1-2009

Identification of WNT/β-CATENIN Signaling Pathway Components in Human Cumulus Cells

Hong-Xing Wang
University of Western Ontario

Francis R. Tekpetey
University of Western Ontario, ftekpete@uwo.ca

Gerald M. Kidder
University of Western Ontario, gerald.kidder@schulich.uwo.ca

Identification of WNT/β-CATENIN signaling pathway components in human cumulus cells

Hong-Xing Wang[1], Francis R. Tekpetey[2, 4], and Gerald M. Kidder[1, 2, 3, 5, 6]

Departments of [1]Physiology and Pharmacology, [2]Obstetrics and Gynaecology, and [3]Paediatrics, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario N6A 5C1; Canada
[4]Reproductive Endocrinology and Infertility Program, London Health Sciences Centre, 339 Windermere Road, London, Ontario, N6A 5A5, Canada.
[5]Children's Health Research Institute, 800 Commissioners Road East, London, Ontario N6C 2V5, Canada

[6]Address for correspondence: Dr. Gerald M. Kidder
Department of Physiology and Pharmacology
Dental Sciences Building, dock 15
The University of Western Ontario
London, Ontario N6A 5C1, Canada
Phone: 1-519-661-3132
Fax: 1-519-850-2562
Email: gerald.kidder@schulich.uwo.ca

Running title : WNT/β-CATENIN pathway in cumulus cells

Signaling via the conserved WNT/β-CATENIN pathway controls diverse developmental processes. To explore its potential role in the ovary, we investigated the expression of WNTs, frizzled (FZD) receptors, and other pathway components in human cumulus cells obtained from oocytes collected for in vitro fertilization. Proteins were detected in cultured cells using immunofluorescence microscopy. Protein-protein interactions were analyzed by means of immunoprecipitation. WNT2, FZD2, FZD3, and FZD9 were identified but WNT1, WNT4 and FZD4 were not detected. WNT2 is co-expressed with FZD2, FZD3, and FZD9. Co-immunoprecipitation using WNT2 antibody demonstrated that WNT2 interacts with both FZD3 and FZD9, but only FZD9 antibody precipitated WNT2. We also identified DVL (disheveled), AXIN, GSK-3β (glycogen synthase kinase-3β), and β-CATENIN. β-CATENIN is concentrated in the plasma membranes. DVL co-localizes with FZD9 and AXIN in the membranes, but GSK-3β has little colocalization with AXIN and β-CATENIN. Interestingly, β-CATENIN is highly co-localized with FZD9 and AXIN. CDH1 (E-cadherin) was also detected in the plasma membranes and cytoplasm, co-localized with β-CATENIN, and CDH1 antibody precipitated β-CATENIN. The results suggest that WNT2 could act through its receptor FZD9 to regulate the β-CATENIN pathway in cumulus cells, recruiting β-CATENIN into plasma membranes and promoting the formation of adherens junctions involving CDH1.

Keywords: ovarian follicle, paracrine signaling, folliculogenesis, gene expression, signal transduction, assisted reproduction

Introduction

WNT genes encode a large family of secreted, cysteine-rich glycoproteins that have been implicated in a variety of cellular processes, such as cell fate specification, cell proliferation, differentiation, survival and apoptosis, polarity, and migration (Wodarz and Nusse, 1998; Hoppler and Kavanagh, 2007). Dysregulation of WNT signaling has been found to cause developmental defects and tumorigenesis (Logan and Nusse, 2004). To date, 19 WNT members have been identified in human. Frizzled (FZD) receptors are a family of seven-transmembrane proteins, 10 of which are encoded in the human genome. The N-terminal extracellular cysteine-rich domain (CRD) has been identified as the WNT-binding domain (Dann *et al*., 2001). The WNT-FZD relationship is well characterized in *Drosophila*, however, the specificity of WNT-FZD interactions remains largely unresolved in vertebrates because of the large number of WNT and FZD genes and the numerous possibilities for WNT-FZD interactions and functional redundancies (Rulifson *et al*., 2000; Hsieh *et al*., 1999).

The canonical WNT pathway is well understood for its ability to regulate cell-cell adhesion and cell cycle control, and β-CATENIN is the central and essential component in this pathway (Mulholland *et al*., 2005). In the absence of WNT activation, β-CATENIN is tightly regulated through phosphorylation at specific N-terminal residues by a destruction complex that includes casein kinase-1α (CK1α), glycogen synthase kinase-3 (GSK3), along with tumor suppressors adenomatous polyposis coli (APC) and AXIN. Phosphorylated β-CATENIN is targeted for rapid ubiquitinylation and degradation in the 26S proteasome (Gordon and Nusse, 2006;

Habas and Dawid, 2005). Disheveled (DVL) is, at present, the only known key intermediate connecting FZD and the downstream β-CATENIN pathway, and may bind directly to the FZD C-terminal region via a PDZ domain (Wong *et al*., 2003). Following WNT binding to the receptor complex consisting of FZD and lipoprotein receptor-related protein 5 and 6 (LRP5/6), the activation of the phosphoprotein DVL recruits AXIN and the destruction complex to the plasma membrane. AXIN is then degraded by proteasomes, which decreases β-CATENIN degradation. The activation of DVL also leads to the destabilization of the destruction complex and inhibition of GSK3, which further reduces the phosphorylation and degradation of β-CATENIN (Gordon and Nusse, 2006; Moon *et al*., 2004). As a result, hypophosphorylated β-CATENIN accumulates in the cytoplasm and is translocated to the nucleus where it interacts with the T-cell factor and lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors to activate the transcription of target genes (Gordon and Nusse, 2006). In addition to its function in the WNT pathway, β-CATENIN also functions as a key component of the cadherin complex, which controls cell-cell adhesion and influences cell migration (Bienz, 2005; Nelson and Nusse, 2004).

Ovarian folliculogenesis requires complex regulatory mechanisms involving both endocrine and intra-ovarian signaling pathways. Recently, WNT signaling has been implicated in ovarian development, oogenesis, and early development. *Wnt4* deficient mice exhibit sex reversal and a paucity of oocytes in the newborn ovary, while mice null for *Fzd4* are infertile and exhibit impaired function of the corpus luteum (Vionio *et al*., 1999; Hsieh *et al*., 2005). Multiple *Wnt* transcripts are localized in the different

compartments of the mouse ovary: *Wnt2* and *Fzd1* are expressed in the granulosa cells while *Wnt4* and *Fzd4* are expressed in the corpus luteum (Ricken *et al*., 2002; Hsieh *et al*., 2002). It has been reported that misregulation of WNT/β-CATENIN signaling in granulosa cells can contribute to granulosa cell tumor development (Boerboom *et al*., 2005).

The preovulatory oocyte is surrounded by several cell layers, known as the cumulus granulosa cells, which are metabolically coupled with each other and the oocyte through intercellular membrane channels (gap junctions) that facilitate exchange of small signaling molecules between the cells (Laird, 2006). The cumulus cells exhibit hormone responsiveness, a gene expression profile, and intercellular signaling distinct from those of the mural (outer) granulosa cell layers, which contribute to the oocyte through the final stage of its development (Russell and Robker, 2007). However, very little is known about the expression or function of the WNT/β-CATENIN signaling pathway in the cumulus cells. This study was undertaken to explore the expression of WNT/β-CATENIN signaling proteins, the interaction of WNTs and FZD receptors, and the possible function of β-CATENIN in human cumulus cells.

Materials and Methods

Cumulus cell culture

The acquisition and use of human cumulus cells was approved by the Health Sciences Research Ethics Board of the University of Western Ontario and all patients gave

informed consent. Cumulus granulosa cells were collected from oocytes being prepared for intracytoplasmic sperm injection (ICSI) as previously described (Wang *et al*., 2008). The cells were washed twice with culture medium consisting of DMEM/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were grown on glass coverslips treated with 0.358 mg/mL collagen (BD Biosciences, Mississauga, ON, Canada) and cultured at 37°C, 5% CO_2 in air for 24 hours. Cumulus cells from individual follicles were cultured separately. All products for this study were purchased from Invitrogen Canada Inc. (Burlington, ON) unless specifically mentioned.

Immunofluorescence microscopy

Information for all antibodies used in this study is listed in Table 1. As a positive control for each antibody, we also used it to stain mouse ovaries by immunohistochemistry. Cells grown on glass coverslips were fixed with pre-chilled methanol/acetone (4:1) at 4°C for 20 min and then rinsed with phosphate-buffered saline (PBS) to prepare them for immunostaining. Briefly, the cells were blocked with washing buffer containing 3% w/v bovine serum albumin (BSA) for 1 h, immunolabeled with primary antibody for 1 h, washed with PBS, and immunolabeled with appropriate secondary antibody for 1 h in the dark. For double-immunolabeling, cells were treated with the first primary antibody for 1 h and then with Alexa Fluor® 594-conjugated secondary antibody for 1 h, followed by treatment with the second primary antibody for 1 h and finally by an Alexa Fluor® 488-conjugated secondary

antibody. Several washes were interposed between the different antibody incubations. Cells were washed in PBS and the nuclei stained with 0.1% Hoechst for 10 min followed by washes with PBS and double distilled H_2O. The coverslips were mounted on slides with Airvol (Air Products & Chemicals, Inc., Allentown, PA, USA) before storage at 4°C. At least 50 patients contributed cumulus cells for this study, with at least 5 coverslips from each patient being analyzed by immunostaining with each antibody. The cells were imaged using a Zeiss (Thornwood, NY) LSM 510 META confocal microscope. Fluorescent signals were captured after excitation with 488, 543, or 730 nm laser lines. Digital images were prepared using Zeiss LSM and Adobe Photoshop 7.0 software.

Co-immunoprecipitation

Cumulus cells from all follicles of one or two patients collected on the same day were pooled for these analyses. Cumulus cells were lysed in immunoprecipitation (IP) buffer [125 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1% Triton-X-100, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1X protease inhibitor cocktail (Roche, Mannheim, Germany)]. Five-hundred μg total proteins were incubated with 3-5 μg of antibody against WNT2, FZD2, FZD3, FZD9 or CDH1 in 1.0 ml IP buffer on a rocker at 4°C overnight. The antibodies were precipitated with 50 μl of Dynabeads® Protein G for 2 hours on a rocker at 4°C. The beads were washed twice with washing buffer (25 mM citric acid, 50 mM Na_2HPO_4, pH 5.0) and then eluted with 0.1 M citrate (pH 2-3). Fifty μg total protein before immunoprecipitation was set as the input sample, while the

sample without antibody incubation was considered as negative control.

Immunoblotting

All samples were boiled for 5 minutes and loaded on 12% SDS-PAGE gels. The proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfout, Buckinghamshire, England). The membrane was blocked with 5% nonfat milk (w/v) in Tris-buffered saline Tween-20 (TBST) for 1 h, and subsequently probed with specific primary antibody other than the immunoprecipitating antibody overnight at 4°C followed by incubation with infrared fluorescent-labeled secondary antibody (Alexa-680 anti-rabbit, anti-goat or anti-mouse). Antibody binding was detected by use of the Odyssey infrared-imaging system (LI-COR Biosciences, Lincoln, NE). The membrane was stripped and re-probed with the same antibody used for immunoprecipitation, then incubated with the corresponding infrared fluorescent-labeled secondary antibody. The immunoblots were processed using LI-COR. Two washes with TBST were inserted between the antibody treatment steps.

Results

Detection of WNTs and FZDs in human cumulus cells

To begin to explore WNT signaling in human cumulus cells, we looked for the expression of WNT1, WNT2, and WNT4. The results showed that WNT2 is expressed in cumulus cells, but WNT1 and WNT4 were not detected (Fig. 1A). The same WNT1 antibody revealed strong immunoreactivity in oocytes and corpora lutea of mouse

ovaries but the WNT4 antibody did not reveal any distinct immunoreactivity (H.-X. Wang and G.M. Kidder, manuscript in preparation). It has been reported that FZD2, FZD3, FZD4 and FZD9 are potential receptors for WNT2 in *Drosophila* or mammalian cells (Wu and Nusse, 2002; Klein *et al*., 2008; Karasawa *et al*., 2002). In our study, FZD2, FZD3, and FZD9 but not FZD4 were detected (Fig. 1B); again, the FZD4 antibody did reveal expression of this protein in mouse oocytes and corpora lutea (H.-X. Wang and G.M. Kidder, manuscript in preparation). FZD2 is localized mainly in the cytoplasm (see also Fig. 2A showing colocalization with cytoplasmic WNT2), whereas FZD3 and FZD9 are localized in the cytoplasm and membranes.

Interaction of WNT2 and its possible receptors

We used double-label immunofluorescence to explore the co-localization of WNT2 and FZDs in human cumulus cells. We found that WNT2 is co-expressed with each of FZD2, FZD3, and FZD9 (Fig. 2). WNT2 co-localizes with FZD2 in the cytoplasm (Fig. 2A) and with FZD3 and FZD9 in both the membranes and cytoplasm (Fig. 2B and C). The possibility of direct interaction of WNT2 with the three receptors was then tested by co-immunoprecipitation experiments. When cell lysates were immunoprecipitated with antibody directed against WNT2, FZD3 and FZD9 were also found in the pellet, but FZD2 was not (Fig. 3A, B, C). Conversely, although WNT2 was precipitated by FZD9 antibody, FZD2 and FZD3 antibodies were ineffective (Fig. 3D). No protein bands were evident in cell lysates not treated with immunoprecipitating antibody (negative controls in Fig. 3A-D).

Expression of WNT2/β-CATENIN signaling proteins in human cumulus cells

As reported previously, FZD9 is activated by WNT2 and functions through the WNT/β-CATENIN pathway (Karasawa *et al*., 2002). DVL, AXIN, GSK-3β, and β-CATENIN are four important molecules in this pathway (Gordon and Nusse, 2006). All four proteins were detected in human cumulus cells by immunofluorescence (Fig. 4). The pattern of immunostaining for DVL, AXIN, and β-CATENIN indicated their localization in both the cell periphery and the perinuclear cytoplasm, whereas that for GSK-3β was clearly cytoplasmic. Co-immunolabeling experiments demonstrated that DVL colocalizes with FZD9 and AXIN in the membranes (Fig. 5A and B) while GSK-3β is localized mostly in the cytoplasm, with little colocalization with AXIN or β-CATENIN (Fig. 5C and D). Interestingly, FZD9 is highly co-localized with β-CATENIN in the cell membranes (Fig. 5E).

Localization of β-CATENIN in cumulus cells

As recently reported, activation of the WNT pathway recruits dephosphorylated β-CATENIN into the plasma membrane where it colocalizes with two members of the destruction complex, APC and AXIN (Hendriksen *et al*., 2008). Our results indicate that AXIN is highly localized with β-CATENIN in the cytoplasm of cumulus cells (Fig. 6A). As a component of adherens junctions, β-CATENIN promotes cell adhesion by binding to the intracellular domain of the transmembrane protein, epithelial cadherin (CDH1) (Bienz, 2005). CDH1 was also detected in human cumulus cells, with some

junction-like plaques being found between the cells (arrows in Fig. 6B). Co-immunoprecipitation confirmed that β-CATENIN interacts with CDH1 in human cumulus cells (Fig. 6C). Co-immunolabeling further confirmed that CDH1 co-localizes with β-CATENIN to a limited extent in the membranes (Fig. 6D).

Discussion

WNTs comprise a large family of secreted glycoproteins that are responsible for key developmental processes including cell proliferation, cell polarity, and cell fate determination. Conventional WNT signaling causes β-CATENIN accumulation in a complex with the transcription factor TCF/LEF that regulates target gene expression (Wodarz and Nusse, 1998; Hoppler and Kavanagh, 2007). Dysregulation of WNT signaling is linked to a range of diseases, most notably cancer (Logan and Nusse, 2004). A few reports have indicated that WNT signaling plays a key role in development of the ovary, and WNT pathway components are normally expressed in ovarian granulosa cells (Vionio *et al*., 1999; Hsieh *et al*., 2005; Ricken *et al*., 2002; Hsieh *et al*., 2002). Aberrant WNT signaling leads to granulosa cell tumorigenesis (Boerboom *et al*., 2005). These findings suggest that WNT signaling is important for follicular development. However, we know little about WNT signaling in human ovarian follicles and its roles in folliculogenesis. It was thus important to explore the expression of WNT signaling components in human cumulus cells.

Previous experiments revealed that WNT2 mRNA is concentrated in the cumulus granulosa cells of rat ovaries and that WNT4 mRNA is expressed in small growing

245 follicles and corpora lutea of mouse ovaries (Ricken *et al*., 2002; Hsieh *et al*., 2002). In the present study, we only detected the expression of WNT2 in human cumulus cells; WNT1 and WNT4 were not detected. Neither of the two different WNT4 antibodies we used, both of which have been successfully used by others, revealed the presence of this protein in mouse ovaries either. We are unaware of any report confirming 250 expression of WNT4 in adult ovaries at the protein level. Therefore, we think it unlikely that WNT4 is present in our human cumulus cell samples, although some uncertainty still exists. Since WNT4 might function to regulate earlier stages of follicle growth, and cumulus cells from ISCI patients are mostly retrieved from large mature follicles, the absence of WNT4 is perhaps not surprising.

255 To verify that the WNT2 signaling pathway is intact in cumulus cells, we looked for the presence of possible receptors. It has been demonstrated that FZD2 and FZD3 interact with WNT2 in *Drosophila* (Wu and Nusse, 2002), that FZD4 is the receptor for WNT2 in rat hepatic sinusoidal endothelial cells and human colon-derived cells (Klein *et al*., 2008; Planutis *et al*., 2007), and that FZD9 is activated by WNT2 in 260 transfected 293 cells (Karasawa et al., 2002). FZD4 was only found in rat but not mouse granulosa cells (Ricken *et al*., 2002; Hsieh *et al*., 2002). Here we confirmed the expression of FZD2, FZD3, and FZD9 but failed to find FZD4 in human cumulus cells. FZD4 plays an essential role in the mouse ovary since *Fzd4* null mice are infertile and exhibit impaired corpus luteum formation and function (Hsieh *et al*., 2005); if FZD4 is 265 similarly involved in human folliculogenesis, its expression must be restricted to the mural granulosa cells or activated only after ovulation. Co-expression and

co-immunoprecipitation experiments demonstrated that WNT2 interacts with FZD3 and FZD9 in human cumulus cells, but since only FZD9 antibody could immunoprecipitate WNT2, it must be considered the most likely receptor candidate. Although the WNT2 antibody had weak ability to precipitate FZD3, two different FZD3 antibodies failed to precipitate WNT2. One possible explanation for these results is that WNT2 associates only weakly or infrequently with FZD3 in cumulus cells. Further study is needed to determine whether FZD3 is another potential receptor for WNT2 in human cumulus cells.

It has been demonstrated that FZD and DVL are essential in both the canonical and noncanonical WNT pathways. In transfected 293 cells, FZD9 is activated by WNT2 and functions through the β-CATENIN pathway (Karasawa *et al*., 2002). β- CATENIN is a key effector of WNT/β-CATENIN signaling and is regulated by the cytoplasmic destruction complex formed by AXIN, GSK-3β, and APC. In this study, we detected the expression of DVL, AXIN, GSK-3β, and β-CATENIN in human cumulus cells. DVL is co-localized with FZD9 and AXIN in the plasma membranes, indicating that FZD9 can activate the cytoplasmic protein DVL and recruit it into the membrane, where it then interacts with AXIN. This interpretation is supported by the observations that overexpression of FZD9 induces the hyperphosphorylation and relocation of mouse DVL-1 from the cytoplasm to the cell membrane, and DVL-2 promotes the recruitment of AXIN to the plasma membrane during WNT signaling (Karasawa *et al*., 2002; Schwarz-Romond *et al*., 2007). Once AXIN is recruited into the membrane, it is then degraded by proteasomes leading to inhibition of the activity of the destruction

complex. The activation of DVL also leads to inhibition of GSK-3β, which further contributes to β-CATENIN accumulation (Moon et al., 2004; Schwarz-Romond *et al*., 2007). These results directly support our finding that GSK-3β is not co-localized with AXIN and β-CATENIN in human cumulus cells.

An interesting finding of our study is that β-CATENIN is mostly localized in the membrane of cumulus cells, and that FZD9 is highly co-localized with β-CATENIN in the plasma membrane. These data indicate that FZD9 will activate β-CATENIN and recruit it into the membrane. In line with this, Hendriksen et al. (2008) have recently shown that endogenous dephosphorylated β-CATENIN appears on the membrane upon WNT3A treatment. This translocation occurs independently of CDH1, and β-CATENIN is co-localized with phospho-LRP6, AXIN, and APC. We also found co-localization of AXIN and β-CATENIN in human cumulus cells. However, the mechanism and function of the recruitment of β-CATENIN into membranes remain unclear. Aside from its function in WNT signaling, β-CATENIN was initially discovered by virtue of its role in forming adherens junctions with CDH1 (Nelson and Nusse, 2004; Brembeck *et al*., 2006). The expression of CDH1/CATENIN complexes had been identified in the rat ovary, suggesting that this complex might play roles in follicle development and luteinization (Sundfeldt et al., 2000). In our study, CDH1 was detected in junction-like plaques between cumulus cells and CDH1 antibody precipitated β-CATENIN; furthermore, CDH1 co-localized with β-CATENIN in some cells. Thus, β-CATENIN in cumulus cells may play a role not only in WNT signaling, but also in forming adherens junctions with CDH1.

In summary, our results are consistent with the possibility that WNT2 signals through its receptor FZD9 to regulate the β-CATENIN pathway in human cumulus cells, potentially recruiting β-CATENIN into the plasma membrane and promoting the formation of adherens junctions involving CDH1. Our recent work with cultured mouse granulosa cells (Wang and Kidder, in preparation) has revealed that WNT2 stimulates proliferation, suggesting that it may also act as a mitogen to promote follicle growth. Thus, WNT2/β-CATENIN signaling might play an important role in human folliculogenesis.

Funding

This work was funded by a grant (reference number MOP-14150) to GMK from the Canadian Institutes of Health Research.

Acknowledgements

We gratefully acknowledge the technical advice and assistance of Lynda Hughes, Kevin Barr, and Dr. Tony Li.

References

Bienz M (2005) β-Catenin: a pivot between cell adhesion and Wnt signalling. Curr Biol 15, R64-67.

Boerboom D, Paquet M, Hsieh M, Liu J, Jamin SP, Behringer RR, Sirois J, Taketo

MM and Richards JS (2005) Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development. Cancer Res 65, 9206-9215.

Brembeck FH, Rosário M and Birchmeier W (2006) Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr Opin Genet Dev 16, 51-59.

Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J and Leahy DJ (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. Nature 412, 86-90.

Gordon MD and Nusse R **(**2006) Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 281, 22429-22433.

Habas R and Dawid IB **(**2005) Dishevelled and Wnt signaling: is the nucleus the final frontier? J Biol 4, 2.

Hendriksen J, Jansen M, Brown CM, van der Velde H, van Ham M, Galjart N, Offerhaus GJ, Fagotto F and Fornerod M (2008) Plasma membrane recruitment of dephosphorylated β-catenin upon activation of the Wnt pathway. J Cell Sci 121, 1793-1802.

Hoppler S and Kavanagh CL **(**2007) Wnt signalling: variety at the core. J Cell Sci 120,

385-393.

Hsieh JC, Rattner A, Smallwood PM and Nathans J (1999) Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. Proc Natl Acad Sci USA 96, 3546-3551.

Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UF, Berger W and Richards JS (2005) Mice null for Frizzled4 (*Fzd4*$^{-/-}$) are infertile and exhibit impaired corpora lutea formation and function. Biol Reprod 73, 1135-1146.

Hsieh M, Johnson MA, Greenberg NM and Richards JS (2002) Regulated expression of Wnts and Frizzleds at specific stages of follicular development in the rodent ovary. Endocrinology 143, 898-908.

Karasawa T, Yokokura H, Kitajewski J and Lombroso PJ (2002) Frizzled-9 is activated by Wnt-2 and functions in Wnt/beta-catenin signaling. J Biol Chem 277, 37479-37486.

Klein D, Demory A, Peyre F, Kroll J, Augustin HG, Helfrich W, Kzhyshkowska J, Schledzewski K, Arnold B and Goerdt S **(**2008) Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway. Hepatology 47, 1018-1031.

Laird DW. (2006) Life cycle of connexins in health and disease. Biochem J 394, 527-543.

Logan CY and Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20, 781-810.

Moon RT, Kohn AD, De Ferrari GV and Kaykas A (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5, 691-701.

Mulholland DJ, Dedhar S, Coetzee GA and Nelson CC (2005) Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? Endocr Rev 26, 898-915.

Nelson WJ and Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303, 1483-1487.

Planutis K, Planutiene M, Moyer MP, Nguyen AV, Pérez CA and Holcombe RF (2007) Regulation of norrin receptor frizzled-4 by Wnt2 in colon-derived cells. BMC Cell Biol 8, 12.

Ricken A, Lochhead P, Kontogiannea M and Farookhi R (2002) Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and

frizzled-4 mRNAs. Endocrinology 143, 2741-2749.

Rulifson EJ, Wu CH and Nusse R (2000) Pathway specificity by the bifunctional receptor frizzled is determined by affinity for wingless. Mol Cell 6, 117-126.

Russell DL and Robker RL (2007) Molecular mechanisms of ovulation: co-ordination through the cumulus complex. Hum Reprod Update 13, 289-312.

Schwarz-Romond T, Metcalfe C and Bienz M (2007) Dynamic recruitment of axin by Dishevelled protein assemblies. J Cell Sci 120, 2402-2412.

Sundfeldt K, Piontkewitz Y, Billig H and Hedin L (2000) E-cadherin-catenin complex in the rat ovary: cell-specific expression during folliculogenesis and luteal formation. J Reprod Fertil 118, 375-385.

Vainio S, Heikkilä M, Kispert A, Chin N and McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. Nature 397, 405-409.

Wang HX, Tong D, El-Gehani F, Tekpetey FR and Kidder GM (2008) Connexin expression and gap junctional coupling in human cumulus cells: contribution to embryo quality. J Cell Mol Med, in press.

Wodarz A and Nusse R. (1998) Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14, 59-88.

Wong HC, Bourdelas A, Krauss A, Lee HJ, Shao Y, Wu D, Mlodzik M, Shi DL and Zheng J (2003) Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. Mol Cell 12, 1251-1260.

Wu CH and Nusse R (2002) Ligand receptor interactions in the Wnt signaling pathway in *Drosophila*. J Biol Chem 277, 41762-41769.

Table I. Details of antibodies used.

Name	Company	Application[a]		
		IF	IB	IP
Goat anti-WNT1	Santa Cruz	1: 100		
Goat anti-WNT2	Santa Cruz	1: 100	1: 200	4.0 μg
Goat anti-WNT4	R&D Systems	1: 100		
Rabbit anti-FZD2	Zymed	1:100	1:400	5.0 μg
Rabbit anti-FZD3	Sigma	1:200	1: 1,000	
Goat anti-FZD3	US Biological		1: 500	3.0 μg
Mouse anti-FZD3	Abnova Corporation		1: 1,000	3.0 μg
Rabbit anti-FZD4	Abcam	1: 200		
Rabbit anti-FZD9	Abcam	1:200	1: 1,000	
Goat anti-FZD9	Santa Cruz	1:100	1: 300	4.0 μg
Rabbit anti-FZD9	MBL International Corp.	1:100	1: 1,000	3.0 μg
Goat anti-DVL	Santa Cruz	1:100		
Rabbit anti-DVL	Abcam	1: 100		
Goat anti-AXIN	Santa Cruz	1:100		
Mouse anti-GSK-3β	Santa Cruz	1: 200		
Rabbit anti-β-CATENIN	Chemicon	1:500	1: 1,000	
Mouse anti-CDH1	Zymed	1:100	1: 500	5.0 μg
Mouse anti-CDH1	Abcam	1: 200	1: 1,000	
Alexa Fluor® 488 goat anti rabbit	Molecular probes	1:500		
Alexa Fluor® 488 goat anti mouse	Molecular probes	1:500		
Alexa Fluor® 488 rabbit anti goat	Molecular probes	1:500		
Alexa Fluor® 594 goat anti rabbit	Molecular probes	1:300		
Alexa Fluor® 594 goat anti mouse	Molecular probes	1:300		
Alexa Fluor® 680 goat anti rabbit	Molecular probes		1: 10,000	
Alexa Fluor® 680 donkey anti rabbit	Molecular probes		1: 10,000	
Alexa Fluor® 680 rabbit anti mouse	Molecular probes		1: 10,000	

[a]IF, immunofluorescence; IB, immunoblotting; IP, immunoprecipitation.

Figure legends

Figure 1. Expression of WNTs and FZDs in human cumulus cells. (A) Strong immunostaining was observed for WNT2, but WNT1 and WNT4 were not detected. (B) Potential WNT2 receptors FZD2, FZD3, and FZD9 were detected but FZD4 was not. Hoechst dye was used for nucleus staining. Scale bars = 50 μm.

Figure 2. Co-immunolabeling for WNT2 and its potential receptors FZD2, FZD3, and FZD9 in human cumulus cells. WNT2 colocalizes with FZD2 (A), FZD3 (B), and FZD9 (C) in individual cells. Hoechst dye was used for nucleus staining. The same magnification was used for all micrographs; scale bar = 50 μm.

Figure 3. Co-immunoprecipitation tests for interaction of WNT2 with its potential receptors. WNT2 antibody immunoprecipitated FZD3 (B) and FZD9 (C) but not FZD2 (A). CoIP: co-immunoprecipitation; IP: immunoprecipitation; IB: immunoblotting. (D) WNT2 was immunoprecipitated with FZD9 antibody but not with FZD2 or FZD3 antibody.

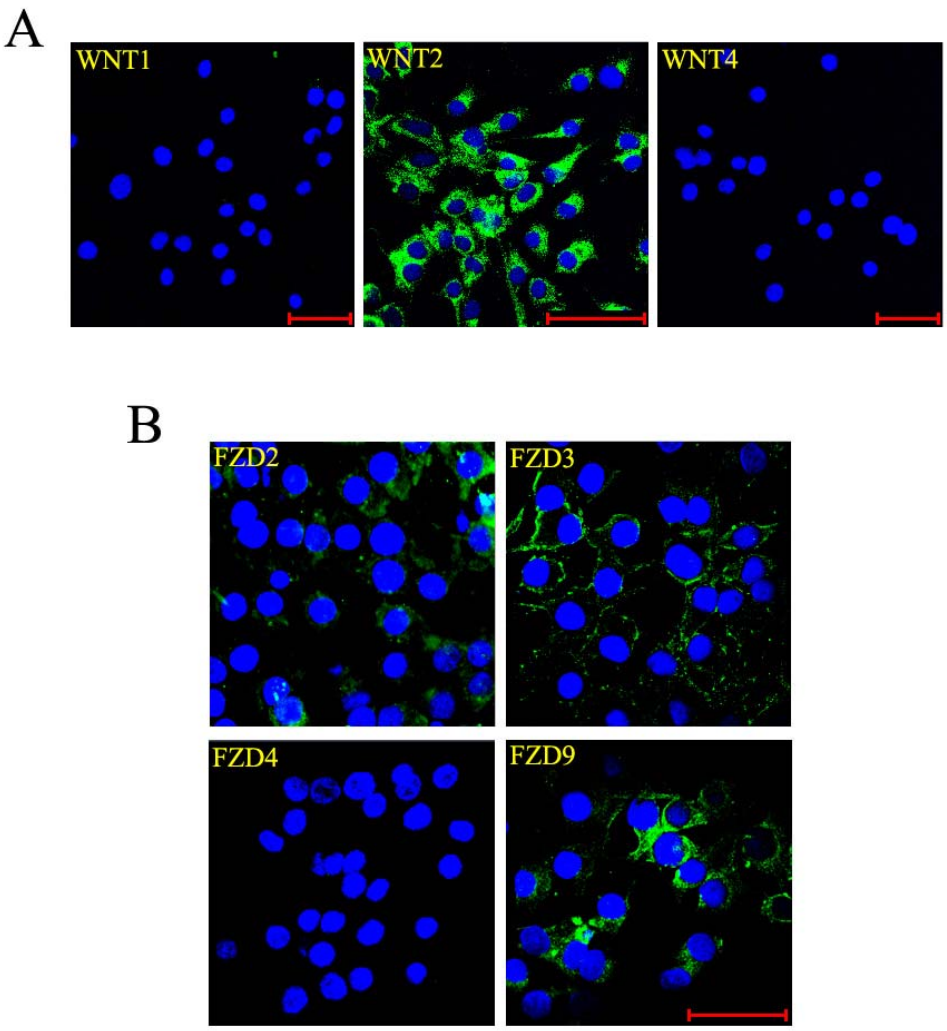
Figure 4. Localization of DVL, AXIN, GSK-3β, and β-CATENIN in human cumulus cells. Arrows indicate putative membrane staining. Hoechst dye was used for nucleus staining. The same magnification was used for all micrographs; scale bar = 50 μm.

Figure 5. Co-expression of WNT/β-CATENIN signaling proteins in human cumulus cells. (A, B) Colocalization of DVL with FZD9 (A) and AXIN (B). (C,D) Co-expression but limited colocalization of GSK-3β with AXIN (C) and β-CATENIN (D). (E) Extensive colocalization of FZD9 and β-CATENIN. Hoechst dye was used for nucleus staining. The same magnification was used for all micrographs; scale bar = 50 μm.

Figure 6. Localization of β-CATENIN in human cumulus cells. (A) Co-localization of AXIN and β-CATENIN. (B) Expression of CDH1 (arrows indicate junction-like plaques). (C) Immunoprecipitation of β-CATENIN with CDH1 antibody. (D) Limited colocalization of CDH1 and β-CATENIN. Hoechst dye was used for nucleus staining. The same magnification was used for all micrographs; scale bar = 50 μm.

Figures

Figure 1



470

Figure 2

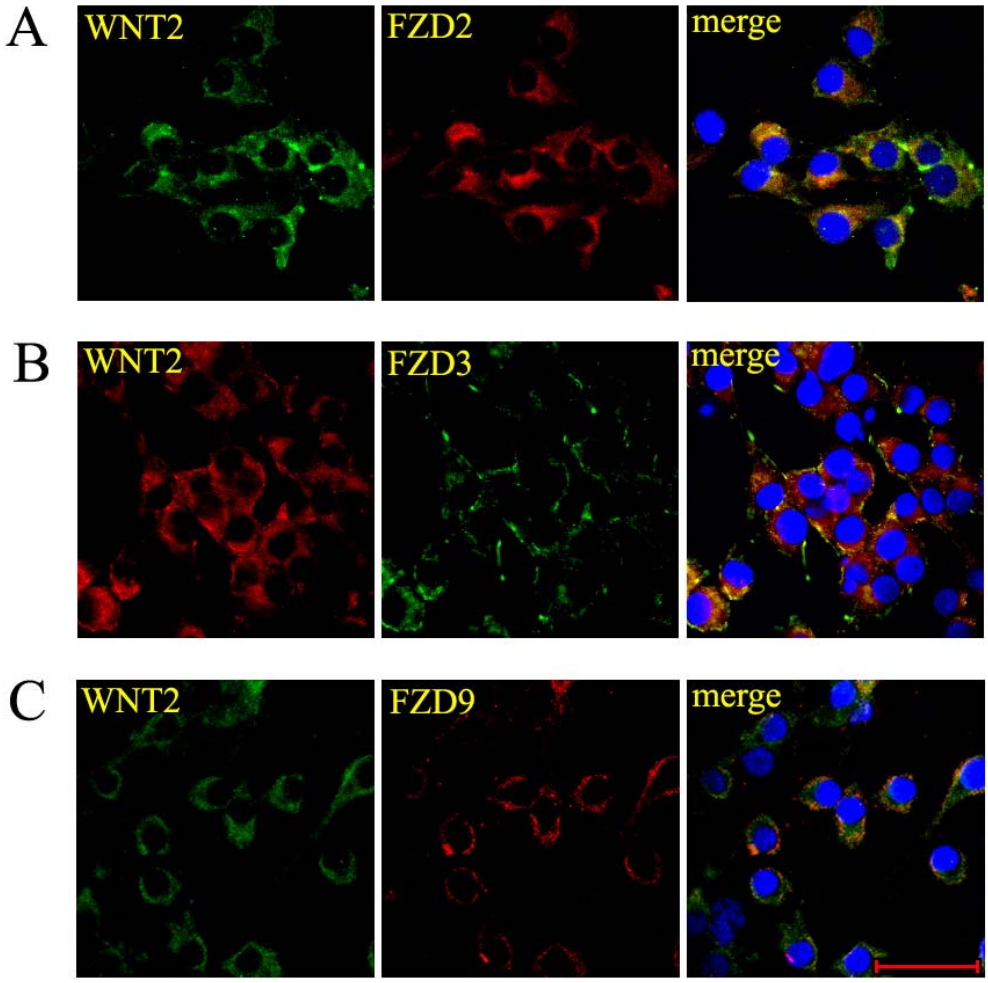
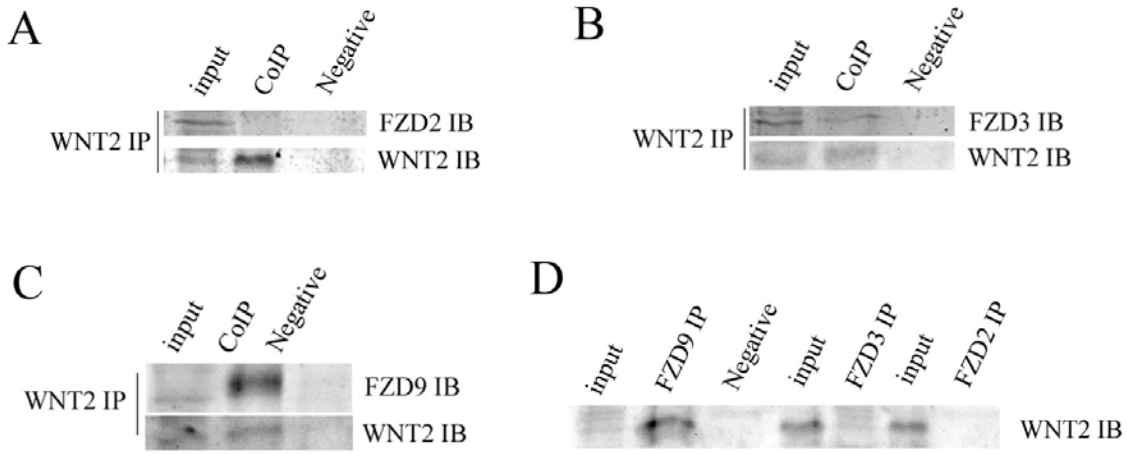


Figure 3


A
input
CoIP
Negative
WNT2 IP
FZD2 IB
WNT2 IB
B
input
CoIP
Negative
WNT2 IP
FZD3 IB
WNT2 IB
C
input
CoIP
Negative
WNT2 IP
FZD9 IB
WNT2 IB
D
input
FZD9 IP
Negative
input
FZD3 IP
input
FZD2 IP
WNT2 IB

475

Figure 4

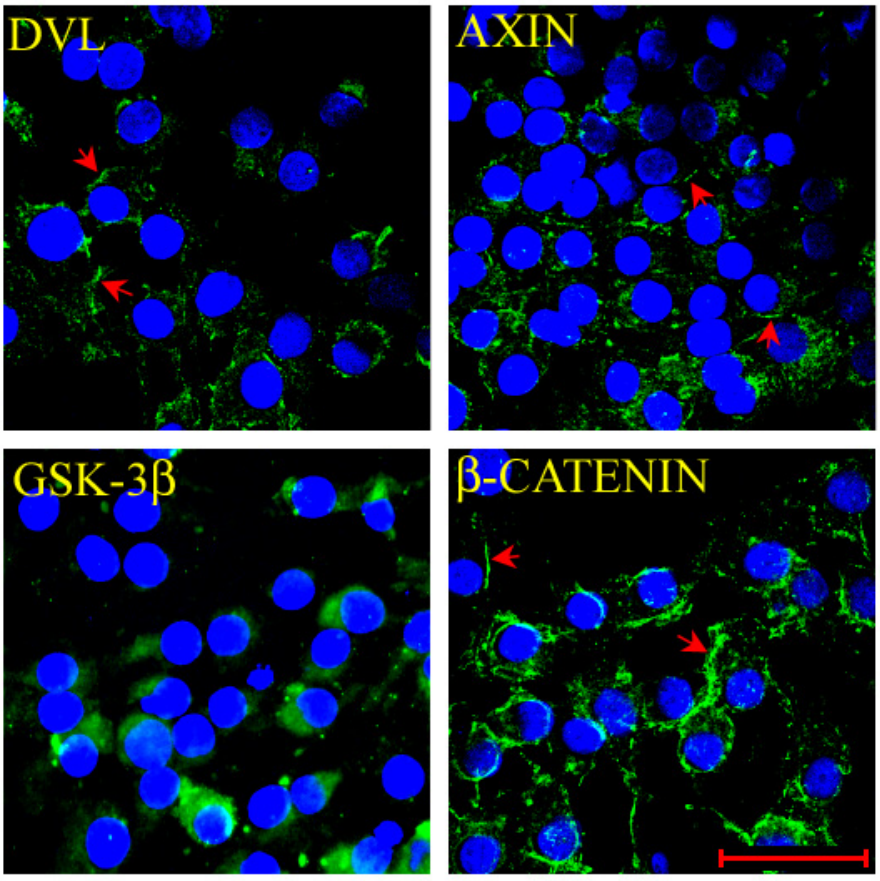
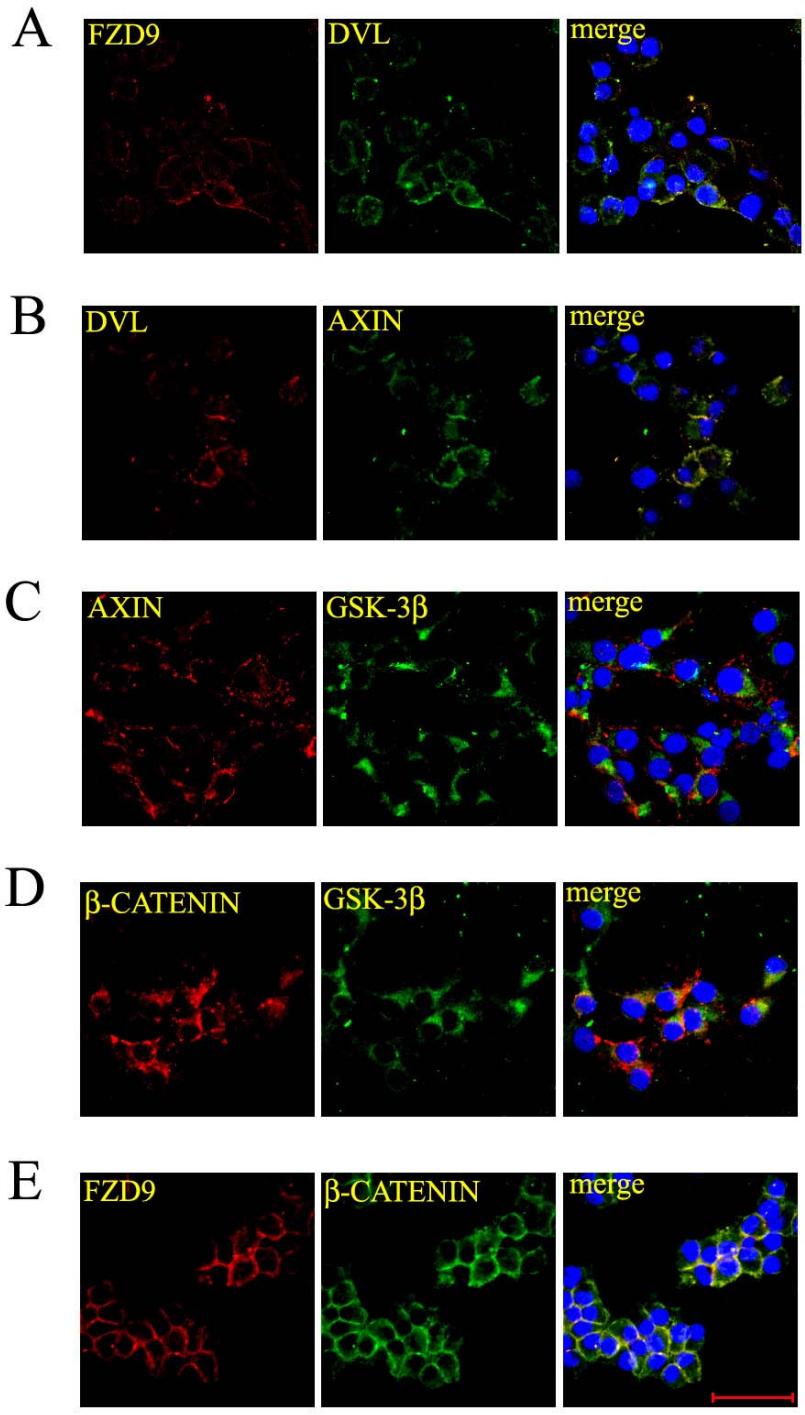
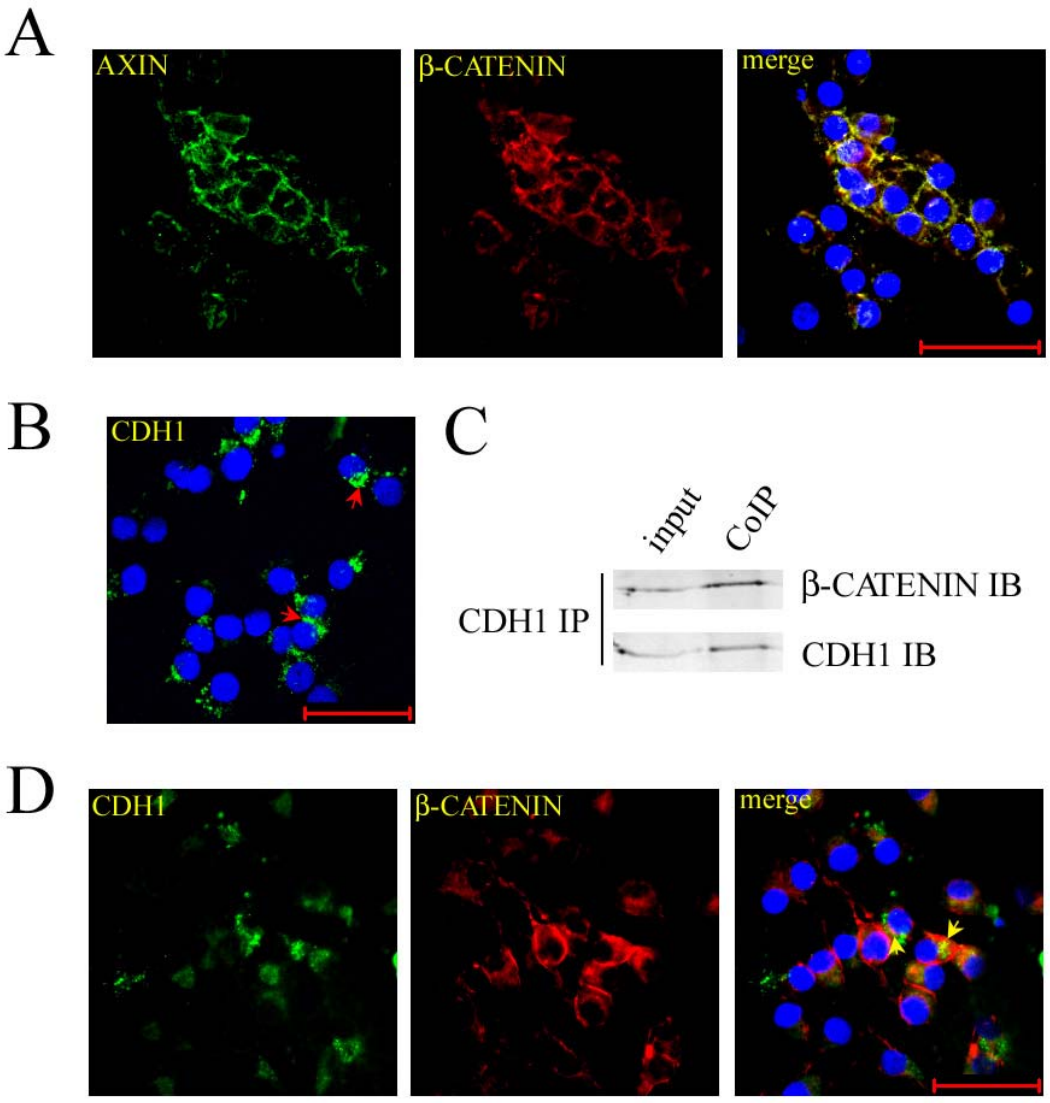


Figure 5


A
FZD9
DVL
merge
B
DVL
AXIN
merge
C
AXIN
GSK-3β
merge
D
β-CATENIN
GSK-3β
merge
E
FZD9
β-CATENIN
merge

480

Figure 6


A
AXIN
β-CATENIN
merge
B
CDH1
C
input
CoIP
CDH1 IP
β-CATENIN IB
CDH1 IB
D
CDH1
β-CATENIN
merge