Running Title: Secretion-independent Wnt signaling

Research article

WNT4 and WNT3A activate cell autonomous Wnt signaling independent of secretion

Deviyani M. Rao, Rebecca L. Ferguson, Matthew J. Sikora

Affiliation: Dept. of Pathology, University of Colorado Anschutz Medical Campus

Corresponding author: Matthew J. Sikora, PhD.; Mail Stop 8104, Research Complex 1 South, Room 5117, 12801 E. 17th Ave.; Aurora, CO 80045. Tel: (303)724-4301; Fax: (303)724-3712; email: matthew.sikora@ucdenver.edu. Twitter: @mjsikora

Funding

This work was supported by R00 CA193734 (MJS) from the National Institutes of Health, and by a grant from the Cancer League of Colorado, Inc (MJS).

Authors' contributions

DMR and MJS conceived of the project and experiments. DMR, RLF, and MJS designed and performed experiments. RLF and DMR developed models for the project. DMR wrote the draft manuscript; all authors read and revised the manuscript, and have read and approved of this version of the manuscript.

Competing interests

The authors have nothing to disclose.

Abstract

Background: Wnt protein secretion and paracrine signaling typically require the enzyme PORCN, suggesting PORCN inhibitors would block Wnt signaling and effectively treat Wnt-dependent forms of cancer. We previously demonstrated that invasive lobular carcinoma (ILC) cell lines depend on WNT4 for proliferation and survival, and hypothesized PORCN inhibition would impede Wnt signaling in ILC. However, PORCN inhibition did not phenocopy WNT4 siRNA knockdown, leading to further investigation of PORCN-independent Wnt signaling.

Methods: ILC cell line MDA MB 134VI (MM134), fibrosarcoma cell line HT1080, and PORCN-knockout HT1080 (HT1080-PKO) were used as Wnt-dependent and Wnt-responsive model systems, with constitutive WNT3A or WNT4 expression. Conditioned medium was used to assess Wnt secretion and paracrine activity, and dependence on the Wnt secretion proteins PORCN and WLS. Secreted Wnt protein activity was compared to recombinant Wnt protein.

Results: The role of PORCN in Wnt secretion and signaling varies in a context-specific manner. PORCN was required for WNT3A secretion and paracrine signaling from HT1080, whereas WNT3A secretion, but not paracrine activity, was PORCN-independent in MM134. WNT4 secretion was PORCN-independent but WLS-dependent in both models. However, secreted WNT4 did not have paracrine activity in any context tested, whereas recombinant WNT4 was able to signal in a paracrine manner. Absent paracrine activity for WNT4, we examined Wnt signaling directly in cells expressing Wnt proteins and identified cell autonomous activation of Wnt signaling, independent of secretion, with both WNT4 and WNT3A. Transfection of Wnt protein activated non-canonical Wnt signaling independent of membrane receptor activation.

Conclusions: PORCN is differentially required for secretion and paracrine activity of WNT4 and WNT3A, specific to the cell type expressing Wnt proteins and the paracrine recipient of Wnt signals. Wnt proteins can activate cell autonomous signaling, independent of PORCN and of secretion. This PORCN-independent mode of Wnt signaling may be critical in cell contexts that are otherwise considered to have dysfunctional Wnt signaling.

Introduction

Wnt signaling is an ancestrally conserved pathway that plays fundamental roles in numerous facets of embryonic development and adult tissue homeostasis. Dysregulation of Wnt signaling is a causative factor for a range of human pathologies, including several forms of cancer (reviewed in [1]). As a result, inhibition of Wnt signaling has become an attractive therapeutic target with ongoing clinical trials, with some strategies focused on inhibiting the action of Wnt proteins [1–3]. Wnt proteins comprise a family of secreted glycoproteins that act as inter-cellular ligands, which stimulate a myriad of signal transduction cascades regulating cellular proliferation, stem cell renewal, cell motility, angiogenesis, and apoptosis [1, 4–6]. Wnt proteins are post-translationally modified by the O-acyltransferase Porcupine (PORCN), which palmitoylates Wnt proteins at conserved serine residues [2, 7, 8]. This lipidation forms a binding motif for interaction with Wntless (WLS), which chaperones Wnt proteins to the plasma membrane for secretion [8–10]. Once secreted, Wnt proteins signal in a paracrine manner, binding nearby receptor complexes. Wnts typically bind a Frizzled (FZD) receptor in conjunction with the LRP5 or LRP6 co-receptor, resulting in second messenger activation (Dishevelled proteins; DVL1/2/3 in humans) and initiation of either canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) signaling [1, 4]. The essential initiating step in Wnt processing is palmitoylation by PORCN, which has prompted development of PORCN inhibitors, including the IWP class of compounds [10], WNT974 (a.k.a. LGK974) [3], and others [2, 11]. These compounds have been shown to block Wnt secretion, inhibit downstream Wnt signaling, and suppress Wnt-driven tumor growth in animal models [3, 12, 13]; WNT974 is also currently in Phase I/II clinical trials for cancer treatment (NCT01351103, NCT02278133). Based on these observations, PORCN inhibitors are an attractive strategy to target Wnt-driven pathologies.

The Wnt protein WNT4 is critical in organogenesis of endocrine organs, regulation of bone mass, and in several steroid hormone-related phenotypes in humans [14–21]. WNT4 dysregulation, including loss-of-function mutations, results in developmental female to male sex reversal and related phenotypes [22–25].

WNT4 polymorphisms have been associated with endocrine dysfunction, reduced bone density with premature skeletal aging, and related phenotypes [26–31]. WNT4 is also critical in mammary gland development, as *Wnt4* knockout in mice prevents progesterone-driven ductal elongation and branching of the mammary gland during pregnancy [32, 33]. In this context, activated progesterone receptor drives expression of *Wnt4* in mammary gland luminal cells resulting in paracrine signaling that supports maintenance of the mammary stem cell niche [6, 34–36].

We recently reported that regulation of *WNT4* expression is co-opted by the estrogen receptor in a subtype of breast cancer, invasive lobular carcinoma (ILC) [37, 38]. Estrogen-driven WNT4 is required in ILC cells for estrogen-induced growth, as well as anti-estrogen resistance [38]. Despite the observed critical roles of WNT4 in both normal and malignant tissues, WNT4 signaling is crudely understood. Both human and murine WNT4 are reported to activate either canonical or non-canonical Wnt signaling in a context and tissue-specific manner (described in above references). Additionally, conflicting reports indicate Wnt4 can or cannot activate canonical Wnt signaling in murine mammary gland [34, 39]. Further, ILC cells lack the capacity to engage canonical Wnt signaling, as β-catenin protein is lost due to the characteristic loss of E-cadherin in ILC [38, 40]. It is also unclear which FZD receptor complexes are utilized by WNT4, although WNT4 is often required for distinct, non-redundant functions, leading to the description of WNT4 as a “problem child” among Wnt proteins (reviewed in [33]). Based on these observations, inhibition of WNT4 signaling upstream of Wnt effector pathways, such as with PORCN inhibitors, is an attractive approach to block WNT4 signaling in a “pathway indifferent” manner.

Since WNT4 is critical for the proliferation and survival of ILC cells, we expected a similar requirement for PORCN. However, treatment of cells with PORCN inhibitors did not suppress growth or survival. These unexpected results initiated further studies into the mechanisms of WNT4 secretion and signaling. In this report, we show WNT4 secretion is mediated by atypical mechanisms. However, our observations

call into question whether secretion is required for Wnt signaling and suggest a novel mechanism by which Wnt proteins, including WNT4, can initiate non-canonical Wnt signaling.

Methods and Materials

Cell culture

HT1080 and PORCN-knockout HT1080 (HT1080-PKO; clone delta-19) were a generous gift from Dr. David Virshup [41], and were maintained in DMEM/F12 (Corning, 10092CV) + 10% FBS (Nucleus Biologics, #FBS1824). HT1080 and HT1080-PKO Wnt over-expression lines were generated by lentiviral transduction of Wnt expression plasmids (see below) and selection of antibiotic-resistant pools, and were maintained in 2.5 μg/mL blasticidin. MDA MB 134VI (MM134; ATCC) and SUM44PE (Asterand) were maintained as described [37]. MM134 overexpression lines were generated by lentiviral transduction of Wnt expression plasmids and selection of antibiotic-resistant pools, and were maintained in 2.5 μg/mL blasticidin. MC3T3-E1 (ATCC) were maintained in MEM Alpha without ascorbic acid (Gibco #A10490-01) + 10% FBS. All lines were incubated at 37°C in 5% CO_2. Cell lines are authenticated annually by the University of Arizona Genetics Core cell line authentication service, and confirmed to be mycoplasma negative every four months. Authenticated cells were in continuous culture <6 months.

Reagents and plasmids

LGK974 was obtained from Cayman Chemical (#14072) and was dissolved in DMSO. WntC59 was obtained from Tocris Biosciences (#5148) and was dissolved in DMSO. Fulvestrant was obtained from Tocris Biosciences (#1047) and was dissolved in EtOH. Recombinant human WNT3A (#5036-WN-010) and WNT4 (#6076-WN-005) were obtained from R&D Systems, and reconstituted per the manufacturer's instructions.

Wnt plasmids used in this publication were a gift from Drs. Marian Waterman, David Virshup and Xi He (Addgene kit #1000000022) [8]. Lentiviral vectors for WNT3A and WNT4 were generated by Gateway Recombination of pENTR-STOP Wnt open reading frames to pLX304, a kind gift from Dr. Bob Sclafani.

Transient transfection assays

HT1080 cells were transfected with Active WNT3A-V5 (G-8) and Active WNT4-V5 (G-10) from Addgene kit #1000000022. The transfection used Lipofectamine LTX Reagent with PLUS Reagent (ThermoFisher, Cat# 15338100) using the manufacturer's instructions.

Protein extraction from conditioned medium

HT1080 or MM134 cells were plated in full medium (as above, 10% FBS). 24hrs later, medium was changed to reduced FBS medium: DMEM/F12 + 2% FBS for HT1080 cells and DMEM/L15 + 5% FBS for MM134 cells. Decreased FBS was required to reduce competition of serum proteins with secreted Wnt proteins during extraction. Conditioned medium was harvested 4-5 days later for HT1080 cells and 6-7 days later for MM134 cells, typically once medium acidification was apparent by phenol red color change. Medium was centrifuged at 300xg for 4min to pellet any cells or debris. The supernatant was then syringe-filtered using a 0.2µm filter. The protein concentration of the conditioned medium was measured using a Pierce BCA Protein Assay Kit (#23225), and medium volumes normalized to total protein were adjusted with sterile-filtered PBS. Strataclean resin (Agilent, #400714) was added to the conditioned media at a ratio of 10µL of resin to 100µg of protein, and vortexed to re-suspend the resin. The medium+resin mixture was incubated, rotating at 4C for 30min, then centrifuged at 425xg for 1min at 4C. The supernatant was then aspirated and the resin was washed using sterile-filtered PBS. The resin was centrifuged again at 425xg for 1min at 4C, and the supernatant was aspirated. An equal volume of 2X Laemmli sample buffer (BioRad, #1610737) was added to the resin to release bound protein, and the slurry was heated at 95C for 5min. Slurry equivalent to 100ug of conditioned medium protein was run on SDS-PAGE gels to detect secreted WNT3A or WNT4 via immunoblotting (below).

Alkaline phosphatase assay

MC3T3-E1 cells were seeded into a 96-well plate at 10,000 cells/well. 24hrs later the cells were treated with either conditioned media (CM) or recombinant WNTs (rWNTs). 72hrs later, alkaline phosphatase activity was assessed, using *para*-Nitrophenyl phosphate (pNp) as a substrate and measuring absorbance at 405nm. Assay protocol is based on Nakamura et al [42] with buffer modifications provided by the recombinant Wnt manufacturer (R&D Systems). A more detailed protocol is available upon request. *para*-Nitrophenyl Phosphate tablets were obtained from Cayman Chemical (cat# 400090).

Proliferation and viability assays

Cell were seeded in a 96-well plate and 24hrs later cells were treated with 100nM Sytox green (Thermo Fisher Scientific, cat# S7020). The plate was then placed into an Incucyte Zoom for 4-5 days where they were imaged every 4hrs at 10x magnification. Cell confluence and cell death (Sytox green-positive counts) were assessed using Incucyte S3 software (v2018A). After time-course completion, total double-stranded DNA was measured by hypotonic lysis of cells in ultra-pure H_2O, with addition of Hoechst 33258 (Thermo Fisher Scientific, #62249) at 1µg/mL in Tris-NaCl buffer (10mM Tris, 2M NaCl; pH 7.4) at equivalent volume to lysate. Fluorescence (360nm ex / 460nm em) was measured on a Bio-Tek Synergy 2 microplate reader.

RNA interference

siRNAs were reverse transfected using RNAiMAX (ThermoFisher) according to the manufacturer's instructions. All constructs are siGENOME SMARTpool siRNAs (Dharmacon): Non-targeting pool #2 (D-001206-14-05), Human *WNT4* (M-008659-03-0005), Human *PORCN* (M-009613-00) and Human *WLS* (M-018728-01-0005).

Gene expression analyses

RNA extractions were performed using the RNeasy Mini kit (Qiagen); mRNA was converted to cDNA on an Eppendorf Mastercycler Pro and using Promega reagents: Oligo $(dT)_{15}$ primer (cat# C110A), Random

Primers (cat# C118A), GoScript 5x Reaction Buffer (cat# A500D), 25mM MgCl2 (cat# A351H), 10mM dNTPs (cat# U1511), RNasin Plus RNase Inhibitor (cat# N261B) and GoScript Reverse Transcriptase (cat# A501D). qPCR reactions were performed with PowerUp SYBR Green Master Mix (Life Technologies, cat # 100029284) on a QuantStudio 6 Flex Real-Time PCR system. Expression data were normalized to *RPLP0*. See below for a list of primer sequences:

qPCR Primers	**Forward**	**Reverse**
RPLP0	CAGCATCTACAACCCTGAAG	GACAGACACTGGCAACATT
WNT4	GCCATTGAGGAGTGCCAGTA	CCACACCTGCCGAAGAGATG
WNT3A	ATGGTGTCTCGGGAGTT	TGGCACTTGCACTTGAG
PORCN	ACCATCCTCATCTACCTACTC	CCTTCATGGCCACAATCA
AXIN2	CTCTGGAGCTGTTTCTTACTG	CTCTGGAGCTGTTTCTTACTG

Immunoblotting

Whole cell lysates were obtained by incubating cells in lysis buffer (1% Triton X-100, 50mM HEPES pH 7.4, 140mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% glycerol; supplemented with Roche protease/phosphatase inhibitor cocktail) for 30min on ice. Cells were centrifuged at 16000xg for 15min at 4C and the resulting supernatant was collected for analysis. Protein concentration was measured using the Pierce BCA Protein Assay Kit (#23225). Standard methods were used to perform SDS-PAGE. Proteins were transferred onto PVDF membranes. Antibodies were used according to manufacturer's recommendations: WNT4 (R&D, MAB4751, clone# 55025), WNT3A (R&D, MAB13242, clone# 217804.2R), DVL2 (Cell Signaling, 3216), DVL3 (Cell Signaling, 3218), pLRP6 (s1490, Cell Signaling, 2568), LRP6 (Cell Signaling, 2560, clone# C5C7), Vinculin (Cell Signaling, cat# 13901S) and V5 (Novus, NB100-62264, clone #SV5-PK1). Secondary antibodies were used according to manufacturer's instruction and were obtained from Jackson ImmunoResearch Laboratories: Goat Anti-Mouse IgG (cat # 115-035-068), Goat Anti-Rabbit IgG (cat# 111-035-045) and Goat Anti-Rat IgG (cat# 112-035-062).

Chemiluminescence was used to detect antibodies and either film or the Licor c-Digit was used to develop the immunoblots. Of note, WNT4 MAB4751 detects a prominent non-specific band at ~50kD in immunoblots from cell lysates. This non-specific target largely precludes detection of WNT4, but cutting immunoblot membranes immediately below a 50kD ladder marker prevents this issue. This non-specific band was not detected in WNT4 immunoblots from conditioned medium. Similarly, in immunoblots of conditioned medium WNT3A MAB13242 detects a prominent non-specific band at ~60kD that precludes detection of secreted WNT3A; cutting membranes above a 50kD ladder marker prevents this issue. This non-specific band was not detected in WNT3A immunoblots from cell lysates.

Protein Transfection

HT1080 wild type cells were seeded into a 24-well plate at 100,000 cell/well. 24hrs later, protein transfection was performed using the Pierce Protein Transfection Reagent (ThemoFisher Cat# 89850). The FITC antibody control was provided with the transfection reagent. All protein transfections were carried out per the manufacturer's instructions, with protein concentrations of 4ug/ml (1ug/24-well). Cells were harvested for whole cell lysate as above 4hr after transfection.

Results

PORCN inhibition does not mimic WNT4 siRNA in lobular carcinoma cells

We hypothesized that since ILC cells are dependent on WNT4 for proliferation and survival [38], inhibition of PORCN would phenocopy WNT4 siRNA by blocking WNT4 secretion and downstream signaling. Proliferation and cell death were monitored by live cell imaging of MM134 (ILC) cells either transfected with siRNA targeting *PORCN* (siPORCN) or treated with PORCN inhibitor (PORCNi) LGK974. Proliferation was compared to untreated cells, and cells treated with the anti-estrogen fulvestrant (Fulv) or transfected with siRNA targeting *WNT4* (siWNT4), both of which strongly suppress growth. Cell death was identified by SyTOX green incorporation. Proliferation results were confirmed at experimental endpoint by dsDNA quantification. As we previously reported, siRNA-mediated *WNT4* knockdown or Fulv halt proliferation, and WNT4 knockdown induces cell death (**Figure 1**). However, neither genetic nor chemical PORCN inhibition had any effect on cell proliferation or survival of MM134 cells (**Figure 1A-C**). Similar results were obtained in ILC cell line SUM44PE (**Supplemental Figure 1**). These data suggest PORCN inhibition is not sufficient to inhibit WNT4 function, and WNT4 signaling likely occurs via PORCN-independent mechanisms.

WNT4 secretion is PORCN-independent but WLS-dependent

Further investigation into mechanisms of WNT4 secretion utilized the HT1080 fibrosarcoma cell line, a well-characterized model for Wnt secretion, signaling, and activity; HT1080 have also been shown to increase DVL activation upon WNT4 over-expression [8]. The role of PORCN in WNT4 secretion was first assessed via transient over-expression of either WNT4 or WNT3A. HT1080 cells were reverse transfected with siPORCN, alone or in combination treatment with one of two PORCNi, WntC59 or LGK974, followed by transfection with V5-tagged WNT4 or WNT3A [8]. Conditioned media (CM) was collected 24hr later, and secreted Wnts were detected by immunoblotting for the V5 tag. As expected, WNT3A secretion was wholly PORCN-dependent in the HT1080 cell line, as either siPORCN or PORCNi blocked secretion (**Figure 2A**). In contrast, siPORCN and PORCNi alone or in conjunction

could not completely suppress WNT4 secretion (**Figure 2A**). To confirm these findings, we assessed WNT4 secretion in both wild-type and PORCN-knockout (HT1080-PKO, clone delta-19, [41]) HT1080 cells following stable over-expression of WNT4 or WNT3A (non-tagged) (**Figure 2B, Table 1**). As observed with transient over-expression, WNT3A secretion is PORCN-dependent and can be detected in conditioned medium from HT1080:W3 but not HT1080-PKO:W3 cells (**Figure 2C**). The same results were obtained when HT1080:W3 cells were treated with LGK974 (**Supplemental Figure 2**). In contrast, WNT4 secretion was detected from both HT1080:W4 and HT1080-PKO:W4. Endogenously expressed WNT4 was also detected in conditioned medium from HT1080 and HT1080-PKO cell lines (**Figure 2C**). Of note, WNT4 secretion from HT1080 did not increase with WNT4 over-expression, despite the increased WNT4 protein present in cell lysate (**Figure 2B**), suggesting WNT4 secretion is an active process that is saturated in HT1080 cells. We observed that secreted WNT4 can be resolved by electrophoresis as a doublet. The larger species was PORCN-dependent and not detected in HT1080-PKO, whereas all endogenous WNT4 shifted to the larger species in HT1080:W3 (**Figure 2C**), potentially due to positive feedback activation of Wnt secretory activity [43]. We observed loss of both species in conditioned media after *WNT4* knockdown by siRNA, confirming both secreted species as WNT4 (**Supplemental Figure 2**). These data indicate that while WNT4 is modified by PORCN, PORCN is not required for WNT4 secretion.

PORCN-mediated palmitoylation of Wnt proteins is commonly described as being required for WLS binding and transport to the cell surface for secretion (see Introduction). However, given reports that WLS may be sufficient for secretion of some Wnt proteins (see Discussion), we examined the requisite of WLS for WNT4 secretion. Knockdown of WLS by siRNA suppressed secretion of both WNT3A and WNT4 from HT1080 (**Figure 2D**), demonstrating that while secretion of WNT3A is dependent on both PORCN and WLS, WNT4 secretion is PORCN-independent but WLS-dependent.

To examine Wnt secretion in the context of ILC, WNT3A and WNT4 over-expression models in MM134 cells were also generated (**Table 1**, **Figure 2E**). Consistent with the above, neither PORCNi nor siPORCN prevented WNT4 secretion, but WNT4 secretion was strongly suppressed by WLS knockdown (**Figure 2F**). We noted that either siPORCN or PORCNi consistently increased WNT4 secretion, suggesting PORCN may repress WNT4 secretion in ILC cells. Unexpectedly, WNT3A secretion was similarly PORCN-independent but WLS-dependent in MM134, as neither siPORCN nor PORCNi suppressed WNT3A secretion (**Figure 2F**). While these data strongly indicate WNT4 secretion is PORCN-independent, they also suggest that Wnt protein processing and signaling may be more broadly atypical in ILC. However, it is unclear if Wnt proteins secreted independent of PORCN are competent to activate paracrine Wnt signaling.

Secreted WNT4 does not activate Wnt signaling pathways

Palmitoylation of Wnt proteins is widely described as necessary for WNTs to activate downstream signaling pathways. Given our observation of PORCN-independent WNT4 secretion, we assessed if WNT4 secreted from either PORCN-active or PORCN-null cells was able to activate paracrine Wnt signaling. HT1080-PKO cells ("receiver" cells) were cultured for 24hrs with conditioned medium from HT1080 WT or PKO, with or without Wnt over-expression. Phosphorylation of DVL2, DVL3, and LRP6, and expression of β-catenin target gene *AXIN2*, in the receiver cells were assessed as markers of activated Wnt signaling. Conditioned medium from HT1080:W3 activated Wnt signaling in the receiver cells by all measures (**Figure 3A-B**). As expected, Wnt signaling activity was not observed using conditioned medium from HT1080-PKO:W3, consistent with a lack of WNT3A secretion in PORCN-null cells (**Figure 2C**). Neither HT1080:W4 nor HT1080-PKO:W4 conditioned media, however, activated Wnt signaling in the receiver cells (**Figure 3A-B**) despite our ability to detect secreted WNT4 from both contexts. Similarly, conditioned medium from MM134:W4 did not initiate Wnt signaling in receiver cells (**Figure 3C-D**). Conditioned medium from MM134:W3 also failed to activate Wnt signaling in receiver

cells, despite the presence of WNT3A in conditioned medium from these cells (**Figure 2F**), indicating that Wnt secretion is not sufficient for paracrine signaling in this cell line.

Since secreted WNT4 in conditioned media was unable to initiate paracrine Wnt signaling, we tested recombinant Wnt proteins WNT3A and WNT4 (rWNT3A and rWNT4) with HT1080-PKO receiver cells. HT1080-PKO cells were treated with 10-500ng/mL rWNT proteins. rWNT3A activated Wnt signaling by all measures, while rWNT4 failed to activate Wnt signaling at any concentration (**Figure 3E-F**). These data suggest that our above observations regarding secreted WNT4 are due to HT1080 being non-responsive to paracrine WNT4. We confirmed the functionality of rWNTs by measuring canonical Wnt signaling activity in 3T3-E1 murine osteoblast cells via induction of alkaline phosphatase (AP) activity (see Materials and Methods). As expected, increasing concentrations of either rWNT3A or rWNT4 increased AP activity (**Figure 4A**) and both rWNT3A and rWNT4 induced DVL and LRP6 phosphorylation in 3T3-E1 cells (**Figure 4B**). Since 3T3-E1 are responsive to paracrine WNT4, we used these cells to assay to conditioned medium from WNT3A and WNT4 over-expressing cells as above. Conditioned medium from HT1080:W3, but not HT1080-PKO:W3, induced AP activity (**Figure 4C**), consistent with a requirement of PORCN for paracrine WNT3A signaling. However, though rWNT4 induced AP activity in 3T3-E1, conditioned medium from neither HT1080:W4 nor HT1080-PKO:W4 induced AP activity (**Figure 4C**). Parallel results were obtained in MM134, as conditioned medium from MM134:W3 induced AP activity in 3T3-E, which could be blocked by PORCNi treatment, and no AP activity was induced with MM134:W4 conditioned medium (**Figure 4D**). The amount of secreted WNT3A and WNT4 was confirmed as being within range of rWNT concentrations that induce AP activity in this assay (**Figure 4E-G**). Notably, commercially available rWNT3A and rWNT4 are produced lacking the N-terminal signal peptide, and both rWNT3A and rWNT4 migrated as a smaller peptide than the corresponding Wnt protein secreted from either HT1080 or MM134 (**Figure 4E-F**). Taken together, secreted Wnt proteins are not equivalent to recombinant proteins, and have distinct

capacities for activating paracrine Wnt signaling. However, while secreted WNT3A activated paracrine Wnt signaling in a context-dependent manner (based on both source and receiver cells), secreted WNT4 was unable to act as a functional paracrine signaling ligand in any tested context.

Secreted or paracrine WNT4 are not required for ILC cell proliferation and viability

Since secreted WNT4 did not activate paracrine Wnt signaling, we hypothesized that WNT4 secretion may not be required for WNT4 function in ILC cells, and examined if blocking WNT4 secretion by WLS knockdown would phenocopy WNT4 knockdown in MM134 cells. MM134 were transfected with siRNAs targeting WNT4, PORCN, or WLS, followed by cell proliferation and death assessment as above. Despite loss of WNT4 secretion, siWLS had no effect on MM134 proliferation or viability (**Supplemental Figure 3A-B**). Similarly, we attempted rescue of WNT4 knockdown with conditioned medium from MM134 (with or without Wnt over-expression) or rWNT protein. MM134 cells were transfected with siWNT4 and 24 hours later treated with conditioned media from MM134 cells or rWNT3A or rWNT4. No exogenous Wnt protein had any effect on the growth inhibition or cell death induced by siWNT4 (**Supplemental Figure 3C-D**). Together with the lack of induction of Wnt signaling by secreted WNT4, these data suggest secretion may ultimately not have a functional role in the activation of Wnt signaling in a WNT4-expressing cell in some contexts.

WNT4 and WNT3A activate cell-autonomous signaling

Our observations suggest WNT4 may activate signaling by a cell-autonomous, rather than paracrine or autocrine, mechanism. We examined Wnt signaling activity (DVL and LRP6 phosphorylation, *AXIN2* expression) directly in HT1080 and HT1080-PKO over-expressing WNT3A or WNT4 (**Figure 5A-B**). As observed in conditioned medium experiments above, both HT1080:W3 and HT1080:W4 had an induction of DVL and LRP6 phosphorylation (**Figure 5A**), but only HT1080:W3 induced *AXIN2* expression

(**Figure 5B**). In HT1080-PKO, over-expression of either WNT3A or WNT4 induced cell-autonomous DVL2 and DVL3 phosphorylation, despite the absence of functional PORCN and the lack of paracrine activity of Wnt proteins in this context (**Figure 3A**). However, consistent with loss of PORCN activity and secretion, Wnt over-expression did not activate LRP6 in HT1080-PKO or induce *AXIN2* expression. WNT3A or WNT4 over-expression in MM134 was not associated with DVL2/3 activation, suggesting cell autonomous Wnt signaling may activate cell-type specific pathways. Also, despite the ability of the MM134 Wnt overexpressing lines to secrete WNT3A and WNT4 **(Figure 2F)**, no phosphorylation of LRP6 was observed, further supporting that secreted Wnt proteins lack the ability to induce paracrine signaling in MM134 (**Supplemental Figure 4**).

As WNT4 is required for bone regeneration and cell proliferation [21], we examined if WNT4 is similarly essential for proliferation and/or viability of HT1080 and HT1080-PKO. We hypothesized that WNT4 might be dispensable in HT1080, due to redundant Wnt signaling. Without functional PORCN driving secretion and paracrine signaling of Wnt family members, HT1080-PKO might become more reliant on cell autonomous PORCN-independent WNT4 signaling. Knockdown of WNT4 partially suppressed proliferation and induced ~21% cell death at 48h post-knockdown in HT1080 (**Figure 5C-D**). In contrast, WNT4 knockdown in HT1080-PKO completely suppressed growth, and cell death was accelerated (~70% cell death at 48h post-knockdown). Cell death induced by WLS knockdown was not equivalent to WNT4 knockdown in either HT1080 or HT1080-PKO, consistent with secretion-independent functions of WNT4. These data support that HT1080-PKO cells are reliant on cell autonomous WNT4 signaling in the absence of paracrine signaling by other family Wnt proteins.

To determine whether cell autonomous Wnt signaling could be activated directly by intra-cellular Wnt protein, we transfected recombinant Wnt protein directly in to HT1080-PKO cells and examined

activation of Wnt signaling as above, 4 hours post-treatment. Of note, these experiments used higher Wnt protein concentrations (~4000ng/mL) than assessed in above paracrine signaling studies (**Figure 3**); rWnt protein is also not identical to Wnt proteins produced endogenously (**Figure 3E-F**). Compared to control transfection with FITC-labeled antibody, transfection of WNT3A or WNT4 activated DVL2/3 phosphorylation in HT1080-PKO (**Figure 5E**). Though paracrine treatment with this high concentration of Wnt protein was sufficient to activate DVL2/3 with either WNT3A or WNT4, LRP6 was only phosphorylated with paracrine WNT3A. This indicates that transfected WNT3A protein activates DVL2/3 independent of extracellular activity, as observed in HT1080-PKO:W3 cells (**Figure 5A-B**).

Discussion

The Wnt modifying enzyme PORCN is considered a gatekeeper for the secretion of Wnt proteins, and thus PORCN inhibition is an approach to broadly block Wnt signaling without targeting cell-type or tissue-specific downstream Wnt pathways. However, while invasive lobular carcinoma (ILC) cells are WNT4-dependent for growth and survival, they are insensitive to PORCN inhibition. PORCN was not required for WNT4 secretion from two distinct cell line models, as secreted WNT4 was detected from cells treated with small molecule PORCN inhibitors and/or *PORCN* siRNA, as well as from PORCN-null HT1080 cells. Secreted WNT4, from cells with or without functional PORCN, was not capable of activating Wnt signaling by paracrine mechanisms in any model tested, despite the ability of recombinant human WNT4 to induce alkaline phosphatase activity and DVL phosphorylation in a context-dependent manner. Similarly, in MM134 ILC cells, secretion of WNT3A was independent of PORCN, but paracrine activation of alkaline phosphatase production in 3T3-E1 cells required functional PORCN. Recombinant Wnt proteins and secreted Wnt proteins were not equivalent on immunoblot, consistent with these distinct activities. Although WNT4 secretion was blocked by siRNA targeting *WLS*, WLS knockdown did not affect ILC cell proliferation or survival. Both WNT3A and WNT4 activate cell autonomous, intra-cellular

signaling independent of secretion. This unique mode of Wnt signaling is likely essential for the survival and proliferation of WNT4-dependent cells.

Extensive data in the literature describe PORCN as an essential component of Wnt signaling (discussed above). Indeed, in our studies, loss of PORCN function ablated all measures of paracrine WNT3A activity, including Wnt receptor activation, second messenger activation, and induction of *AXIN2* expression. However, in support of our observations of PORCN-independent Wnt secretion and signaling, previous studies have reported PORCN-independent Wnt signaling in other systems. Nusse and colleagues first reported PORCN-independent secretion and activity in studies of *Drosophila* WntD [44]. WntD is secreted at high levels in fly tissues and cell culture models, independent of both Porcupine (PORCN homolog) and Wntless (WLS homolog), and ablation of either Porcupine or Wntless did not affect WntD signaling in fly tissues. WntD utilizes the early secretory pathway protein Rab1 GTPase (RAB1A homolog) for secretion, which represented a distinct and novel secretion mechanism versus Porcupine-mediated secretion of Wingless (WNT1 homolog). PORCN-independent Wnt secretion and signaling was also observed in a study of human primary cells by Richards et al [45]. Neither PORCN-inhibitor IWP-2 nor *PORCN* siRNA knockdown suppressed secretion of any endogenously expressed Wnt proteins from CD8+ T-cells (Wnts 1, 3, 5B, 10B) or astrocytes (Wnts 1, 3, 6, 7A, 10A, 16). Wnt proteins secreted from IWP-2-treated cells were functional in conditioned medium experiments, but PORCN-independent secretory mechanisms were not characterized. Further studies have shown that PORCN may differentially regulate the activity of individual Wnt proteins. In HEK293T cells, over-expression of PORCN and WLS together enhanced secretion of WNT1 (compared with WLS alone) but suppressed WNT1-induced paracrine or autocrine activation of β-catenin, versus WLS over-expression alone [46]. This was not observed with WNT3A, indicating PORCN specifically suppressed canonical signaling activity of WNT1, despite driving WNT1 secretion. Wnt-specific Porcupine functions have also been reported in Zebrafish [47]. Knockdown of *porcn* in Zebrafish embryos resulted in defects related to loss of non-canonical signaling and suppressed secretion of Wnt5a. Conversely, canonical Wnt signaling

was not altered by *porcn* knockdown, and secretion of Wnt3a was not impaired. These reports, together with our results on WNT3A and WNT4, indicate the roles of PORCN and WLS in Wnt modification, secretion, and signaling are context-dependent across individual Wnt proteins in a cell-type specific manner.

This observed context-dependence includes not only the specific "receiver" cells in question (likely best understood in the context of differentially expressed FZD receptors), but also includes the source of the Wnt protein. For example, WNT3A secreted from HT1080 robustly activated Wnt signaling in 3T3-E1 or HT1080-PKO cells, and PORCN was required for both secretion and paracrine activity. In contrast, WNT3A secreted from MM134 cells activated Wnt signaling in 3T3-E1 but not HT1080-PKO cells, and PORCN was required for paracrine activity but not secretion. Furthermore, we observed that recombinant Wnt proteins were more promiscuously able to activate signaling than secreted Wnt proteins. For example, whereas rWNT4 activated 3T3-E1 cells, WNT4 secreted from neither HT1080 nor MM134 activated 3T3-E1 Wnt signaling. This highlights that care needs to be taken when using recombinant Wnt proteins, as some rWnt proteins may represent a specific Wnt species that may or may not be active in the context of interest. Similarly, rWnt proteins may have distinct functional capacity not reflected by their endogenous counterpart Wnt protein in a given context. Additionally, studies with rWNT proteins do not capture potential contributions of autocrine or cell autonomous Wnt signaling.

Our observations indicate WNT4 and WNT3A likely signal via at least three distinct mechanisms: 1) as a secreted protein with PORCN modification; 2) as a secreted protein without PORCN modification; 3) by a cell autonomous mechanism independent of secretion. This may offer explanation for the myriad of context-dependent signaling pathways activated by WNT4, including activating canonical β-catenin activity [14, 34, 48], repressing β-catenin-driven transcription [49, 50], or activating non-canonical Wnt signaling pathways [21, 51]. For example, PORCN-driven palmitoylation may mediate the ability of WNT4 to act via canonical vs non-canonical Wnt signaling, and/or via cell autonomous mechanisms.

Additionally, the differences we observed between recombinant and secreted WNT4 indicate differential protein processing may guide Wnt proteins to activate distinct signaling pathways. The commercially available recombinant Wnt proteins used in our study lack the N-terminal signal peptide (residues 1-22 for WNT4), however, Wnt signal peptides may have important roles in regulation of signaling activity [52]. The WNT4 signal peptide has uniquely high percentages of arginine (14%) and serine (18%) compared to other human Wnts (average 5% and 9%, respectively), whereas WNT3A has no charged or polar residues in its signal peptide. Mutation in the signal peptide (L12P) of WNT4 has also been linked to Mayer-Rokitansky-Küster-Hauser syndrome [23]. The L12P mutant functions as a dominant negative inhibitor and suppresses the activity of wild-type WNT4 when co-expressed. Although the L12P mutant protein is not palmitoylated, it is secreted and does not prevent secretion of wild-type protein. These observations are consistent with our findings of PORCN-independent WNT4 secretion. The mechanism of dominant-negative activity has not been described but suggests distinct forms of WNT4 may drive cell autonomous Wnt signaling. Identifying the sequence and protein modifications of potential WNT4 species is an important future direction.

To our knowledge, this is the first report of PORCN-independent Wnt activity that is also independent of secretion. The studies discussed above showed Wnt proteins secreted in PORCN-independent manners were active in paracrine signaling models, although it was not reported whether Rab1-mediated secretion of WntD was ultimately required for signaling [44]. We observed that secreted WNT4 was unable to activate paracrine signaling in any model system tested, and that cell autonomous activation of DVL proteins could only be achieved by directly expressing or transfecting WNT3A or WNT4 protein in cells. Cell autonomous DVL phosphorylation appeared to be independent of membrane receptor activation, as increased phospho-LRP6 was not seen in WNT4 over-expressing cells or cells transfected with WNT4 protein. Similarly, either paracrine or cell autonomous WNT3A activated DVL2/3, but only paracrine WNT3A activated LRP6. For both WNT3A and WNT4, cell autonomous signaling is likely activating non-canonical signaling mechanisms exclusively, as induction of *AXIN2* expression was not associated

with DVL activation in these contexts. Further, it is unclear whether DVL are the primary executors of downstream signaling, as DVL2/3 were not activated in MM134:W3 or MM134:W4 cells, and activation of DVL2/3 was not specific to WNT4 transfection in HT1080-PKO. Defining the mechanisms by which Wnt proteins activate cell autonomous signaling is a critical part of ongoing studies.

ILC may represent a unique context for paracrine Wnt signaling, as we observed that both WNT3A and WNT4 could be secreted from MM134 cells in a PORCN-independent but WLS-dependent manner. This atypical Wnt processing (in particular for WNT3A) may be related to broader Wnt signaling dysfunction in ILC cells. The genetic hallmark of ILC is loss of E-cadherin (*CDH1*) [40], which leads to dysfunction of catenin proteins, including activation of p120 catenin [53], or in-activation of β-catenin. E-cadherin loss in ILC leads to a loss of β-catenin protein in both patient tumors and cell lines [38, 40], and as a result β-catenin-driven TOP-Flash reporter activity cannot be activated in ILC cells [38]. This catenin protein dysfunction was previously postulated as being linked to PORCNi sensitivity, and ILC patients were specifically included in a trial of WNT974 (NCT01351103). This trial opened in 2011, but by 2015 ILC patients were removed from the inclusion criteria. It is unclear whether this is due to accrual problems or a lack of efficacy, as neither have been specifically reported for ILC patients on this trial, although our data suggest PORCNi are unlikely to have clinical efficacy for ILC. This highlights the importance of defining the unique context for Wnt signaling in ILC, in particular for WNT4, based on our prior findings [38]. Our laboratory has begun to characterize WNT4-driven signaling in ILC cells, which may be mediated by PORCN-independent, cell autonomous WNT4 signaling.

Strengths of our study include the use of diverse cell line models to investigate Wnt secretion and paracrine activity of Wnt proteins, which allowed us to identify the context-dependent Wnt signaling described herein. We used primarily non-tagged Wnt expression constructs, which eliminated previously described complications with altered processing and reduced activity for tagged Wnt proteins. Wnt signaling activity was measured by diverse yet redundant pathway readouts that facilitated our study of

context-dependent Wnt signaling activities. Notably, the cell line models used for Wnt secretion were both cancer-derived, and thus it is unclear how our observations will translate to normal tissue, developmental, or *in vivo* contexts. We observed identical processing and secretion for endogenously expressed WNT4 as for over-expressed WNT4 (neither model expresses endogenous WNT3A), but over-expression was required to facilitate signaling experiments. As such, future studies will need to determine the contribution of endogenous Wnt protein levels to activating the signaling pathways discussed herein.

Conclusions

The secretion and paracrine activity of Wnt proteins is heavily context-dependent, as WNT3A and WNT4 present with differing dependence on PORCN for secretion and paracrine activity in distinct model systems. Secretion of WNT4 is PORCN-independent and WLS-dependent, but WNT4 did not present paracrine activity in cells otherwise dependent on WNT4, indicative of cell-autonomous, secretion-independent activity. Both WNT4 and WNT3A presented cell autonomous activity via non-canonical Wnt signaling, independent of secretion. These studies identify a PORCN-independent mode of Wnt signaling which may be of particular importance in contexts with otherwise presumed Wnt signaling dysfunction.

References

1. Nusse R, Clevers H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell. Elsevier Inc.; 2017 Jun 1;169(6):985–999. PMID: 28575679
2. Madan B, Ke Z, Harmston N, Ho SY, Frois AO, Alam J, et al. Wnt addiction of genetically defined cancers reversed by PORCN inhibition. Oncogene. 2016 Apr 10;35(17):2197–2207. PMID: 26257057
3. Liu J, Pan S, Hsieh MH, Ng N, Sun F, Wang T, et al. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc Natl Acad Sci U S A. 2013 Dec 10;110(50):20224–9. PMID: 24277854
4. van Amerongen R. Alternative Wnt Pathways and Receptors. Cold Spring Harb Perspect Biol. 2012 Oct 1;4(10):a007914–a007914. PMID: 22935904
5. Loh KM, van Amerongen R, Nusse R. Generating Cellular Diversity and Spatial Form: Wnt Signaling and the Evolution of Multicellular Animals. Dev Cell. 2016 Sep 26;38(6):643–655. PMID: 27676437
6. Brisken C, Hess K, Jeitziner R. Progesterone and Overlooked Endocrine Pathways in Breast Cancer Pathogenesis. Endocrinology. 2015 Oct;156(10):3442–50. PMID: 26241069
7. Miranda M, Galli LM, Enriquez M, Szabo LA, Gao X, Hannoush RN, et al. Identification of the WNT1 residues required for palmitoylation by Porcupine. FEBS Lett. 2014 Dec 20;588(24):4815–4824. PMID: 25451226
8. Najdi R, Proffitt K, Sprowl S, Kaur S, Yu J, Covey TM, et al. A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. Differentiation. Elsevier; 2012 Sep;84(2):203–13. PMID: 22784633
9. Bänziger C, Soldini D, Schütt C, Zipperlen P, Hausmann G, Basler K. Wntless, a Conserved Membrane Protein Dedicated to the Secretion of Wnt Proteins from Signaling Cells. Cell. 2006 May 5;125(3):509–522. PMID: 16678095
10. Coombs GS, Yu J, Canning CA, Veltri CA, Covey TM, Cheong JK, et al. WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. J Cell Sci. 2010 Oct 1;123(19):3357–3367. PMID: 20826466
11. Proffitt KD, Madan B, Ke Z, Pendharkar V, Ding L, Lee MA, et al. Pharmacological Inhibition of the Wnt Acyltransferase PORCN Prevents Growth of WNT-Driven Mammary Cancer. Cancer Res. 2013 Jan 15;73(2):502–507. PMID: 23188502
12. Hayashi M, Baker A, Goldstein SD, Albert CM, Jackson KW, McCarty G, et al. Inhibition of porcupine prolongs metastasis free survival in a mouse xenograft model of Ewing sarcoma. Oncotarget. 2017 Oct 3;8(45).
13. Agarwal P, Zhang B, Ho Y, Cook A, Li L, Mikhail FM, et al. Enhanced targeting of CML stem and progenitor cells by inhibition of porcupine acyltransferase in combination with TKI. Blood. 2017 Feb 23;129(8):1008–1020. PMID: 28011678
14. Li Q, Kannan A, Das A, Demayo FJ, Hornsby PJ, Young SL, et al. WNT4 acts downstream of BMP2 and functions via β-catenin signaling pathway to regulate human endometrial stromal cell differentiation. Endocrinology. 2013 Jan;154(1):446–57. PMID: 23142810
15. Itäranta P, Chi L, Seppänen T, Niku M, Tuukkanen J, Peltoketo H, et al. Wnt-4 signaling is involved in the control of smooth muscle cell fate via Bmp-4 in the medullary stroma of the developing kidney. Dev Biol. 2006 May 15;293(2):473–83. PMID: 16546160
16. Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, Kojima A, et al. R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. Hum Mol

Genet. 2008 May 1;17(9):1278–1291.

17. Chang J, Sonoyama W, Wang Z, Jin Q, Zhang C, Krebsbach PH, et al. Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. J Biol Chem. 2007 Oct 19;282(42):30938–48. PMID: 17720811

18. Prunskaite-Hyyryläinen R, Skovorodkin I, Xu Q, Miinalainen I, Shan J, Vainio SJ. Wnt4 coordinates directional cell migration and extension of the Müllerian duct essential for ontogenesis of the female reproductive tract. Hum Mol Genet. 2016 Mar 15;25(6):1059–73. PMID: 26721931

19. Strochlic L, Falk J, Goillot E, Sigoillot S, Bourgeois F, Delers P, et al. Wnt4 Participates in the Formation of Vertebrate Neuromuscular Junction. Klymkowsky M, editor. PLoS One. 2012 Jan 12;7(1):e29976.

20. Caprioli A, Villasenor A, Wylie LA, Braitsch C, Marty-Santos L, Barry D, et al. Wnt4 is essential to normal mammalian lung development. Dev Biol. 2015 Oct;406(2):222–234.

21. Yu B, Chang J, Liu Y, Li J, Kevork K, Al-Hezaimi K, et al. Wnt4 signaling prevents skeletal aging and inflammation by inhibiting nuclear factor-κB. Nat Med. 2014 Sep;20(9):1009–17. PMID: 25108526

22. Bernard P, Harley VR. Wnt4 action in gonadal development and sex determination. Int J Biochem Cell Biol. 2007 Jan;39(1):31–43. PMID: 16905353

23. Philibert P, Biason-Lauber A, Rouzier R, Pienkowski C, Paris F, Konrad D, et al. Identification and functional analysis of a new WNT4 gene mutation among 28 adolescent girls with primary amenorrhea and müllerian duct abnormalities: a French collaborative study. J Clin Endocrinol Metab. 2008 Mar;93(3):895–900. PMID: 18182450

24. Vainio S, Heikkilä M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. Nature. 1999 Feb 4;397(6718):405–9. PMID: 9989404

25. Biason-Lauber A, De Filippo G, Konrad D, Scarano G, Nazzaro A, Schoenle EJ. WNT4 deficiency—a clinical phenotype distinct from the classic Mayer–Rokitansky–Kuster–Hauser syndrome: A Case Report. Hum Reprod. 2007 Jan;22(1):224–229. PMID: 16959810

26. Powell JE, Fung JN, Shakhbazov K, Sapkota Y, Cloonan N, Hemani G, et al. Endometriosis risk alleles at 1p36.12 act through inverse regulation of CDC42 and LINC00339. Hum Mol Genet. 2016 Sep 15;25(22):5046–5058. PMID: 28171565

27. Rahmioglu N, Macgregor S, Drong AW, Hedman AK, Harris HR, Randall JC, et al. Genome-wide enrichment analysis between endometriosis and obesity-related traits reveals novel susceptibility loci. Hum Mol Genet. 2015 Feb 15;24(4):1185–1199. PMID: 25296917

28. Mafra F, Catto M, Bianco B, Barbosa CP, Christofolini D. Association of WNT4 polymorphisms with endometriosis in infertile patients. J Assist Reprod Genet. 2015 Sep 3;32(9):1359–1364. PMID: 26139156

29. Zhang G, Feenstra B, Bacelis J, Liu X, Muglia LM, Juodakis J, et al. Genetic Associations with Gestational Duration and Spontaneous Preterm Birth. N Engl J Med. 2017 Sep 21;377(12):1156–1167. PMID: 28877031

30. Zmuda JM, Yerges-Armstrong LM, Moffett SP, Klei L, Kammerer CM, Roeder K, et al. Genetic analysis of vertebral trabecular bone density and cross-sectional area in older men. Osteoporos Int. 2011 Apr 9;22(4):1079–1090. PMID: 21153022

31. Zheng Y, Wang C, Zhang H, Shao C, Gao L-H, Li S-S, et al. Polymorphisms in Wnt signaling pathway genes are associated with peak bone mineral density, lean mass, and fat mass in Chinese male nuclear families. Osteoporos Int. 2016 May 5;27(5):1805–1815. PMID: 26733379

32. Brisken C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, et al. Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev. 2000 Mar

15;14(6):650–4. PMID: 10733525

33. Alexander CM, Goel S, Fakhraldeen S a., Kim S. Wnt signaling in mammary glands: plastic cell fates and combinatorial signaling. Cold Spring Harb Perspect Biol. 2012 Oct 1;4(10). PMID: 22661590

34. Rajaram RD, Buric D, Caikovski M, Ayyanan A, Rougemont J, Shan J, et al. Progesterone and Wnt4 control mammary stem cells via myoepithelial crosstalk. EMBO J. 2015 Mar;34(5):641–652. PMID: 25603931

35. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, et al. Progesterone induces adult mammary stem cell expansion. Nature. 2010 Jun;465(7299):803–807. PMID: 20445538

36. Meier-Abt F, Milani E, Roloff T, Brinkhaus H, Duss S, Meyer DS, et al. Parity induces differentiation and reduces Wnt/Notch signaling ratio and proliferation potential of basal stem/progenitor cells isolated from mouse mammary epithelium. Breast Cancer Res. 2013 Apr 29;15(2):R36. PMID: 23621987

37. Sikora MJ, Cooper KL, Bahreini A, Luthra S, Wang G, Chandran UR, et al. Invasive lobular carcinoma cell lines are characterized by unique estrogen-mediated gene expression patterns and altered tamoxifen response. Cancer Res. 2014 Mar 1;74(5):1463–74. PMID: 24425047

38. Sikora MJ, Jacobsen BM, Levine K, Chen J, Davidson NE, Lee A V, et al. WNT4 mediates estrogen receptor signaling and endocrine resistance in invasive lobular carcinoma cell lines. Breast Cancer Res. Breast Cancer Research; 2016 Sep 20;18(1):92. PMID: 27650553

39. Kim YC, Clark RJ, Pelegri F, Alexander CM. Wnt4 is not sufficient to induce lobuloalveolar mammary development. BMC Dev Biol. 2009 Jan;9:55. PMID: 19878558

40. Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, et al. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. Cell. 2015 Oct 8;163(2):506–519. PMID: 26451490

41. Proffitt KD, Virshup DM. Precise Regulation of Porcupine Activity Is Required for Physiological Wnt Signaling. J Biol Chem. 2012 Oct 5;287(41):34167–34178. PMID: 22888000

42. Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F. p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. Exp Cell Res. 1999;250(2):351–363. PMID: 10413589

43. Glaeser K, Urban M, Fenech E, Voloshanenko O, Kranz D, Lari F, et al. ERAD-dependent control of the Wnt secretory factor Evi. EMBO J. 2018 Feb 15;37(4):e97311. PMID: 29378775

44. Ching W, Hang HC, Nusse R. Lipid-independent secretion of a Drosophila Wnt protein. J Biol Chem. 2008 Jun 20;283(25):17092–8. PMID: 18430724

45. Richards MH, Seaton MS, Wallace J, Al-Harthi L. Porcupine is not required for the production of the majority of Wnts from primary human astrocytes and CD8+ T cells. PLoS One. 2014;9(3):e92159. PMID: 24647048

46. Galli LM, Zebarjadi N, Li L, Lingappa VR, Burrus LW. Divergent effects of Porcupine and Wntless on WNT1 trafficking, secretion, and signaling. Exp Cell Res. Elsevier; 2016;347(1):171–83. PMID: 27492485

47. Chen Q, Takada R, Takada S. Loss of Porcupine impairs convergent extension during gastrulation in zebrafish. J Cell Sci. 2012 May 1;125(Pt 9):2224–34. PMID: 22357957

48. Lyons JP, Mueller UW, Ji H, Everett C, Fang X, Hsieh J-C, et al. Wnt-4 activates the canonical beta-catenin-mediated Wnt pathway and binds Frizzled-6 CRD: functional implications of Wnt/beta-catenin activity in kidney epithelial cells. Exp Cell Res. 2004 Aug 15;298(2):369–87.

PMID: 15265686

49. Jordan BK, Shen JH-C, Olaso R, Ingraham HA, Vilain E. Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. Proc Natl Acad Sci U S A. 2003 Sep 16;100(19):10866–71. PMID: 12949260

50. Bernard P, Fleming A, Lacombe A, Harley VR, Vilain E. Wnt4 inhibits beta-catenin/TCF signalling by redirecting beta-catenin to the cell membrane. Biol Cell. 2008 Mar;100(3):167–77. PMID: 17976036

51. Louis I, Heinonen KM, Chagraoui J, Vainio S, Sauvageau G, Perreault C. The signaling protein Wnt4 enhances thymopoiesis and expands multipotent hematopoietic progenitors through beta-catenin-independent signaling. Immunity. 2008 Jul 18;29(1):57–67. PMID: 18617424

52. Willert K, Nusse R. Wnt proteins. Cold Spring Harb Perspect Biol. 2012 Sep 1;4(9):a007864. PMID: 22952392

53. Schackmann RCJ, van Amersfoort M, Haarhuis JHI, Vlug EJ, Halim VA, Roodhart JML, et al. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. J Clin Invest. 2011 Aug;121(8):3176–3188. PMID: 21747168

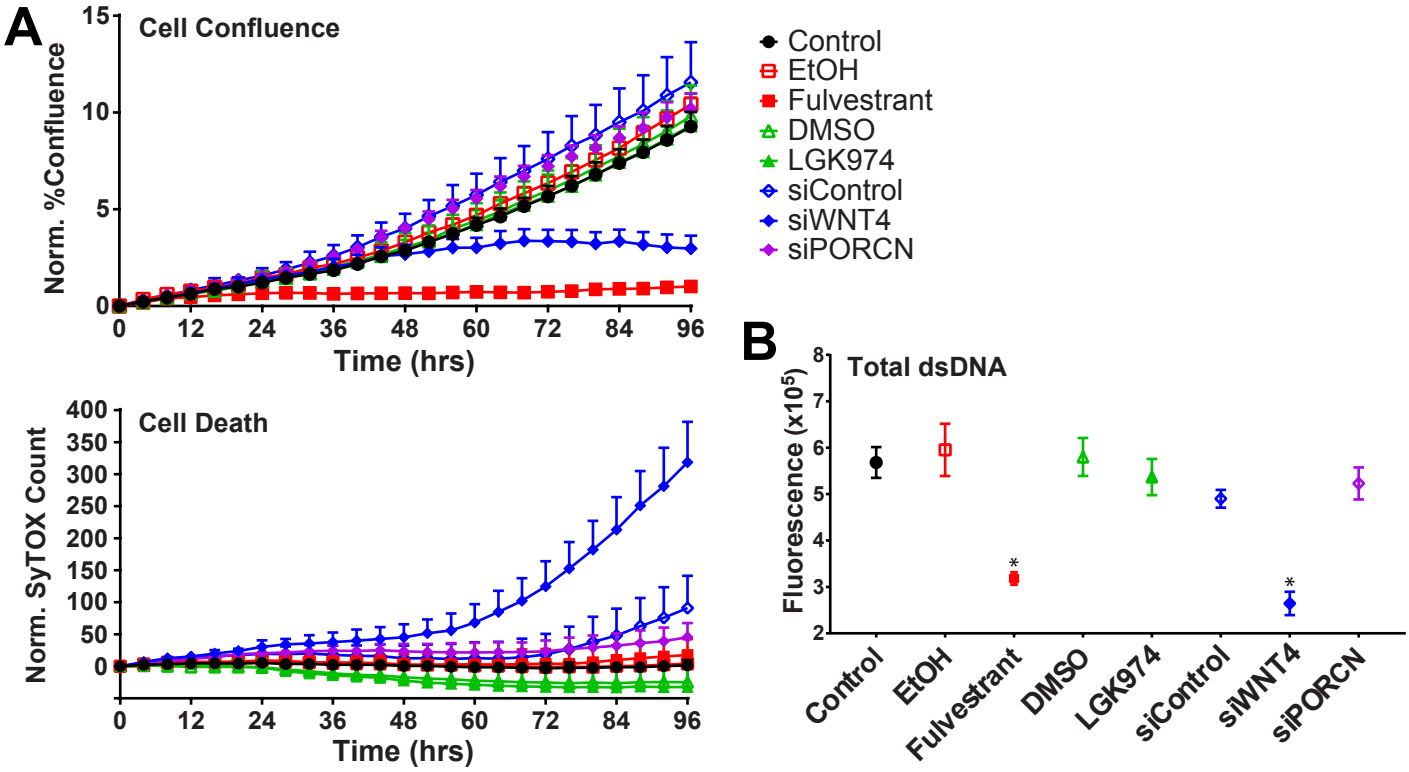


Figure 1. Inhibition or knockdown of PORCN does not phenocopy knockdown of WNT4 in MM134
(A), MM134 cells were transfected with siRNA or treated with fulvestrant (100nM), LGK974 (10nM), or 0.1% vehicle (EtOH or DMSO) at time 0, prior to live cell imaging for proliferation (phase-contrast confluence) and death (SyTOX green incorporation). Points represent mean of 6 biological replicates ±SD. **(B),** Total double-stranded DNA was measured from assays in (A) at timecourse completion. *, $p<0.05$, ANOVA with Dunnett's multiple correction. Results in A,B are representative of three independent experiments.

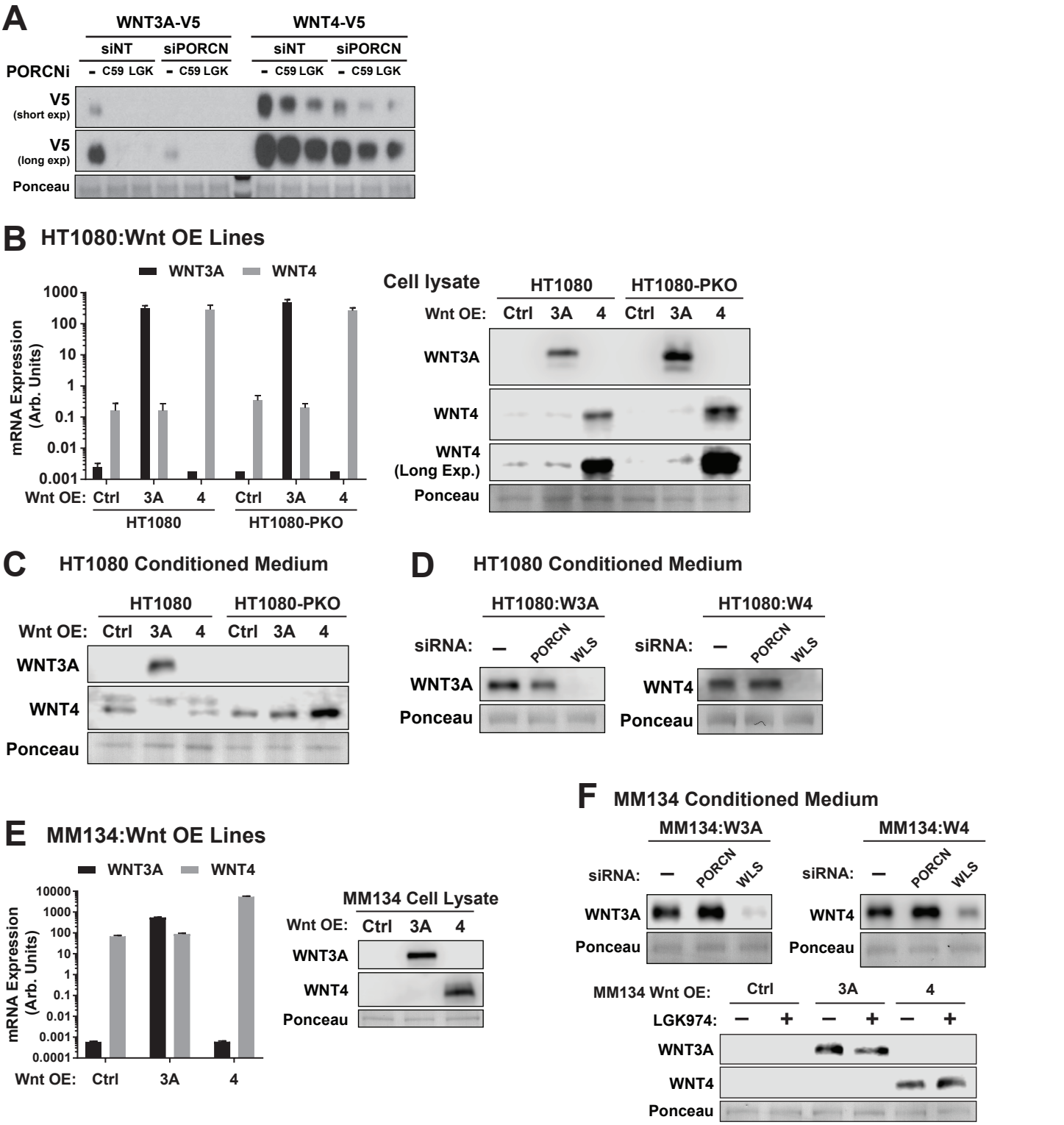


Figure 2. WNT4 secretion is PORCN-independent, but WLS-dependent.
(A), HT1080 cells were transfected sequentially with siRNA or V5-tagged WNTs as indicated and then treated with 1uM PORCNi (LGK974 or WNTC59). Total protein was extracted from conditioned medium after 24hrs for immunoblotting. **(B),** HT1080 constitutive Wnt over-expression cell lines were created as detailed in Methods and Materials. Left, qPCR for *WNT3A* and *WNT4*. Right, immunoblot for cellular expression of WNT3A and WNT4. Over-expression shows endogenous WNT4 expression. **(C),** HT1080 medium was conditioned for 5 days as described in Materials and Methods, prior to total protein extraction for immunoblot. **(D),** HT1080 cells were transfected with siPORCN or siWLS, and after 24hrs, medium was changed and conditioned for 5 days. Total protein was extracted from medium as above for immunoblot. **(E),** MM134 constitutive Wnt over-expression cell lines were created as detailed in Methods and Materials. Left, qPCR for *WNT3A* and *WNT4*. Right, immunoblot for cellular expression of WNT3A and WNT4. Endogenous WNT4 could not be visualized here due to the level over over-expression. **(F),** MM134 cells were transfected with siPORCN or siWLS, and after 24hrs, medium was changed and conditioned for 7 days. Total protein was extracted from medium as above for immunoblot.

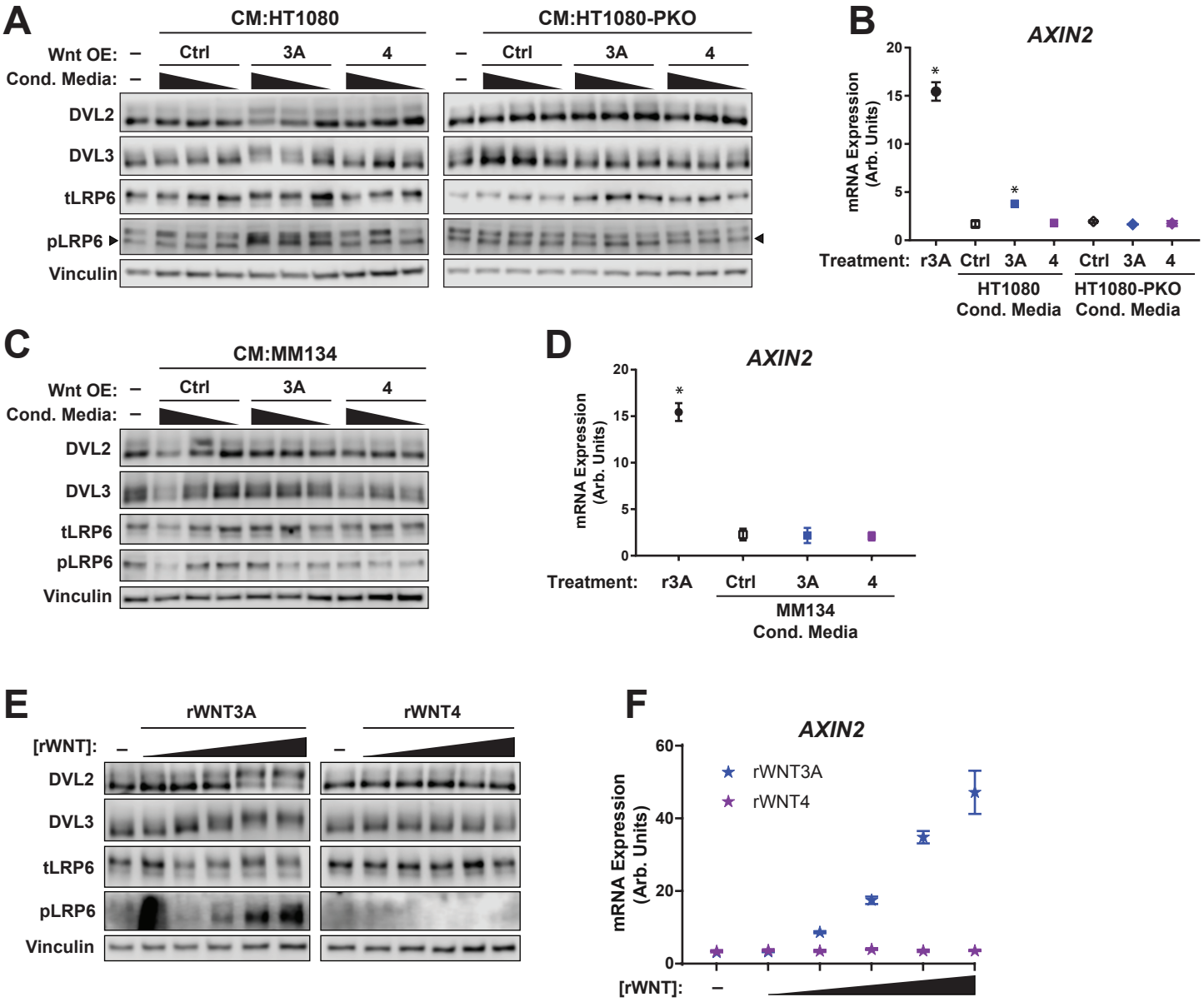
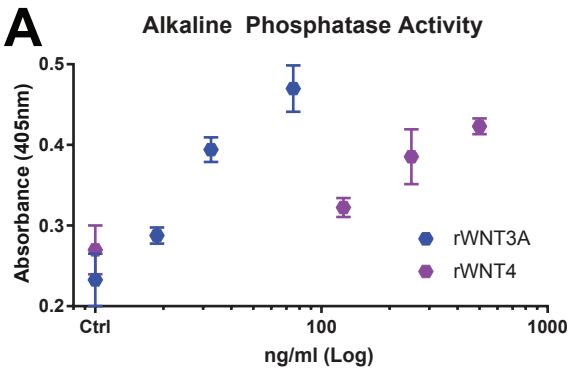
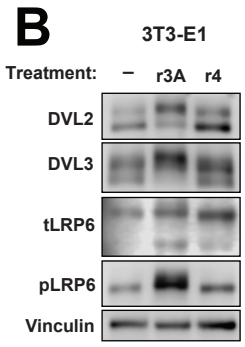
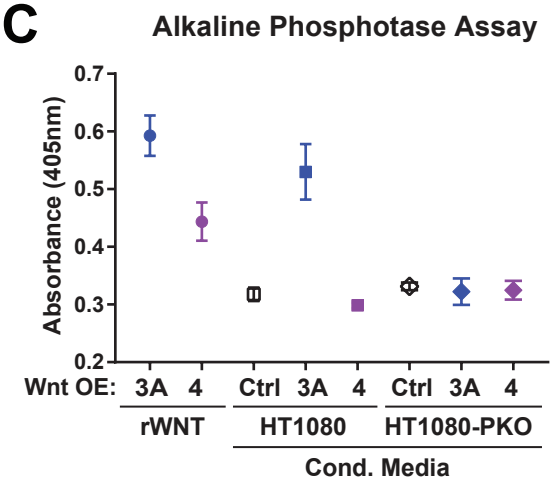
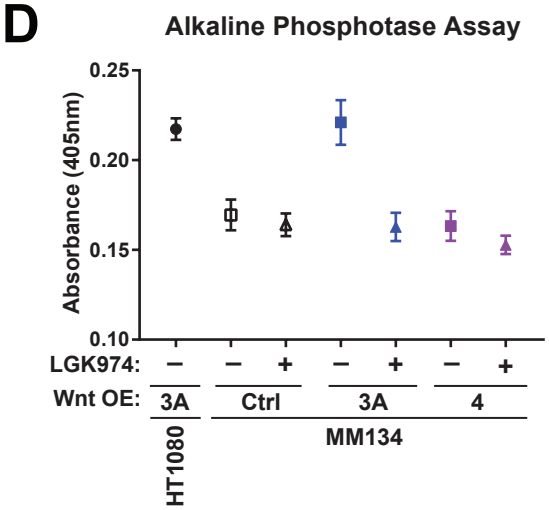


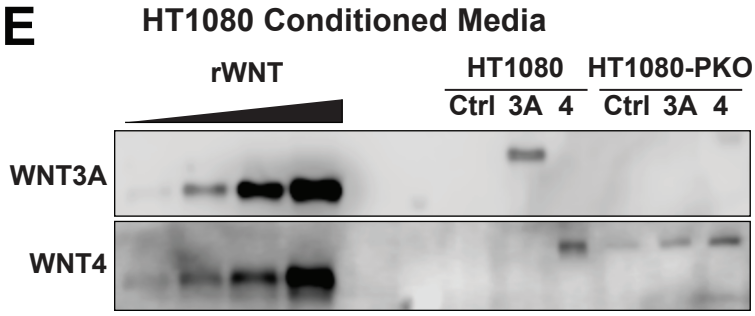
Figure 3. Secreted WNT4 does not activate Wnt signaling in a paracrine manner.
HT1080-PKO control cells were treated for 24 hours with either conditioned media (CM, at 50%, 25% or 12.5% final volume supplemented with fresh medium) from: **(A-B),** HT1080 cell lines; **(C-D),** MM134 cell lines; or **(E-F),** with recombinant WNT protein (at concentrations of 10ng/ml, 50ng/ml, 100ng/ml, 250ng/ml, or 500ng/ml). **(A,C,E),** Immunoblots of whole cell lysates from the treated HT1080-PKO cells were run and probed for DVL2, DVL3, total LRP6 and phosphorylated LRP6. **(B,D,F),** mRNA from the treated HT1080-PKO cells was extracted for qPCR for *AXIN2* mRNA expression levels, vs *RPLP0*. (B,D), cells treated with 62.5ng/mL rWNT3A as a positive control. *, $p<0.05$ vs Ctrl, ANOVA with Dunnett's multiple correction.

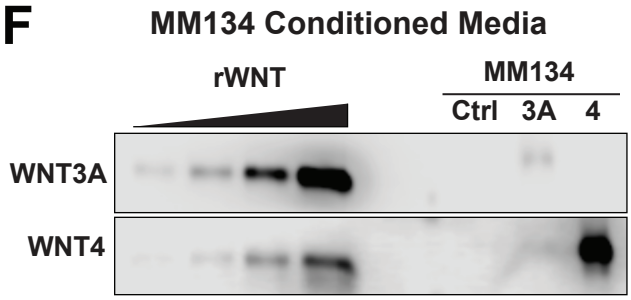












G

	Concentration (ng/ml)	
Cell Line	**sWNT3A**	**sWNT4**
HT1080 Control	-	-
HT1080 WNT3A	62	-
HT1080 WNT4	-	190
HT1080-PKO Control	-	42
HT1080-PKO WNT3A	-	55
HT1080-PKO WNT4	-	64
MM134	-	-
MM134 WNT3A	157	-
MM134 WNT4	-	1808

Figure 4. Secreted Wnt protein differentially induces alkaline phosphatase activity in 3T3-E1 versus recombinant Wnt protein. (A,C-D), MC3T3-E1 cells were plated into a 96-well plate and 24hrs later were treated with **(A),** rWNT3A (0, 18.75, 32.5, 75ng/ml) or rWNT4 (0, 125, 250, 500ng/ml), **(C),** rWNT3A (62.5ng/ml), rWNT4 (250ng/ml) or 50% conditioned media (CM) from HT1080 cell lines, or **(D),** 50% CM from HT1080 or MM134 cell lines that were treated with or without 10nM LGK974. **(B),** 3T3-E1 cells were treated for 24hrs with 300ng/ml of either rWNT3A or rWNT4. Immunoblots of whole cell lysates were probed for DVL2, DVL3, total LRP6 and phosphorylated LRP6. **(E-F),** Serial dilutions of rWNTs (2.5, 5, 10, 20ng) were run on an immunoblot alongside CM collected from HT1080 or MM134 cell lines. Blots were probed for either WNT3A or WNT4, respectively. **(G)**, Estimated concentrations of secreted WNTs (sWNT) present in CM based on linear regression of rWNTs. Volume of CM loaded per well varied from 25-70uL, normalized based on total protein concentration (see Materials and Methods).

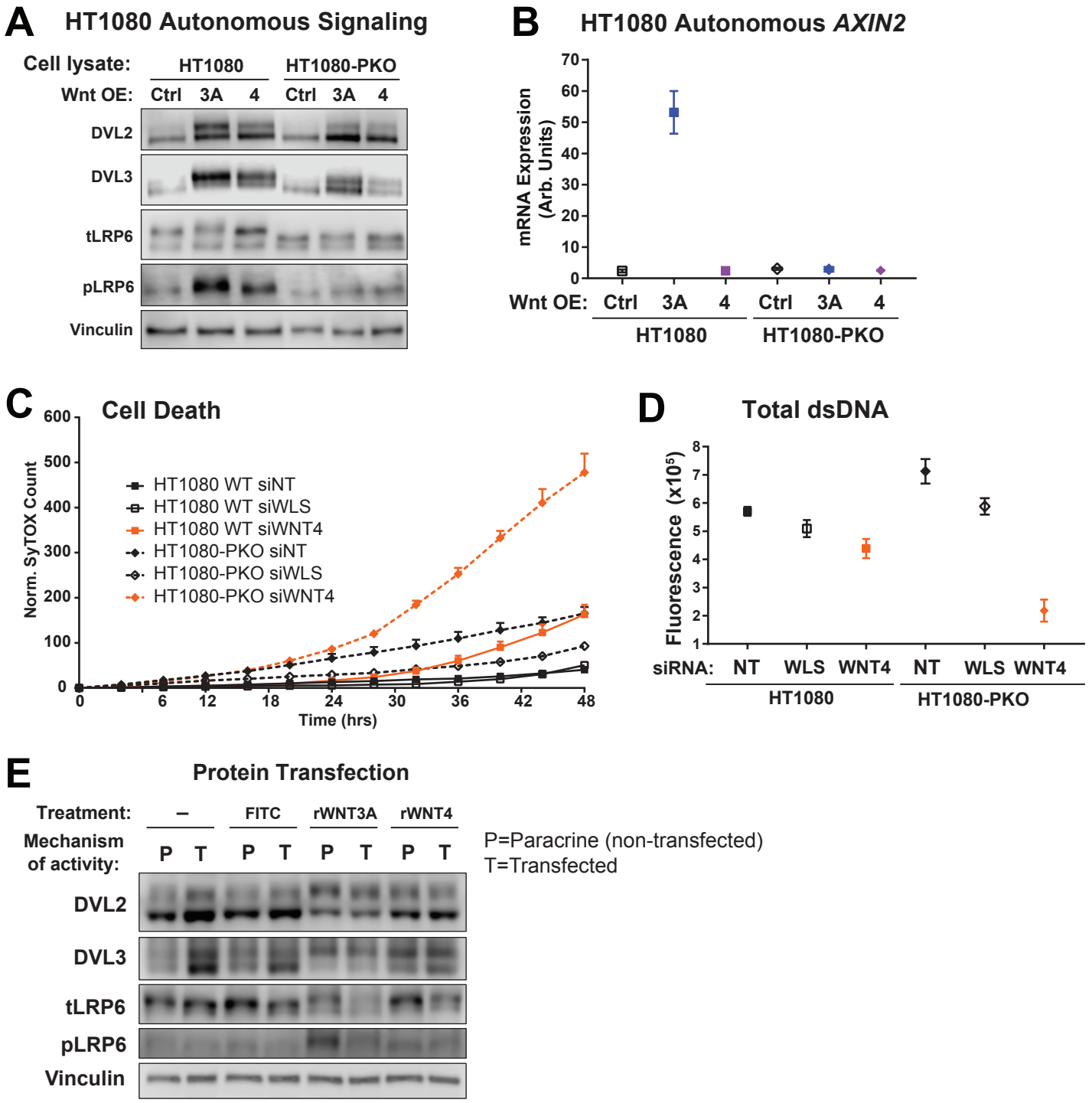


Figure 5. WNT4 and WNT3A have cell autonomous activity independent of PORCN.
(A), Whole cell lysates from HT1080 cell lines were harvested and immunoblotted for DVL2, DVL3, total LRP6 and phosphorylated LRP6. **(B),** mRNA from HT1080 cell lines was extracted and qPCR was performed to determine expression levels of *AXIN2.* **(C-D),** HT1080 cell lines transfected with siControl, siWLS, or siWNT4. **(C),** Cells were live cell imaged for cell death (SyTOX green) and **(D)**, total double-stranded DNA was measured at timecourse completion. **(E),** HT1080 control cells were treated or transfected with 1µg of either a FITC-labeled antibody, rWNT3A or rWNT4. Whole cell lysates were harvested 4hr post-transfection and immunoblotted for DVL2, DVL3, total LRP6, or phosphorylated LRP6.

Table 1 - Model Systems.

	WNT Overexpression (Wnt OE)		
Cell Line	**WNT3A**	**WNT4**	**PORCN Activity**
HT1080	-	-	Normal
HT1080:W3	Yes	-	Normal
HT1080:W4	-	Yes	Normal
HT1080-PKO	-	-	Inactive
HT1080-PKO:W3	Yes	-	Inactive
HT1080-PKO:W4	-	Yes	Inactive
MM134	-	-	Normal
MM134:W3	Yes	-	Normal
MM134:W4	-	Yes	Normal