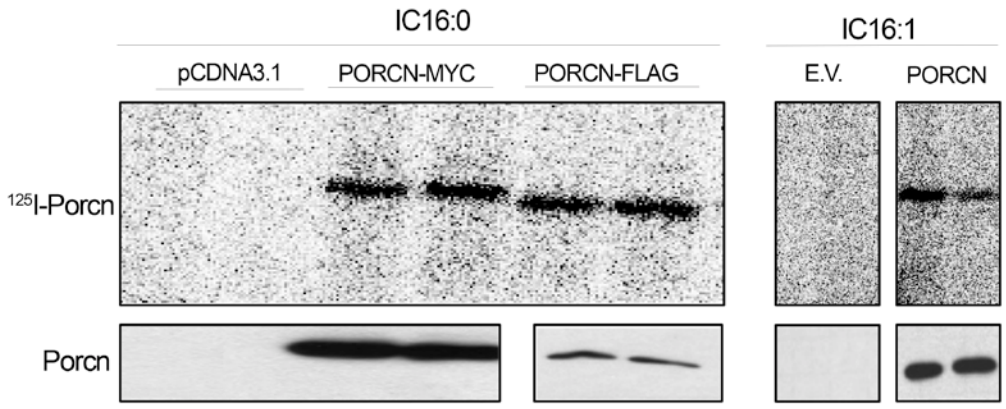

Figure 4.1 Porcn incorporates palmitate and palmitoleic acid.

COS-1 cells expressing myc- or Flag-tagged Porcn (or empty vector as control) were labeled for 6 hrs with IC16:0 or IC16:1, lysed and IPed as indicated. Palmitate incorporation was detected by phosphorimaging (top panel). Bottom panel: IP/Western Blot.

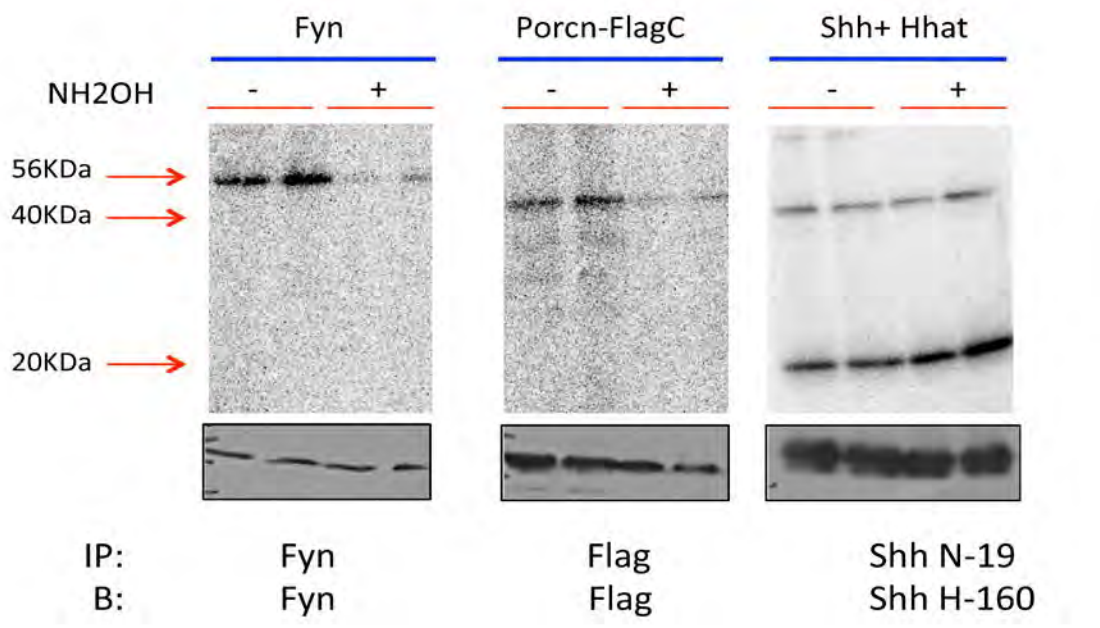

Figure 4.2 IC16 incorporation into Porcn is sensitive to hydroxylamine treatment

COS-1 cells expressing Fyn, Porcn-FLAG or Shh and HHAT were labeled with IC16 for 5 hrs, lysed and immunoprecipiated with the indicated antibodies. Porcn, Shh and Fyn immunoprecipitates containing ^{125}I -IC15:0 were treated with 1M Tris or NH_2OH pH 7.0 for 1hr at RT, and analyzed by SDS-PAGE followed by phosphorimaging or Western Blot.

CONCLUDING REMARKS

This work has greatly expanded our understanding of Porcn-mediated Wnt acylation. When this thesis research was begun, it was not clear whether Porcn was a *bona fide* acyltransferase, whether it catalyzed attachment of one or two different fatty acids, palmitate (16:0) and palmitoleate (16:1), to Wnt proteins, or where the active site was located within the protein. We undertook the challenging task of uncovering the mechanism by which Porcn recognizes and transfers fatty acids to Wnt proteins. Our analysis defines Porcn's requirements for fatty acid substrates and suggests that Porcn is capable of generating a varied population of Wnt proteins with different acyl groups. We showed that Porcn transfers MUFAs but not saturated fatty acids onto Wnt proteins. In the cell, MUFA's are produced by Stearoyl CoA Desaturase (SCD) and blocking SCD activity abrogates Wnt acylation, secretion and signaling activity. These findings place SCD as a novel intermediary during Wnt biogenesis and establish an unprecedented connection between Wnt signaling and fatty acid metabolism. In addition, a comprehensive structure-function analysis of highly conserved residues in Wnt and Porcupine led to the identification of a consensus sequence for Porcn-mediated Wnt acylation and an initial map of the active site of Porcn. These findings lay the groundwork for the study of Porcn activity *in vitro* and to explore SCD as an alternative target to block Wnt signaling activation in normal and disease states.

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