Precise Regulation of Porcupine Activity is Required for Physiological Wnt Signaling

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Background: Wnts control development and differentiation; their secretion may be regulated.

Results: All Wnt activity absolutely requires post-translational modification by the O-acyl transferase PORCN, whose abundance or activity modulates signaling tightly and over a large dynamic range.

Conclusion: Subtle changes in PORCN activity have major consequences and cause the human disease Focal Dermal Hypoplasia.

Significance: PORCN is a key regulator of all Wnt signaling.

SUMMARY

Gradients of diverse Wnt proteins regulate development, renewal, and differentiation. Porcupine (PORCN) is a membrane-bound O-acyl transferase (MBOAT) that is required for post-translational modification of all Wnts to enable their transport, secretion, and activity. Mutations in PORCN are associated with Focal Dermal Hypoplasia (FDH), while gene deletion causes embryonic lethality in mice. To study the protein in more detail, zinc finger nucleases were used to edit the PORCN genomic locus, establishing two HT1080 fibrosarcoma clones null for PORCN activity that facilitate the study of PORCN structure and function. We establish that PORCN is a key non-redundant node for the regulation of global Wnt signaling, because PORCN null cells are completely incapable of autocrine Wnt signaling. The strength of Wnt signaling is exquisitely sensitive to PORCN expression with a dynamic range of at least 3 orders of magnitude, suggesting PORCN activity is a key modulator of all Wnt ligand activity. Consistent with this, we find that multiple FDH-associated mutants have only subtle alterations in enzyme activity yet are associated with a severe FDH phenotype. These studies support an essential regulatory role of PORCN in shaping Wnt signaling gradients.

Wnts are secreted protein ligands that direct the activation of diverse signaling pathways, of which β-catenin mediated transcription is the best known and understood. Multiple Wnt-triggered pathways coordinately regulate key developmental processes as well as adult tissue homeostasis and repair, and are dysregulated in diverse diseases including cancer, malformation syndromes, osteoporosis, diabetic retinopathy, and pulmonary fibrosis (1-5). Wnts act locally and regionally, with both quantitative and qualitative regulation of downstream signaling dependent on local Wnt concentration (6,7). Different Wnts can have opposing actions in the same tissue. The local Wnt concentration and balance of varying Wnt activities at any given target cell is dependent on diverse factors including transcription and translation rates, post-translational modification, secretion, and extracellular transport, and thus these are topics of active investigation (reviewed in (8,9).

Careful control of global Wnt activity may be critical for development and homeostasis, as
changes in the core pathway can affect diverse Wnt-dependent signaling pathways. The rate of Wnt secretion is one potential control point for Wnt signaling. While there are 19 distinct Wnt genes and multiple Wnt receptors, there appears to be a single conserved and essential Wnt biogenesis pathway. This core pathway includes porcupine (PORCN), ER to Golgi transit promoting p24 proteins (10,11), the Wnt chaperone Wntless (WLS, Evi, GPR177 (12-14), and the retromer complex and sorting nexins, which are necessary for recycling WLS (15). The first step in Wnt protein production is translation in the endoplasmic reticulum, followed by post-translational modification by the ER-resident enzyme PORCN (porcupine) (16). PORCN is a membrane-bound O-acyl transferase (MBOAT), and it catalyzes the palmitoylation of the serine corresponding to S209 of WNT3A (17,18). This modification is absolutely required for the next step in Wnt secretion, binding to the carrier protein WLS. In addition, palmitoylation is essential for Wnts’ ability to interact with Frizzled receptors at the cell surface (19-21). While Wnts were reported to be palmitoylated at a conserved cysteine corresponding to C77 of WNT3A (22,23), in the crystal structure of Xenopus Wnt8 in complex with Fz8-CRD the C77 equivalent residue was instead engaged in a disulfide bond. (21). This disulfide formation likely explains why mutations of C77 caused decreased activity of Wnt. It is notable that all Wnts examined from diverse organisms, with the sole exception of Drosophila WntD, require PORCN or at least maintain the serine corresponding to S209 of WNT3A. WntD does not contain this serine (replaced by glutamine), and is secreted independently of either Porcn or Wls, though it retains dependence on the p24 protein opm (11).

Little is known about the regulation of PORCN expression and palmitoyl-transferase activity. PORCN is a member of the MBOAT family (24), and in most species it appears to be a single copy X-linked gene most closely related (but with <35% identity) to a family of lysophospholipid acyltransferases {Hishikawa:2008dq} and no evidence for functional redundancy in Drosophila, zebrafish, mice, or humans (16,25-28). The enzyme is expressed in most tissues of the body, with four tissue-specific splice variants of unknown significance (29,30). Because of the importance of Wnt signaling in human disease, small molecule inhibitors of PORCN have been developed that inhibit Wnt signaling in cultured cells and in intestinal organoids (31,32).

Consistent with its critical role in Wnt signaling, germline PORCN mutations are associated with the X-linked dominant genetic disease Focal Dermal Hypoplasia (FDH, OMIM 305600) (33,34). The disease occurs primarily in heterozygous females, due to location of PORCN on the X chromosome, and its phenotypic manifestations are variable due to patterns of X-inactivation. Complete deletion of PORCN is embryonic lethal in mice and also presumably humans, although post-zygotic mutations do occur in males and cause FDH, but allow survival. Heterozygous deletion of PORCN in mice also recapitulates the phenotypes seen in FDH (26-28). FDH is associated with defects in ecto-mesodermally derived tissues, leading to superficial skin striation and atrophy, fat herniation, and papillomas of the mucous membranes, as well as more severe defects such as limb shortening and syndactyly. Combined with the knowledge that the known function of PORCN is in Wnt modification, it is presumed, but not yet proven, that FDH is ultimately a disease of Wnt malfunction. Whether there is a discernible genotype-phenotype correlation is not clear (35). Over 100 different PORCN mutations have been reported in FDH (https://grenada.lumc.nl/lovd2/amc). Some PORCN mutations in FDH patients are clearly loss of function, as they are small or large deletions and/or cause premature protein termination. However, a number of PORCN alterations are missense mutations that fall in residues of unknown function. As PORCN may have a role beyond the Wnt pathway (36), it is also possible that some of the FDH mutations in PORCN do not alter the Wnt signaling pathway.

In this report we have used zinc finger nucleases to functionally delete PORCN from the human (male) fibrosarcoma cell line HT1080. There appears to be no functional redundancy for Wnt palmitoylation, as these cells absolutely require PORCN for any Wnt secretion or signaling. Further, we use these cells to demonstrate that physiologically relevant expression levels of PORCN can fine-tune Wnt signaling over a large dynamic range. While all FDH mutants tested alter Wnt signaling, importantly, a subset of mutations demonstrates near normal activity until titrated to very low but physiologically relevant levels. Splice variants of PORCN displace small changes in activity
similar to those seen in some FDH mutants. These findings suggest that in humans, small changes in Wnt gradients due to subtle mutations in PORCN can have major phenotypic effects.

EXPERIMENTAL PROCEDURES

Reagents

HT1080 cells were acquired from the American Type Culture Collection (VA, USA) and grown in DMEM (Nacali Tesque, Japan) containing 4.5 g/L glucose, pen/strep, 10% FBS, and 1 mM sodium pyruvate in a humidified 37 °C atmosphere. The zinc finger nuclease was purchased from Sigma-Aldrich (St. Louis, MO, USA), and is composed of an array of 6 zinc fingers upstream and 5 zinc fingers downstream of a 5 bp spacer cut site at the beginning of exon 9 (Fig. 1A). Antibody to WNT3A was a kind gift from Shinji Takada. Antibody against HA tag is from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (SC-7392). Dvl2 antibodies (used 1:500) were from Santa Cruz Biotechnology (#13974). Surveyor mutation detection kit was from Transgenomic (Omaha, NE, USA). Super8xTopflash reporter was a gift from Randy Moon (37). pGK-WNT3A plasmid was provided by Karl Willert. Other Wnt expression constructs were prepared as part of the Open Source Wnt project (38). pMKIT-3xHA-mPORCN-D as well as isoforms B, C, and D were gifts from Tatsuhiko Kadowaki. Xenopus laevis PORCN (isoform A) expression vector (clone ID 6638385) was purchased from Open Biosystems/Thermo Scientific (Lafayette, CO, USA). Point mutants and small deletions in PORCN plasmids were created with Stratagene QuikChange site directed mutagenesis kit (La Jolla, CA, USA). DNA sequencing was performed by AITBiotech (Singapore). The human tissue array mRNA set was from Clontech, lot #1001243A.

Zinc Finger Nuclease Mediated PORCN Deletion

In order to delete PORCN, HT1080 cells were transfected in 6-well dishes with 2 μg each of ZFN 1 and ZFN 2, creating the heterodimeric endonuclease. 48 hours after transfection, genomic DNA was isolated, and a 382 bp fragment of PORCN surrounding exon 9 was amplified using forward primer: TTT CTT CTC TGC TGC CTT CC and reverse primer: ACA CTT CTG TCT TGG CCG TT. This product was subjected to the transgenomic Cell1-based endonuclease assay to detect gene editing. Following confirmation of significant editing within the pooled population, single cells were cloned by limiting dilution into 96-well plates. After ~2 weeks growth, gDNA stabilizing lysate was prepared using Lyse-and-go reagent (Promega, Madison, WI, USA). This lysate was used for amplification of the same 382 bp region surrounding exon 9. A 13% polyacrylamide TBE gel was used to resolve products, and mutant clones were identified by faster migration. Two clones with significant deletion were isolated, in addition to an isogenic clone with no modification in the PORCN gene.

Wnt Secretion and SuperTOPFlash Assays

To monitor Wnt secretion into culture media, cells were grown in 6-well plates and transfected with 1 μg WNT3A, along with mCherry and 100 ng of 3xHA-mPORCN-D encoding plasmids as indicated. Six hours after transfection, media was changed to low-serum 2% FBS at half volume (1 mL). Lipofectamine 2000 was used for transfections (Life Technologies, Carlsbad, CA, USA). For STF assays, experiments were performed in 24-well plates, transfecting 800 ng total plasmid per well, composed of 50 ng Wnt, 100 ng mCherry, 550 ng SuperTOPflash, and up to 100 ng PORCN plasmid (pMSCV-puro empty vector used as filler). Lysates were prepared in PBS with 0.6% IGEPA-C630, and firefly luciferase was measured with luciferase assay kit according to manufacturer recommendations (Promega), using a Tecan Infinite M200 plate reader (San Jose, CA, USA).

Dvl2 Mobility Shift Assay

For measurement of Dvl2 mobility shift, transfection in 24-well dishes was with 200 ng of each Wnt, 600 ng mCherry, and 10 ng mPORCN-D expression plasmids. Lysates were prepared in 100 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% IGEPA-C630, complete protease inhibitor cocktail (Roche) and analyzed by SDS-PAGE on 10% gels followed by Western blotting to detect Dvl2.

qPCR

RNA was isolated using the RNEasy kit (Qiagen), reverse transcribed with the i-script RT kit (Biorad), and quantified on a Biorad CFX96 real time cycling machine using the Ssofast evagreen PCR assay (Biorad). Primers
used for AXIN2 were F: CTC CCC ACC TTG AAT GAA GA and R: TGG CTG GTG CAA AGA CAT AG. Primers for PORCN for qPCR were: F: GGA GGC TCG GGT TGC CAT CCT and R: GCC CCT CGC ATC TTG TGG TCA. Primers for ACTB were: F: AAG GAT TCC TAT GTG GGC GAC G and R: GCC TGG ATA GCA ACG TAC ATG G. Primers for GUSB were F: TTT CTC ACA AAG GTC ACA GG, R: CGT CCC ACC TAG AAT CTG CT. Primers for PLA2G4A were F: TTC TTT GGC ACT CTA GCG TCT, R: GAA TTC TCC GGA GCT GAA AA. Primers for PORCN reverse transcription in exons 6-7 and 10 were: F: CTC CTT CGC AAC AAG AAA CG, R: AGT GGC TTG GAC ACC GTG CTG.

PORCN Null MEF Isolation and Assays

Mice with loxP sites surrounding exon3 of PORCN were acquired from the lab of Janet Rossant. MEFs were isolated at day 14.5 from a homozygous and hemizygous cross. Briefly, fetal heads and internal organs were removed and remaining tissue was minced, then trypsinized to yield a single-cell suspension. These cells were grown in DMEM and spontaneously immortalized by serial passaging and growth to high density with several changes of media. After immortalization, PORCN was deleted by adenoviral Cre-GFP (Vector Biolabs, Philadelphia, PA, USA) expression and validated by genomic and RT-PCR. Adenoviral GFP expression was used as control. To perform SuperTOPflash assays in PORCN null MEFs, 2 x 10^6 cells per data point were transfected by nucleofection using VPD-1004 MEF 1 nucleofector kit with an Amaxa nucleofector I (Lonza).

RESULTS

Creation of PORCN Null Cells

In order to perform rigorous characterization of PORCN function and test the hypothesis that all FDH mutants affect the Wnt signaling pathway, we chose to create PORCN null HT1080 fibrosarcoma cancer cells using zinc finger nuclease technology. HT1080 cells were selected because they are derived from mesoderm, the germ layer that is most affected in FDH, male and hence hemizygous for PORCN, readily transfected, and are highly responsive to Wnt ligands. While they normally express PORCN, their growth rate is unaffected by inhibition of PORCN by RNAi and small molecule inhibitors of PORCN (36). Despite some reports indicating that HT1080 cells have an autocrine Wnt signaling loop (39,40), we were unable to detect any endogenous SuperTOPflash (β-catenin activated TCF/LEF transcriptional reporter) (STF) activity without the co-expression of Wnt.

A zinc finger nuclease targeting exon 9 of PORCN was generated to create double-strand breaks in and therefore inactivate the PORCN gene (Fig. 1A). Exon 9 is predicted to encode a membrane-spanning region. Its importance in PORCN function is indicated by the fact that two missense mutations, a single amino acid insertion, and three frameshift mutations as well as mutations in splice donor and acceptor sites occur in exon 9 in FDH patients (35). Additionally, as the catalytic active site of PORCN exists within exon 11, a frameshift in the coding sequence anywhere upstream is expected to disrupt enzymatic activity.

Single-cell dilution cloning allowed for the recovery of two separate ZFN-modified HT1080 sub-lines with defined 12 and 19 base pair deletions (hereafter referred to as Δ12 and Δ19), along with an isogenic unedited wild-type clone (Fig. 1B). The 12 base pair deletion of Δ12 extends into the intron upstream of exon 9, thus removing the AG splice acceptor, while the Δ19 deletion is fully exonic, causing a predicted frameshift. RT-PCR analysis and sequencing of the mRNA in this region reveals that the Δ12 clone transcript lacks exon 9 (and hence one predicted membrane-spanning domain), while the Δ19 clone produces two transcripts, one with an internal deletion and hence a frame shift, and one that skips exon 9 (Fig. 1C). Quantitative RT-PCR of PORCN mRNA in edited cells showed that the Δ12 clone has a slightly reduced transcript level, while Δ19 has only ~25% remaining, likely due to nonsense-mediated RNA decay (Fig. 1D). To ensure that the observed phenotypes were due to mutation of the PORCN gene and not a second site mutation, the two clones were tested side-by-side and found to behave identically in all subsequent assays. As anticipated, inactivation of PORCN in these cells had no effect on cell growth or viability (data not shown).

PORCN is Essential and Non-Redundant for Wnt Ligand Activity

Given that PORCN is a member of the MBOAT multigene family, one important question is whether there is redundancy of PORCN function. To test this, PORCN null or
wild-type cells were transfected with plasmid encoding WNT3A, and culture media and lysates were subsequently isolated to probe for Wnt secretion. As seen in Figure 2A, PORCN null cells were able to express WNT3A as well as their wild-type counterparts, but could not effectively secrete WNT3A into the media. Secretion of WNT3A was rescued by expression of mouse 3xHA epitope tagged PORCN-D, indicating the secretion defect was due to the mutation of the PORCN gene (Fig. 2D). Even more dramatically, PORCN null cells were entirely unable to increase signaling from the STF reporter in the presence of transected WNT3A expression plasmid, while wild-type cells responded with a > 200 fold increase in luciferase activity (Fig. 2B). While PORCN null cells could not produce active WNT3A, they remained fully competent to respond to exogenous Wnt from conditioned medium produced from PORCN positive cells (Fig. 2C).

MBOAT proteins have a putative catalytic histidine (H341) residue that in PORCN and other MBOAT proteins (ghrelin O-acyl transferase, GOAT) is required for activity. Neither Wnt secretion nor β-catenin signaling was rescued by PORCN with an active site (H341A) mutation, confirming both the importance of H341 to MBOAT enzyme activity and showing that the secretion and activity defect was due to a loss of enzyme activity. As has been reported previously (e.g. (6)), there was not a direct correlation between detectable Wnt secretion and signaling activity, as even 1 ng of PORCN-encoding plasmid (per well in a 24-well culture dish, typical of assays and results to follow) could robustly rescue STF activity, but was not sufficient to produce immunologically detectable WNT3A in the conditioned medium (Fig. 2D). This reinforces the conclusion that the detection of free Wnt in culture media is an insensitive measure of active Wnt. We conclude that mutation or skipping of PORCN exon 9 creates cells null for PORCN catalytic activity, and that active PORCN is absolutely essential for production of active Wnts in HT1080 cells.

**Stringent Control of Wnt Signaling by PORCN**

PORCN expression and splicing patterns vary widely between tissues, during development (26,29,30), and in some disease states(36,41). This suggests that PORCN expression regulates the strength of Wnt signaling independent of Wnt gene expression. To test this experimentally, a saturating amount of WNT3A plasmid was co-transfected with increasing amounts of PORCN plasmid into PORCN-null cells. Impressively, as little as one picogram of PORCN plasmid (mixed with carrier plasmid) was sufficient to restore detectable expression from the STF reporter. This single picogram of PORCN plasmid, transfected into a 24-well dish containing ~150,000 cells, is calculated to be about one expression plasmid per cell. The Wnt/β-catenin signal output varied linearly with PORCN expression over 3 orders of magnitude (Fig. 3A). This broad dynamic response to PORCN indicates that tissues could finely modulate Wnt signaling by varying PORCN activity. Analysis of the dynamic range of signal mediated by Wnt at a saturating concentration of PORCN revealed, as expected, that levels of the Wnt also produce a strong control over resulting signal. To test this further, we examined the relative contributions of PORCN and Wnt expression to overall signal output. Between 400 pg and 50 ng of WNT3A expression plasmid was transfected in different combinations with between 100 pg and 3 ng of PORCN plasmid. Figure 3B shows that each of these variables has a similar amount of control over signal output. This exquisite control of Wnt signaling is not simply a function of the synthetic β-catenin responsive STF reporter, as the natural target gene AXIN2 demonstrates a similar dose-response curve, albeit we are comparing mRNA in one case and a reporter enzyme in another (Fig. 3C). Thus, fine-tuning of both high and moderate response Wnt/β-catenin target genes can be similarly modulated by the abundance of both Wnt ligand and its activator, PORCN. This strongly suggests that the strength of Wnt signaling in development and disease will be critically dependent on both PORCN and Wnt protein abundance.

**Analysis of Conserved Amino Acids in PORCN and Influence on Activity**

Sequence conservation in PORCN suggests several amino acid residues that are likely to be crucial to its acyltransferase activity (24). The MBOAT family contains highly conserved residues including N306, S337 and H341 of PORCN-D. In the MBOAT protein ghrelin O-acyl transferase, which transfers octanoate onto the appetite-stimulating hormone ghrelin, the asparagine and histidine equivalent to PORCN N306 and H341 are absolutely required for enzyme activity (42). PORCN is predicted to palmitoylate Wnt either co-translationally or
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post-translationally, so that in either case the catalytic site is likely to be in the membrane or in the lumen of the ER. However, in most models of PORCN topology, N306 and S337 are predicted to be in the cytoplasm, while most FDH point missense mutants are predicted to be in the lumen of the ER (Fig. 4). Thus, it was important to test if N306 and S337 are indeed critical to PORCN function in the same manner that H341 demonstrably is. In our assay, unlike its role in GOAT, the N306A and S337A PORCN mutants were virtually identical to wild-type PORCN in their ability to restore Wnt/β-catenin activity to PORCN-null HT1080 cells (Fig. 5A). These mutants were fully active with several Wnts regardless if they were β-catenin activating or not (Fig. 5B, D). Confirming that their activity was not an artifact of the HT1080 cell line, these mutants also fully restored Wnt/β-catenin signaling activity in MEFs deleted for PORCN (Fig. 5C). This was not an artifact of mammalian PORCN, because independent mutation of the N306 and S337 sites in Xenopus PORCN had no discernible effect on activity. (Fig. 5E). Thus, these highly conserved, putative cytoplasmic loop residues may play an evolutionarily important role distinct from direct involvement in Wnt acyltransferase activity.

**PORCN Splice Variants Differ Globally in Signaling Activity**

PORCN exists as four different isoforms due to the alternative splicing of exons 7 and 8. This results in splice variants B, C, and D with the addition of 7, 5, or 12 amino acids with net positive charge not present in variant A (six of the 12 added residues are R or K). This charged patch is predicted to reside in the ER lumen (Fig. 4). Tissue-specific expression patterns of these isoforms have been reported (30). One possibility is that the isoforms allow selectivity for specific Wnts; alternatively, they could globally modulate activity, or they could confer sensitivity to different regulatory pathways. To differentiate between these options, we tested the ability of each splice variant to restore Wnt/β-catenin signaling activity in the PORCN-null HT1080 cells. There is no detectable signaling activity for any of these Wnts in the absence of PORCN (38). As Figure 5F shows, the different splice variants globally affect Wnt ligand activity and show more subtle differences between Wnts. For example, isoform B has a reduced capacity to signal relative to isoforms C and D for Wnts 1, 2, and 3. Most notably, isoform A (lacking both exons 7 and 8) is approximately half as active as the other splice variants. This suggests the basic region missing in isoform A globally upregulates PORCN enzymatic activity. As isoform A is the predominant variant expressed in kidney, spleen, uterus, testis, and corpus callosum(30), these tissues may down-regulate global Wnt signaling by alternative splicing of PORCN mRNA.

**Analysis of PORCN Mutations Associated with FDH**

Inherited and post-zygotic mutations in the PORCN gene cause the disorder Focal Dermal Hypoplasia (33,43). Since the initial association was discovered, over 100 mutations have been identified (34,35, https://grenada.lumc.nl/LOVD2/amc). It is presumed but not yet demonstrated that all of these mutations cause disease through a loss of PORCN enzymatic function and hence loss of Wnt signaling. Consistent with this, several cases of FDH are associated with either large deletions in the PORCN gene, or early nonsense mutations predicted to entirely eliminate PORCN expression. However, in just over 20 phenotypically similar patients, the disease is associated with small missense or in-frame deletions in PORCN coding sequence. Whether and how these mutations affect PORCN function is less clear. In order to address this experimentally, we re-created 18 of the disease-associated point mutations and small deletions within the coding sequence of a 3xHA-mPORC-D plasmid, highlighted in Figure 4. The point mutations associated with PORCN are largely predicted to exist in intramembrane space or aligned on the same predicted side of the ER membrane. As such, the FDH mutation-prevalent side of PORCN is predicted to be in the ER lumen ((44), Fig. 4).

Knowing that the PORCN expression level would have a significant effect on overall signal, we tested the ability of WNT3A to activate STF signaling in the absence or presence of 300 pg of PORCN plasmid, with the goal of measuring activity in the middle of dynamic range. Due to the limits of antibody sensitivity, we measured protein abundance of the mutants by transfecting 100 ng of plasmid encoding the PORCN mutants (Fig. 6A). We find that FDH mutants fall into three categories (Table 1). Class 1 mutants show low expression and low activity, indicating these mutations affect protein stability. Five of 18
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mutants tested fall into this category. Class 2 mutants (5/18) are well expressed but have low specific activity (<50% of wild-type). We presume these mutants affect enzyme function. Consistent with this, the FDH mutant H341Y, affecting the putative active site residue, is in this class. A third, unexpectedly common class of mutants (8/18) have both significant Wnt/β-catenin signaling activity (≥50% of wild-type) and detectable PORCN protein expression. Consistent with our work, the S136F mutant was recently reported to also have significant activity, using a different assay method (28). Remarkably, patients with these high-activity disease-associated mutations appear to be phenotypically indistinguishable from null mutations (44-47).

We tested if the class 3 mutants might be less active at very low but still physiologically relevant PORCN expression levels. As shown in Figure 6B, this is what we observed: reduced amounts of class 3 FDH mutant PORCN had increasingly reduced activity compared to the equivalent amount of wild-type PORCN plasmid. For instance, the mutant V258E had ~50% WT activity when 100 ng plasmid was transfected, but the activity quickly fell to <10% of WT when just 300 pg was transfected. PORCN with L331R, L347P, or A369V mutations surprisingly had near wild-type activity at high expression levels, but the relative activity is reduced when the more physiologically relevant 100 pg PORCN plasmid is used. Interestingly, for several of these disease mutations, the reductions in activity are similar to those seen when splice variant A is compared with splice variants B-D. These results were generalizable to other Wnts, as we found that an active (L331R) and inactive (V258E) FDH mutant behaved consistently with five additional Wnts (Fig. 6C). Finally, we found that the S136F and L331R mutants are also capable of activating β-catenin-independent signaling, while the H341Y mutant is not (Fig. 6D).

We wished to ascertain if our picogram-level transfections produced physiologically-relevant levels of PORCN mRNA. We recognize that this is an imperfect measure as the PORCN expression construct does not have native 5' and 3' UTRs and will vary in translation efficiency. By performing qRT-PCR to compare absolute PORCN mRNA abundance in a panel of human tissue samples, in HT1080 cells, and exogenously expressed in HT1080 cells by transient transfections, we calculate that 2.5 ng transfected PORCN expression plasmid results in approximately endogenous mRNA levels in HT1080 cells. (Fig. 7 and Supplemental Fig. 1). HT1080 cells have relatively high expression of PORCN mRNA compared to many of the tissues analyzed. Thus, transfection of lower quantities of PORCN, i.e. in the 100 pg range, is likely to reflect PORCN abundance in many physiological settings. Since several FDH mutants (S136F, L331R, L347P) have near 50% of wildtype activity when 100 pg expression vector is transfected, our data strongly suggest that the complete FDH phenotype can be caused by as little as a 50% decrease in PORCN activity.

DISCUSSION

In this study, we confirm that PORCN has the non-redundant function of regulating human Wnt activation and secretion. We show using both sensitive and endogenous reporters that Wnt signaling can be robustly and dynamically regulated by PORCN gene expression. We confirm that all known missense and in-frame deletions in the PORCN gene found in the disease FDH alter PORCN function in Wnt/β-catenin signaling. Strikingly, one common class of mutations causes only two-fold reductions in Wnt/β-catenin signaling, and then only at low but physiologically relevant levels of gene expression. Notably, these reductions are similar to those seen comparing normal PORCN splice variants. These findings indicate that subtle alterations in PORCN activity and hence Wnt activation are sufficient to dysregulate development in sensitive tissues, specifically in the mesodermal derived tissues that are most affected in FDH.

Here we have used zinc finger nuclease technology to create PORCN null cells. By targeting a double-strand break to the PORCN gene in this manner, we were able to rapidly create two independent clones of HT1080 cells with unique small deletions ultimately disrupting PORCN activity. This validated zinc finger nuclease is now available to create similar deletions in other human cell types. Our results, combined with those in a recent manuscript (38) show definitively and for all Wnts tested (both β-catenin stabilizing and “non-canonical”) that PORCN is absolutely required for their signaling activity. Previous studies have come to the same conclusions in a more limited fashion, using siRNA (48), small molecule inhibitors (31), gene trap and assay in embryonic stem cells (26), or loxP targeting and assay with ES cells or MEFs.
Some reports have indicated that Wnts can be secreted without PORCN activity. One recent study defines a novel β-catenin independent lengthening of cells that occurs even in the presence of PORCN inhibition, or when using an S224 mutant of WNT1 that cannot be palmitoylated, conditions under which Wnt secretion into culture media is observed (49). Yet another report indicated that WNT3A could be easily detected in media, while free extracellular WNT1 was undetectable, despite having similar or better signaling capacity (50). Instead, the authors observed WNT1 associated with an extracellular matrix. We find that there is a non-linear relationship between Wnt that is easily detected in culture media compared with an amount sufficient to activate STF signaling; even when Wnt is not detectable in media, there can be enough active Wnt to produce very robust β-catenin signaling activity. Clearly, there are experimental differences that affect Wnt secretion. One difference between our results—where no Wnt is observed in culture media in absence of PORCN—and those results indicating PORCN-independent Wnt secretion, may be the quantity of plasmids transfected. In our hands, 1-10 ng WNT3A plasmid saturates signaling to the STF reporter, while other studies often use 1 μg or more of Wnt expression plasmid, possibly allowing Wnt secretion through other lower affinity pathways. In addition, other cell types (e.g. neuronal-derived HEK293 cells) may release non-palmitoylated Wnts more readily. This relationship between Wnt palmitoylation, general secretion and ultimate signaling activity is complex and nuanced, and as such we would stress caution in over-reliance on Wnt secretion as an assay method. Most importantly, the activity that we measure in the form of STF activity, AXIN2 mRNA level, and Dvl2 phosphorylation absolutely depends on PORCN expression. This is entirely consistent with the recent crystal structure of XWnt8 in complex with Fz8, showing that the palmitoyl moiety forms a major contact between Wnt and its receptor (21).

We made the unexpected observation that the MBOAT highly conserved residues N306 and S337 are not required for PORCN activity on Wnts, and validated this result using both MEF cells with deletion of PORCN exon 3, and Xenopus PORCN. The N306 site in particular is completely conserved in PORCN going back to the primitive eumetazoan Trichoplax and its PORCN-like protein (51). That mutation of this residue does not affect Wnt/β-catenin signaling indicates additional complexities to PORCN structure and function that are not yet understood. A separate function of PORCN has been described that promotes cancer cell proliferation independently of acyltransferase activity, as a catalytic mutant PORCN (H341A) was able to rescue siRNA mediated cell growth inhibition in breast cancer cells (36). Additionally, PORCN is most closely related to acyltransferases that act on lysophospholipid species (52) and was originally suggested to not modify Wnt directly, but rather an associated sugar due to this homology (24). PORCN may retain acyltransferase activity towards lipids, or other proteins not detected in our current assays. Thus, identifying additional PORCN functions is an area of active investigation.

Our finding that PORCN can rescue Wnt signaling capacity at very low concentrations and then provide a linear relationship between expression level and STF signal capacity over at least three orders of magnitude provides several insights. First, PORCN represents a critical and non-redundant node for the control of Wnt signaling; second, due to this apparent importance, it is likely that there are control mechanisms in place to regulate PORCN activity, such as post-translational modification, subcellular localization and degradation pathways. This data also provides support to the notion that small-molecule targeting of PORCN would be an attractive means to disrupt all Wnt signaling in various Wnt-associated disease conditions. It will be interesting to learn, however, whether treatment of Wnt-high disorders with PORCN inhibitors could maintain a therapeutic window, considering the role Wnt/β-catenin signaling plays in adult stem cell homeostasis (53).

Our data show that FDH with a typical phenotype can be due to mutations that produce only a 50% decrease in PORCN activity. In the setting of development, where all 19 Wnts may be expressed, and multiple Wnts can be expressed from a single cell, this modest decrease in signal evidently leads to dramatic effects on a subset of tissues responding to complex morphogen gradients. The defects associated with FDH originate in this crucial developmental period, and our results indicate that mutations such as L331R, with a 50% decrease in activity, have equivalent phenotypic effects as more clearly deleterious mutations like H341Y (45,46). This is consistent with the
concept that 50% changes in morphogen gradients are biologically critical in the developing organism. However, the degree of severity associated with FDH is also a function of X chromosome inactivation, making such direct comparisons more complicated. We recently reported that PORCN has additional functions that require PORCN protein but not PORCN enzyme activity (36). Hence, some manifestations of FDH could result from Wnt-independent pathways. While the many early termination mutations associated with FDH may be compounded by this non-Wnt function for PORCN, it is less likely that the point mutants discussed here suffer these additional disruptions, as catalytically inactive PORCN maintained this moonlighting function. Still, it serves as a reminder that many proteins have more than one function. We are also aware that we have relied on WNT3A-initiated β-catenin signaling as our primary measure of FDH mutant PORCN activity; it remains possible that these mutations could have more significant effects when considering all of the potential Wnts and pathways both canonical and non-canonical.

In summary, we have used PORCN null cells in order to study the effects of PORCN mutation on Wnt signaling capacity. In so doing, we have uncovered exquisite control of Wnt signaling through this acyltransferase. Further, we have used these null cells to confirm that mutations associated with FDH have reduced activity compared to normal, consistent with the conclusion that FDH is a disease of Wnt malfunction. These null cells will be useful for further studies into mutational effects on activity, and will help increase understanding of PORCN catalytic activity.

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**FOOTNOTES**

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4The abbreviations used are: PORCN, porcupine; FDH, Focal Dermal Hypoplasia; ZFN, zinc finger nuclease; MBOAT, membrane bound O-acyl transferase; WLS, Wntless; GOAT, ghrelin O-acyl transferase

**FIGURE LEGENDS**

**Figure 1.** Zinc finger nuclease mediated deletion of PORCN from HT1080 cells. A. Graphic representation of ZFN targeting strategy. Capital letters indicate exonic coding sequence, lighter gray 5 base pair spacer is the site of initial cleavage. B. Sequence of genomic DNA at PORCN locus for two edited clones. Not shown is a third unedited clone. C. PORCN cDNA. Region around exon 9 was amplified, and bands were excised and sequenced. The Δ19 clone upper band matches deletion seen in B, while lower band sequence lacks exon 9. The Δ12 band also lacks exon 9. D. Quantitative PCR for PORCN. Results have been normalized to actin levels and error bars represent SD.

**Figure 2.** PORCN null cells cannot activate WNT3A. A. PORCN null cells do not secrete WNT3A. Cloned cells indicated were transfected with WNT3A, and culture media and lysates were prepared 24 hours later. Western blot was performed to detect WNT3A. Left: No PORCN. Right: 3xHA tagged mPORCN-D was co-transfected with Wnt, and rescues secretion. B. Cells were transfected with
SuperTOPFlash reporter in the absence or presence of WNT3A. mCherry transfection was used to ensure equivalent transfection. 24 hours after transfection, lysates were prepared and probed for firefly luciferase. C. Luciferase assay as in B. Δ12 cells shown. WNT3A-conditioned media (WCM) was added in right bar and is able to activate signaling. D. WT PORCN can rescue Wnt secretion and STF activity (number shown at bottom indicates fold signal above background), but there is a non-linear relationship between detectable secreted Wnt and STF activity. The catalytic active site mutant PORCN H341A (100 ng transfected) cannot rescue secretion or STF activity. Duplicates are shown.

Figure 3. PORCN governs a broad dynamic range of Wnt signaling. A. In a 24-well dish, Δ19 cells were transfected with WNT3A, SuperTOPFlash, mCherry transfection control, and amounts of 3xHA mPORCN-D plasmid as indicated. When titrating PORCN, 50 ng WNT3A was used; when titrating WNT3A, 3 ng PORCN was used. Luciferase assay was performed 24 hours after transfection. B. Same as A, except with variable Wnt expression. C. Δ19 Cells were treated as in A, without transfection of STF. Instead, the natural reporter gene AXIN2 was measured by qRT-PCR. 24 hours after transfection, RNA was isolated and probed for AXIN2, using actin (ACTB) as control. Error bars represent SD.

Figure 4. Schematic representation of PORCN structure and topology model. FDH mutations studied in this report are highlighted in yellow, red and green, while the highly conserved residues N306 and S337 are highlighted in white on black. Splice variants resulting from selective inclusion of exons 7 and 8 are indicated by lines between amino acids, and resulting different protein sequences shown below. Exon 9 is highlighted in light blue with the ZFN binding site indicated. Colored amino acid residues indicate mutations associated with FDH. Colors indicate classification: yellow – low protein expression; red – loss of activity and near-normal expression; green – normal activity and expression at higher transcription levels. N-terminus is at the left side, and the predicted ER luminal side is below. Only missense mutations are indicated here. We have borrowed heavily from a previous publication in constructing this model(44).

Figure 5. Highly conserved residues N306 and S337 of PORCN are not required for Wnt activation, and PORCN splice variants exert subtle control of activation. A. Wnt/β-catenin reporter (STF) assay assessing PORCN mutants N306A and S337A relative to WT. Δ19 cells were transfected with the following plasmids: 50 ng WNT3A expression, 100 ng mCherry expression, 550 ng SuperTOPFlash reporter, and wildtype or mutant 3xHA-mPORCN-D expression plasmids as indicated. B. STF assay of N306A and WT PORCN plasmids (300 pg transfected) comparing multiple Wnts as indicated. C. PORCN null mouse embryonic fibroblasts were used to confirm results for N306A mutant PORCN. MEFs were nucleofected with plasmids as indicated in Materials and Methods. 24 hours after transfection, luciferase activity was measured. This assay was performed with multiple replicates twice with similar results. D. Conserved PORCN residues are not required for non-canonical Wnt signaling to Dvl2. The indicated PORCN expression constructs were co-expressed in PORCN-null HT1080 cells with WNT4, and Dvl2 mobility shift was subsequently assessed (Materials and Methods).E. Conserved residues in Xenopus PORCN equivalent to N306 and S337 are not required for Wnt/β-catenin signaling. STF assay was performed in Δ19 cells transfected with 50 ng WNT3A, 100 ng mCherry, 650 ng STF reporter, and the indicated 500 pg Xenopus PORCN-A expression constructs. F. STF assay was performed after transfection of 50 ng of the indicated Wnt, in combination with 300 pg each of 3xHA-mPORCN splice variants A-D as indicated. Data is presented as % maximal signal obtained for each Wnt (the PORCN isoform producing maximal signal varies). Fold activity is dependent on Wnt transfected. Maximal signals: WNT1, 122x; WNT2, 24x; WNT3, 302x; WNT3A, 147x; WNT6, 21x; WNT7A, 3x; WNT7B, 4x; WNT9A, 5x; WNT9B, 10x; WNT10B, 52x. Expression of isoform mutants from a separate experiment with 100 ng transfection per well is shown below (duplicates).

Figure 6. Focal Dermal Hypoplasia associated PORCN mutations disrupt activity. A. SuperTOPFlash assay of ability of different PORCN mutants to rescue signaling mediated by WNT3A. Top: all wells receive 50 ng WNT3A, 100 ng mCherry, 300 pg of 3xHA-mPORCN-D variant indicated, and 650 ng SuperTOPFlash reporter. Bottom, for analysis of expression levels, all PORCN variants were
transfected in a separate experiment at 100 ng. B. For select FDH mutant PORCN variants, TOPflash assay was performed while titrating down the total amount of PORCN from 100 ng to 100 pg. Results are displayed as percent of WT signaling activity at the corresponding equivalent transfection quantity. Δ12 cells were used in this experiment. C. STF assay was performed in Δ19 cells transfected with 50 ng of Wnt indicated, along with 100 ng mCherry, 650 ng STF reporter, and 300 pg 3xHA-mPORCN-D variant indicated. Results are displayed as % of signal obtained from indicated Wnt when co-transfected with WT PORCN. D. Dvl2 mobility shift induced by WNT4.

**Figure 7.** *PORCN* expression levels vary across human tissues, and HT1080 cells have high expression. A human tissue mRNA panel was used to assess relative levels of *PORCN* message in HT1080 cells related to these various tissues. PORCN mRNA expression is plotted normalized to input RNA quantity (open bars) or normalized to a combination of *GUSB* and *PLA2G4A* abundance (grey bars).
Table 1

| Class 1: Low expression (presumed protein stability mutant) | Class 2: Expression but low activity (mutations primarily affect MBOAT activity) | Class 3: near-normal specific activity (citation) |
|-----------------------------------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------|
| G60R                                                      | V258E                                                                           | S136F (44)                                       |
| G168R                                                     | S297L                                                                           | L331R (46)                                       |
| Δ349-355                                                  | H341Y                                                                           | L347P (45)                                       |
| Δ355-360                                                  | W439X                                                                           | E361V (47)                                       |
| R365Q                                                     | W448X                                                                           | A369V (45)                                       |
|                                                           |                                                                                  | A374P (45)                                       |
|                                                           |                                                                                  | C385R (44)                                       |
|                                                           |                                                                                  | W439R (44)                                       |
Figure 1

A.

Exon 9 (Active Site in Exon 11)

B.

Δ19

Δ12

C.

cDNA

D.

Relative PORCN mRNA

WT Δ19 Δ12
Figure 2

A. 

|         | EV         | mPORC-D |         |
|---------|------------|---------|---------|
| Wnt3a- Media | [Image]    | [Image] |         |
| Wnt3a- Lysate | [Image]    | [Image] |         |

B. 

|         | WT | Δ12 | Δ19 |
|---------|----|-----|-----|
| Relative β-catenin activity | 1000 | 100 | 10 |

C. 

|         | TOPFlash | TOPFlash + Wnt3a | TOPFlash + WCM |
|---------|----------|-----------------|----------------|
| Relative β-catenin activity | 10 | 1 | 5 |

D. 

|         | Wnt3a- Media | Wnt3a- Lysate | HA-PORCN |
|---------|---------------|---------------|----------|
| Wnt3a  | 0.5 μg PORCN | 1 μg PORCN | 1 μg PORCN |
|         | None | None | None |
| Rel. Luc | 1 | 1 | 481 |

|         | 1 μg | 100 ng | H341A |
|---------|------|--------|-------|
| Rel. Luc | 929 | 1 |

Porcupine Tightly Regulates Wnt Signaling
Figure 3

A. 

![Graph showing Relative Fold Signaling vs. Log plasmid (g) for PORCN and Wnt3a.]

B. 

![Graph showing Relative Fold Signaling vs. picograms PORCN plasmid for different concentrations of Wnt3a: 50 ng, 10 ng, 2 ng, and 0.4 ng.]

C. 

![Bar chart showing Relative Axin2 mRNA vs. picograms PORCN plasmid for different concentrations.]

Porcupine Tightly Regulates Wnt Signaling
Porcupine Tightly Regulates Wnt Signaling

Figure 5

A. Relative TOPFlash

![Graph showing Relative TOPFlash](image)

B. Fold TOPFlash

![Graph showing Fold TOPFlash](image)

C. Relative TOPFlash

![Graph showing Relative TOPFlash](image)

D. Fold TOPFlash

![Graph showing Fold TOPFlash](image)

E. Relative TOPFlash

![Graph showing Relative TOPFlash](image)

F. % of Maximal Signal

![Graph showing % of Maximal Signal](image)
Figure 6

A. % of WT Activity

B. % of WT Activity

C. % of WT Activity

D. WNT4 +

Porcupine Tightly Regulates Wnt Signaling
Figure 7

Porcupine Tightly Regulates Wnt Signaling

- **Tissue Type**: HT1080 Cells, bone marrow, brain, cerebellum, colon, fetal brain, fetal liver, heart, liver, lung, prostate, salivary glands, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, trachea, uterus.

- **Relative Transcript**: 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256.

- **Transfected**: 10 ng, 1 ng, 0.1 ng.

- **Graph Comparison**: PORCN vs. PORCN:(GUSB,PLA2G4A)
