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**CO-LOCALIZATION OF POLYCYSTIC OVARY SYNDROME CANDIDATE GENE
PRODUCTS IN HUMAN THECA CELLS SUGGESTS NOVEL SIGNALING PATHWAYS**

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science Virginia Commonwealth University, 2019

By

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LIST OF ABBREVIATIONS AND SYMBOLS

AP-2	Adaptor protein -2
APPL-1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
C9orf3	Chromosome 9 Open Reading Frame 3
cAMP	Cyclic adenosine monophosphate
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
DENND1A	<u>D</u> ifferentially <u>E</u> xpressed in <u>N</u> ormal and <u>N</u> eoplastic cells Domain Containing 1A
FSHR	Follicle Stimulating Hormone Receptor
GNL/GNA	<i>Galanthus nivalis</i> lectin/ <i>G. nivalis</i> agglutinin
hCG	Human chorionic gonadotropin
INSR	Insulin Receptor
LH	Luteinizing hormone
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor)
PBS	Phosphate Buffer Saline
RAB5B	Ras-related protein Rab-5B
YAP1	yes-associated protein 1
ZNF217	Zinc finger protein 217

ABSTRACT
CO-LOCALIZATION OF POLYCYSTIC OVARY SYNDROME CANDIDATE GENE PRODUCTS IN HUMAN THECA CELLS SUGGESTS NOVEL SIGNALING PATHWAYS

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Polycystic ovary syndrome (PCOS) is the leading cause of anovulatory infertility, and the most common endocrinopathy of women of reproductive age. Genome-wide association studies (GWAS) identified a number of loci associated PCOS in different ethnic populations, including women with Asian and European ancestry. Replication studies have confirmed some of these associations. Among the loci identified are those located near the LH receptor gene (*LHCGR*), a clathrin-binding protein gene (*DENND1A*) that also functions as a guanine nucleotide exchange factor, and the gene encoding RAB5B, a GTPase and protein involved in vesicular trafficking. The functional significance of one of these GWAS candidates (*DENND1A*) was supported by our discovery that a truncated protein splice variant of *DENND1A* termed DENND1A.V2, is elevated in PCOS theca cells, and that forced expression of DENND1A.V2 in normal theca cells increased *CYP11A1* and *CYP17A1* expression and androgen synthesis, a hallmark of PCOS. PCOS GWAS loci could be assembled into a functional network that contributes to altered gene expression in ovarian theca cells, resulting in increased androgen synthesis. This

thesis demonstrates the localization of LHCGR, DENND1AV.2 and RAB5B proteins in various cellular compartments in normal and PCOS theca cells. hCG and forskolin stimulation affects the distribution and co-localization of DENND1A.V2 and RAB5B in various cellular compartments This cytological evidence supports the PCOS gene network concept, and raises the intriguing possibility that LHCGR activation, via a cAMP-mediated process, promotes the translocation of DENND1A.V2 and RAB5B-containing vesicles from the PCOS theca cell cytoplasm into the nucleus, resulting in increased transcription of genes involved in androgen synthesis.

CHAPTER 1

INTRODUCTION

POLYCYSTIC OVARY SYNDROME (PCOS)

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy of reproductive age women and the leading cause of anovulatory infertility. It is characterized by hyperandrogenism (increased levels of testosterone), anovulation, which leads to infertility, and the characteristic presence of multiple small subcortical follicular cysts, which detectable by transvaginal ultrasound (1,2). PCOS is often accompanied by metabolic disturbances including insulin resistance and hyperinsulinemia (1,2). The etiology of PCOS is thought to be rooted in genetic and environmental factors (3)

PCOS INCIDENCE AND DETECTION

PCOS is believed to be prevalent in about 5-7% of reproductive age women(4–7), but these numbers may vary according to the diagnostic criteria. The three common diagnostic criteria used for the detection of PCOS were formulated by the: National Institutes of Health (NIH) Criteria, the Rotterdam Criteria, and the Androgen Excess Society (AES) (8). The NIH criteria include a combination of hyperandrogenism along with oligo/amenorrhea anovulation (infrequent or absence of menstruation), the Rotterdam Criteria includes two out of three features; polycystic ovarian morphology (PCOM) seen on ultrasound and the two criteria mentioned in the NIH guidelines, whereas the AES criteria includes hyperandrogenism along with oligomenorrhea or PCOM or both (8).

PCOS TREATMENT

There are a variety of treatment options available, and the standard treatment is oral contraceptives, if the woman is not seeking to become pregnant. For infertility, competitive aromatase inhibitors (e.g., Letrozole), which inhibit estrogen biosynthesis and result in an acute elevation of FSH, or estrogen receptor antagonists like clomiphene citrate, which also increases FSH, or FSH injections are effective in inducing follicular development in most women (9). These agents have been used along with other medications including dexamethasone or metformin (9).

As many women with PCOS are overweight, leading to metabolic problems and insulin resistance, weight loss and use of insulin sensitizing agent like metformin seems to yield positive results. Recent studies show that a combination of myo-inositol and D-chiro-inositol in a ratio of 10:1 is effective in improving the metabolic and hormonal aspects of women with PCOS (10).

Other research has shown that natural product like *Tetragonia tetragonioides* (TTK), a medicinal plant which is being used for a long time to treat diabetic, inflammatory, and female-related disorders, can be used as a treatment for PCOS (11). This study shows that TTK extract had significantly reduced forskolin stimulated androgen production in NCI-H295R cells, and also reduced serum LH, testosterone and follicular cysts in a letrozole-induced PCOS mouse model (11). Thus, TTK could in future be possibly used in the treatment of PCOS.

Currently, the only long term solutions to treat PCOS are lifestyle and dietary changes along with weight loss. Thus, understanding the mechanism by which PCOS is initiated and progresses is key to finding better ways to treat PCOS.

PCOS CAUSE AND PATHOPHYSIOLOGY

The pathophysiology underlying PCOS is not yet known, but it believed to be a multifactorial disorder, which may result from various environmental and genetic factors. Family-based and twin studies revealed a significant genetic contribution to PCOS (12,13). To understand the possible mechanisms involved in PCOS several different genome-wide association studies were performed in different demographics, with an aim to understand and possibly find a treatment for it. These genome-wide association studies (GWAS) have yielded more than 20 loci located near putative PCOS genes, including *DENND1A*, *LHCGR*, *FSHR*, *ZNF217*, *YAP1*, *INSR*, *RAB5B*, and *C9orf3*, being most critical among others (14–18). A number of these loci have been replicated in GWAS or targeted genotyping studies in different populations(19,20). However, the specific variants associated with the PCOS phenotype or their functional significance remain to be identified for most of the PCOS candidates.

***DENND1A* AND PCOS**

Among the candidate genes, the *DENND1A* locus at 9q22.32, was identified in both Asian and European ancestry populations in GWAS and replication studies (19,20). *DENND1A* is a member of the connectenn family that encodes a clathrin-binding protein localized to coated pits, a clustering point for plasma membrane receptors (21). The DENN

(Differentially Expressed in Normal and Neoplastic cells) domain encodes a guanine nucleotide exchange function, which was previously shown to interact with RAB35, a small GTPase (21). The splice variant of DENND1A, termed DENND1A.V2, encodes a truncated molecule retaining the DENN and clathrin-binding domains, but lacks the proline-rich domain which interacts with the adapter protein, growth factor receptor-bound protein 2, and contains a unique 33 amino acid C-terminus (Fig. 1) (22,23). Examining the protein-coding potential of the V1 and V2 mRNAs showed that they are identical through the end of exon 20 and then diverge. V1 continues with canonical exons 21 and 22 to yield a 1009 AA open reading frame, while V2 lacks the sequences encoded by exons 21 and 22 and has a different coding sequence that is responsible for a 559 AA open reading frame with a unique C-terminal 33 AA (Fig. 2) (4). These structural differences presumably have functional significance. Remarkably, DENND1A.V2 is elevated in theca cells of women with PCOS (7). Moreover, forced expression of DENND1A.V2 in normal human theca cells in culture resulted in increased expression of *CYP17A1* and production of androgens, whereas knockdown of DENND1A.V2 mRNA in PCOS theca cells reduced *CYP17A1* expression and androgen secretion, further supporting the notion that DENND1A.V2 contributes to hyperandrogenism in PCOS (7).

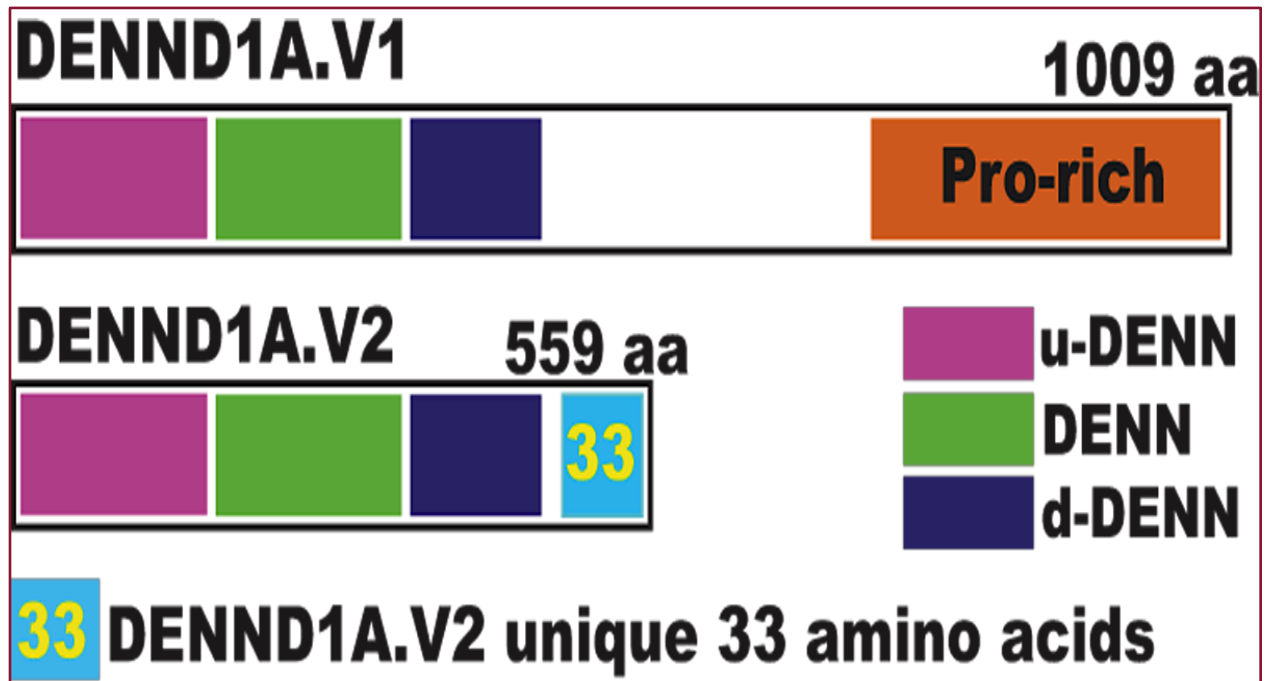


Figure 1. Schematic showing variants of *DENND1A*. The figure shows different domains, of variants of *DENND1A*: A 1009 amino acids *DENND1A.V1* with a proline rich tail and 559 amino acids *DENND1A.V2* with a unique 33 amino acids. Here, u-DENN and d-DENN are regions upstream and downstream of the DENN domain respectively.

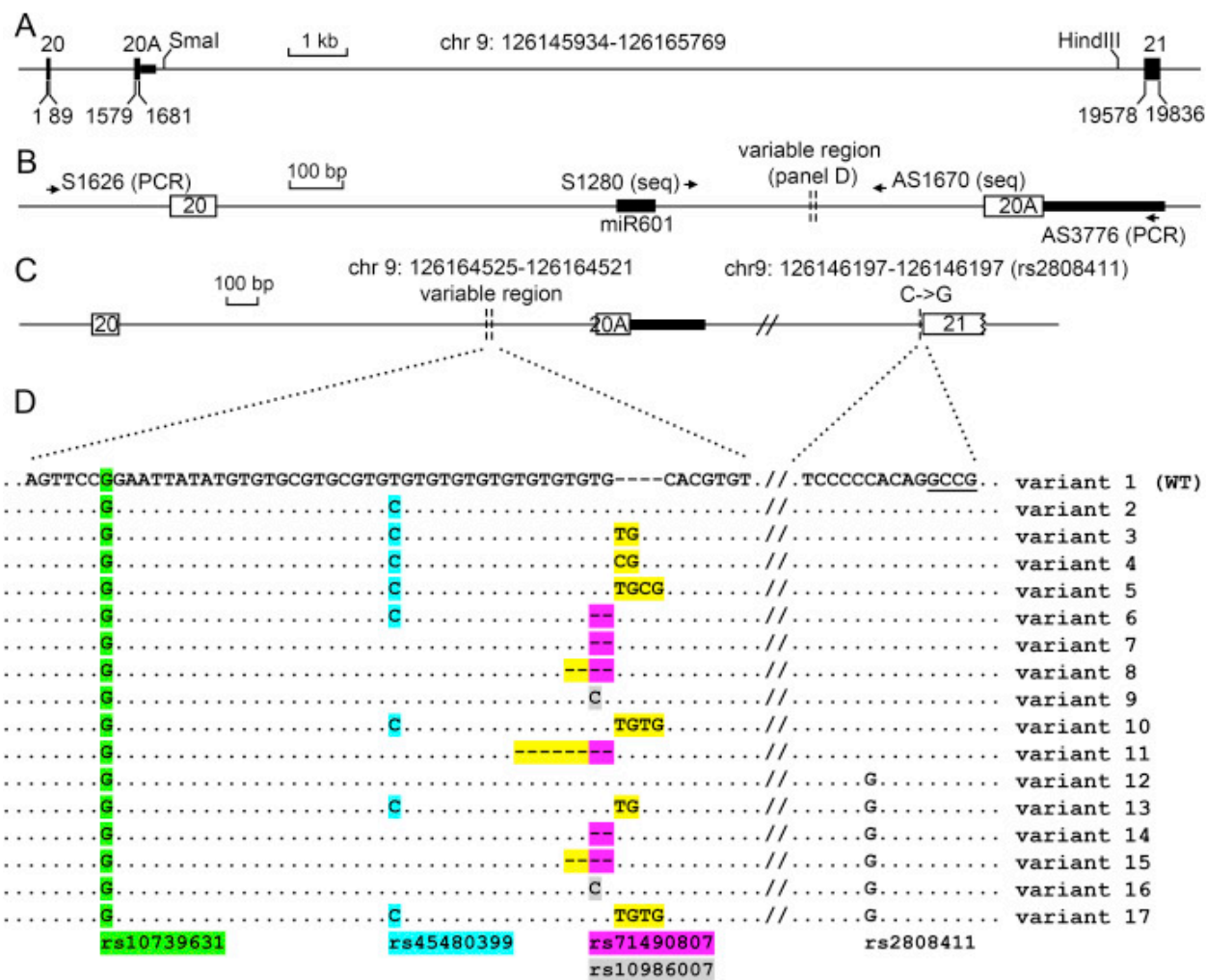


Figure 2. Diagram of the 3' end of the DENND1A gene and splicing variants identified taken from 'Tee MK, Speek M, Legeza B, Modi B, Teves ME, McAllister JM, Strauss JF, Miller WL'. Alternative splicing of DENND1A, a PCOS candidate gene, generates variant 2. *Mol. Cell. Endocrinol.* 2016;434:25–35 . A. Scale diagram of ~20 kb spanning DENND1A exons 20 and 21; bases are numbered starting with the first base of exon 20 (chr 9:126145934) designated as base 1; exons are shown as boxes. The intronic regions between exons 20 and 20A and between exons 20A and 21 are 1490 bp and 17,897 bp, respectively. B. Magnified diagram of the region between exons 20 and 20A; note the difference in

the scale bars in panels A and B. The 3' end of miR601 (shown as a heavy black line) is 282 bp upstream of the hypervariable region. The heavy black line following exon 20A represents the DNA encoding the 3' untranslated region of DENND1A V2 mRNA.

***RAB5B* AND PCOS**

Among the other loci identified in PCOS GWAS studies *RAB5B* is a prominent one. *RAB5B* is an isoform of *RAB5*, which functions as a member of endocytic pathway and is localized in the early endosomal compartments (24). All the three isoforms of *RAB5* have highly conserved phosphorylation consensus sites and have an 80% identity. It is speculated that these three isoforms can be differentially phosphorylated by different kinases, and this could vary the function of the three isomers (24).

In different GWAS studies (7,25,26), it has been found that *RAB5B* is associated with PCOS. *RAB5B* participates in endosome formation and endocytic cycling between plasma membrane and early endosomes. It is possible that it could interact with the DENN domain of *DENND1A*, which is another important candidate protein for PCOS and is even involved in endocytosis.

***LHCGR* AND PCOS**

LHCGR which stands for Luteinizing Hormone/Choriogonadotropin Receptor, is another important gene that came up in GWAS studies (7,27,28). This gene codes for the receptor for both luteinizing hormone and choriogonadotropin. *LHCGR* belongs to a G- protein coupled receptor-1 family whose activity is managed by the G-proteins that activate

adenylate cyclase. It has been found that polymorphisms involved in the *LHCGR* gene are linked with PCOS and other infertility disorders (27,29).

LH stimulation is known to be required for the excess ovarian androgen levels in PCOS and RAB5 proteins are known to play a role in endocytosis and gonadotropin receptor signaling. LHCGR stimulation by hCG is thought to activate the adenylate cyclase/ cyclic-AMP pathway ultimately leading to increased androgen production (29). In a previous study it was found that when human theca cells were treated with forskolin - a stimulator of adenylate cyclase, which ultimately activates the cAMP pathway, it leads to an increase of androgen production (7). Thus, it can be speculated that hCG and forskolin could induce steroidogenesis by a similar pathway.

It was even seen that *DENND1A* plays an important role in steroidogenesis, and it was seen that overexpression/ knockdown of *DENND1A.V2* in theca cells, results in augmented/ reduced *CYP17A1* expression and androgen secretion (7). Thus, it is possible to speculate an interaction between LHCGR and *DENND1A.V2*, which are two important proteins linked to PCOS.

HYPOTHESIS

RAB5B and LHCGR, along with *DENND1A*, form a network that might contribute to the hyperandrogenemia of PCOS. RAB5B and *DENND1A*, which are both involved in the early endocytosis processes and recycling, maybe involved in LHCGR internalization and processing, somehow leading to the characteristic over production of androgens, which is an important aspect in PCOS.

To explore this three protein network model, we examined the localization of DENND1A.V2-containing compartments and those containing LHCGR and RAB5B. This thesis reports the first studies on the localization of DENND1A.V2, evidence for interaction among compartments containing LHCGR and RAB5B, and the unexpected translocation of DENND1A.V2 and RAB5B proteins into the nucleus of theca cells, raising the intriguing possibility that LHCGR may initiate alterations in gene expression via vesicular trafficking and nuclear regulation of transcription of genes involved in androgen synthesis.

CHAPTER 2

MATERIALS & METHODS

Cell culture

Chinese hamster ovary (CHO) cells

CHO cells were cultured in tissue culture dishes (60×15 mm, Corning, NY) for Western blot studies and Falcon 8 well chamber tissue culture slide for immunofluorescence studies. Cells were grown in DMEM, high glucose pyruvate medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, Catalogue number: 10437028), 1% glutamine, and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA). At 60-70 % confluency cells were transfected with pCMV6-XL5 (OriGene Technologies, Inc. Rockville, MD) for DENND1A.V2 expression (the vector has a CMV promoter, ampicillin resistance marker for selection in prokaryote host, and the DENND1A.V2 gene is inserted in MCS between two BamHI sites), empty vector control (pCMV-BAM); pGFP2-N2-myc-hLHCGR (kindly provided by Dr. Deborah Segaloff) (30) using Continuum (Gemini Bio-products, West Sacramento, CA). The plasmid DNA to Continuum ratios were: DENND1A.V2 (5:1); LHCGR (2.5:1); and pCMV-BAM (5:1). Cells were harvested or fixed 48 h after transfection. In some experiments, 48 h after transfection cells were washed with DMEM medium with serum to remove the transfection medium. Cells were treated with hCG (Sigma- Aldrich, St. Louis, MO) at a concentration of 1 IU/ml for 0, 10, 30, 60, 90, 120, and 150 min prior to fixation.

Human theca cells

Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, following informed consent under a protocol approved by the Institutional Review Board of The Pennsylvania State University College of Medicine. As a standard of care, oophorectomies were performed during the luteal phase of the cycle. Theca cells from normal cycling and PCOS follicles were isolated and grown as we have as previously reported in detail (31,32). The theca cell preparations used in these studies have been described and characterized previously (31–39). The steroidogenic phenotypes of the normal and PCOS theca cells have been reported to result from the inherent properties of the cells, rather than the cycle phase at the time that they were isolated (33,35,40). PCOS and normal ovarian tissue came from age-matched women, 38–40 year old. The diagnosis of PCOS was made according to National Institutes of Health (NIH) consensus guidelines (8,41) which include hyperandrogenemia, oligoovulation, polycystic ovaries, and the exclusion of 21 α -hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels (33,35,40). Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21–35 days, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and pelvic pain. Experiments comparing PCOS and normal theca were performed using fourth-passage (31–38 population doublings) theca cells isolated from individual size-

matched follicles obtained from age-matched subjects, in the absence of in vivo stimulation. The use of fourth-passage cells allowed us to perform multiple experiments from the same patient population, and were propagated from frozen stocks of second passage cells in the media described above. The passage conditions and split ratios for all normal and PCOS cells were identical. Cells were grown until sub-confluent and treated with and without 20 μ M forskolin for 16 h in defined serum-free media. Similar treatments with 1IU/ml hCG were performed for 0, 10, 30, 60, 90, 120, and 150 min prior to fixation.

Rabbit polyclonal DENND1A.V2 antibody (rabbit 256 antibody)

A rabbit polyclonal antibody against a 20 amino acid peptide ([C]-QKSITHFAAKFPTRGWTSSSH) that is specific to DENND1A.V2, was generated by Thermo's Custom Antibody Service. The specificity of this rabbit polypeptide antibody for the 62 kDa DENND1A.V2 protein was validated by Western blotting and antibody neutralization with the DENND1A.V2 peptide.

Production of mouse monoclonal antibodies to DENND1A.V2 peptide

Mouse monoclonal antibodies to the same DENND1A.V2 peptide used for the monoclonal antibody generation using standard procedures (7) BALB/c mice were immunized using KLH-coupled peptide in RIBI adjuvant. Immunizations were delivered both subcutaneously and intra-peritoneally in volumes of 0.05 ml per site per mouse per

immunization. Immunizations were given bi-weekly for 3 weeks. The final booster immunization was given as KLH-peptide in saline. Three days after the final booster immunization, mice were anesthetized using ketamine/xylazine and spleen and lymph nodes were removed following exsanguination. Single cell suspensions of immune cells were prepared and fused with P3X63-Ag8.653 myeloma cells for the production of hybridomas. Supernatants from cultures of hybridomas were screened by ELISA for reactivity to the DENND1A.V2 peptide, and to BSA-peptide. Positive cultures were isolated for expansion and cloning. Clones producing reactive antibodies in ELISA were adapted to serum free conditions using Sigma serum-free culture media. High titer antibodies were produced using BD Biosciences cell line devices, purified using Protein A/G columns (Pierce, Rockford, IL). Antibody specificity was established by Western blotting and antibody neutralization with the DENND1A.V2 peptide.

Peptide neutralization experiments

Primary antibody was neutralized by preincubation with the DENND1A V2 specific peptide ([C]-QKSITHFAAKFPTRGWTSSSH; ChinaPeptides, Shanghai, China) for 30 min at room temperature. The peptide concentration was varied from 1 mg/ml to 0.1 µg/ml.

Immunoblotting

Transfected cells were lysed in cold RIPA buffer (Tris (50 mM), NaCl (150 mM), NP40 (1%), SDS (0.1%), deoxycholic acid (0.5 %), EDTA.Na₂ (1 mM), pH adjusted to 8) for 30 min at 4°C. Thirty µg protein per lane was loaded onto 10% SDS-PAGE gels,

electrophoretically separated, and transferred to PVDF membranes (Millipore, Billerica, MA) by semi-dry transference. Membranes were blocked for 1 h in 5% milk-TTBS (BIO-RAD, Hercules, CA) and then incubated overnight with anti-DENND1A.V2 polyclonal rabbit antibody (256 rabbit antibody: dilution 1:1,000), mouse monoclonal anti-DENND1A.V2 specific antibodies clones P2F5.7 and P5F9.4 (dilution 1:1,000), monoclonal IgG1 control antibody (dilution 1:1,000), or anti-DENND1A rabbit antibody (Aviva Systems Biology, Corp San Diego, CA: dilution 1:1,000). After several washes in 1X Tris buffer saline (TBS) supplemented with 0.02% Tween 20, the membranes were incubated with secondary antibodies anti-rabbit IgG or anti-mouse horseradish-peroxidase labelled (1:2,000 dilution) for 1 h at room temperature. Protein was detected with Super Signal Chemiluminescent Substrate (Pierce, Waltham, MA). β -actin antibody (Cell Signaling, Danvers, MA) was used as loading control to assure relatively equivalent amounts of protein were present amongst sample types.

Immunofluorescence (IF)

Transfected cells were fixed with 4% formalin for 1 h, washed with PBS twice and blocked with a blocking serum, containing 10% goat serum, 3% BSA and 0.2% Triton X-100. The cells were then incubated with anti-DENND1A.V2 rabbit polyclonal antibody (7) (dilution 1:100), mouse monoclonal anti-DENND1A.V2 specific antibodies clones P2F5.7 and P5F9.4 (dilution 1:50), and same isotype monoclonal IgG1 control antibody (dilution 1:50). Other antibodies used for IF were (Table 1) anti-RAB5B goat antibody (LifeSpan BioSciences, Seattle, WA), which is a specific antibody for RAB5B and does not interact with RAB5A and RAB5C, and anti-LHCGR mouse antibody (Novus Biologicals,

Centennial, CO). For detection of primary antibodies, the cells were incubated with secondary antibody (anti-mouse Alexa Fluor 594 labeled, anti-rabbit Cy3-labeled, anti-rabbit Alexa Fluor 488 labeled, anti-goat Alexa Fluor 488 labeled, or anti-mouse Alexa Fluor 488 labeled) for 1 h. In some experiments, before mounting the slides a Lectin (GNL/GNA)-Cy3-labeled (bioWORLD, Dublin, OH) was used to identify Golgi vesicles. The cells were then washed with PBS and mounted with VectaMount with DAPI (Vectorsheld, Vector Laboratories, Inc., Burlingame, CA), and then sealed with nail polish.

Table 1: Commercial Antibodies used

Sr. no.	Antibody type	Antibody Name	Dilution	Company	Catalog Number
1	Primary	LHR Antibody	IF: 1/50	Novus Biologicals, Centennial, CO	NBP2-52504
2	Primary	RAB5B	IF: 1/50 WB: 1/500	LifeSpan BioSciences, Seattle, WA	LS-C204235
3	Primary	β -Actin	WB: 1/2000	Cell Signaling Technology, Danvers, MA	#4967
4	Secondary	anti-mouse Alexa Fluor 488 labeled	IF: 1/3000	Jackson ImmunoResearch Laboratories, West Grove, PA	715-547-003
5	Secondary	anti-goat Alexa Fluor 488 labeled	IF: 1/3000	Jackson ImmunoResearch Laboratories, West Grove, PA	705-545-147
6	Secondary	anti-rabbit Alexa Fluor 488 labeled	IF: 1/3000	Jackson ImmunoResearch Laboratories, West Grove, PA	711-546-152
7	Secondary	anti-rabbit Cy3-labeled	IF: 1/5000	Jackson ImmunoResearch Laboratories, West Grove, PA	111-165-144

8	Secondary	<i>Galanthus nivalis</i> Lectin (GNL/GNA) - Cy3	30 µg/ml for 20 min	bioWORLD, Dublin, OH	21761108-1
9	Secondary	Anti-rabbit IgG horseradish peroxidase, linked whole antibody	WB: 1/2000	GE Healthcare, UK limited, Chicago, IL	NA934V
10	Secondary	Anti-mouse IgG horseradish peroxidase, linked whole antibody	WB: 1/2000	GE Healthcare, UK limited, Chicago, IL	NA931V

Imaging

Images were captured by Zeiss LSM 700 confocal laser-scanning microscope, and processed by Fiji(42) and CellProfiler software(43) (NIH). In order to confirm the results, z-stacks were taken to span across the cell. High resolution images were also captured by N-SIM microscope and reconstructed by NIS-Elements AR software.

Image analysis

The nuclear/cytoplasm staining ratios were calculated using CellProfiler version-3.1.5 software(43). Composite images were split into RGB panels and saved individually using Fiji(42). “Identify Primary Objects” module was used to select the nuclear area from the images. “Identify Secondary Objects” module was used to select the cells. This method finds dividing lines between clumped objects where the image stained for secondary objects shows a change in staining. A global threshold strategy which, calculates a single threshold value of the input image and uses that value to differentiate between foreground and background, was used to identify the secondary objects as the background of the image was relatively uniform. Then, “Identify Tertiary Objects” module was used to

identify the cytoplasm of each cell. The inputs to this module were “Edited Nucleus” and “Edited Cells”. The area from the “Edited Cells” was subtracted from that of the “Edited Nucleus” to get the object Cytoplasm. Fluorescence intensities of the selected nucleus and cytoplasm areas were determined and exported to an excel file using the module “Measure Object Intensity”. Finally, “Mean Intensity Data” was used to calculate the Nucleus/Cytoplasm ratio. Results were then expressed in a graph using InfoStat software (44). The ‘Coloc’ module of the Ziess Zen Black edition 2012, was used to estimate colocalization. The cellular areas were selected manually for this purpose, and the colocalization coefficients were used for data analysis. The colocalization analysis in this software is done on a pixel by pixel basis and thresholds (called as crosshairs in this software), were set according to the guidance given in the “Acquiring and Analyzing Data for Colocalization Experiments in AIM or ZEN Software” manual. This method gives us two colocalization coefficients, one coefficient for the colocalization of Protein A vs Protein B, and another coefficient for Protein B vs Protein A. These values quantify how much protein A colocalizes with protein B and vice versa (these two coefficients can have different values, as the amounts of protein A and B in the analyzed area can be different).

Statistical methods

Statistical analysis were performed using InfoStat (44) software. One way ANOVA and Newman-Keuls as post-hoc test were used to determine if there was any significant difference between the Nucleus/Cytoplasm ratios in normal and PCOS theca cells, with/without the forskolin treatment.

CHAPTER 3

RESULTS

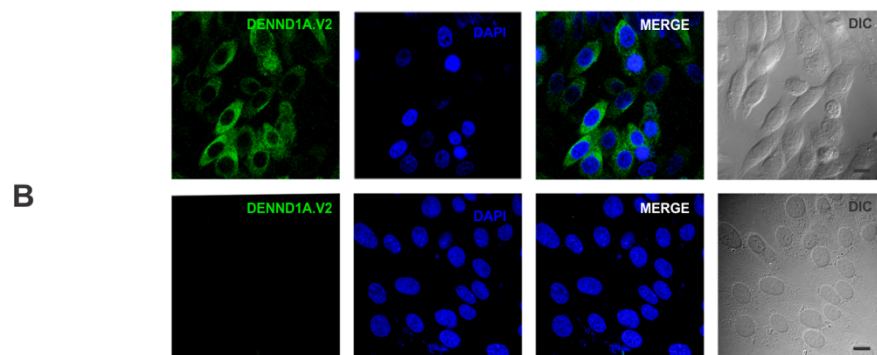
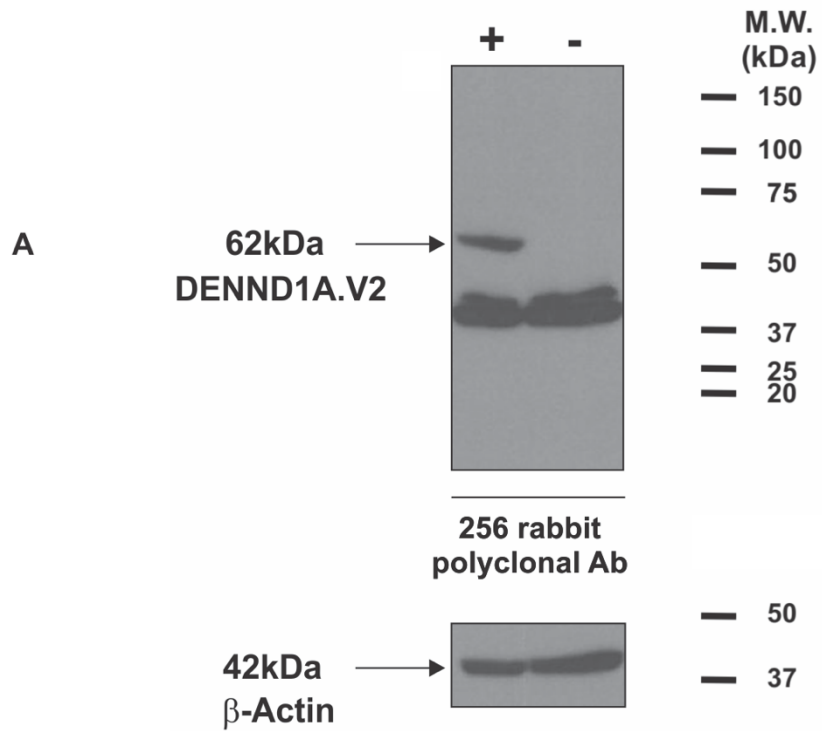
PART A: Polyclonal Antibody

Specificity of the polyclonal anti-DENND1A.V2 antibody

The specificity of the polyclonal anti-DENND1A.V2 antibody was evaluated by western blotting using whole cell lysates of CHO cells transfected with a vector expressing DENND1A.V2 (Fig. 3A). The antibody reacted with a protein band of the expected molecular weight (62 kDa) in extracts of DENND1A.V2 transfected cells, but not in empty vector transfected cell extracts. In both cases a non-specific band of lower molecular weight was detected. Additionally, the antibody was also tested in CHO cells transfected with DENND1A.V1 or DENND1B.V1 expressing vectors. It did not recognize V1 protein suggesting that it is specific to DENND1A.V2 protein (Fig. 4) The localization of DENND1A.V2 by IF was examined in CHO cells transfected with a plasmid expressing human DENND1A.V2. As shown in Figure 3B, DENND1A.V2 was localized in the cytoplasm of transfected cells, but no signal was detected in untransfected or empty vector transfected cells (data not shown). The anti-DENND1A.V2 signal was completely suppressed when IF was carried out with peptide-neutralized antibody (Fig. 3B).

IF of normal human theca cells and PCOS theca cells revealed DENND1A.V2 present in a punctate pattern in the cytoplasm and the nucleus. The IF signals were completely suppressed when studies were carried out with neutralized antibody at peptide concentrations of 10 µg/ml and 1 µg/ml (Fig. 3C). The significant nuclear localization in

human theca cells differs from the pattern seen in transfected CHO cells (Fig. 3B).



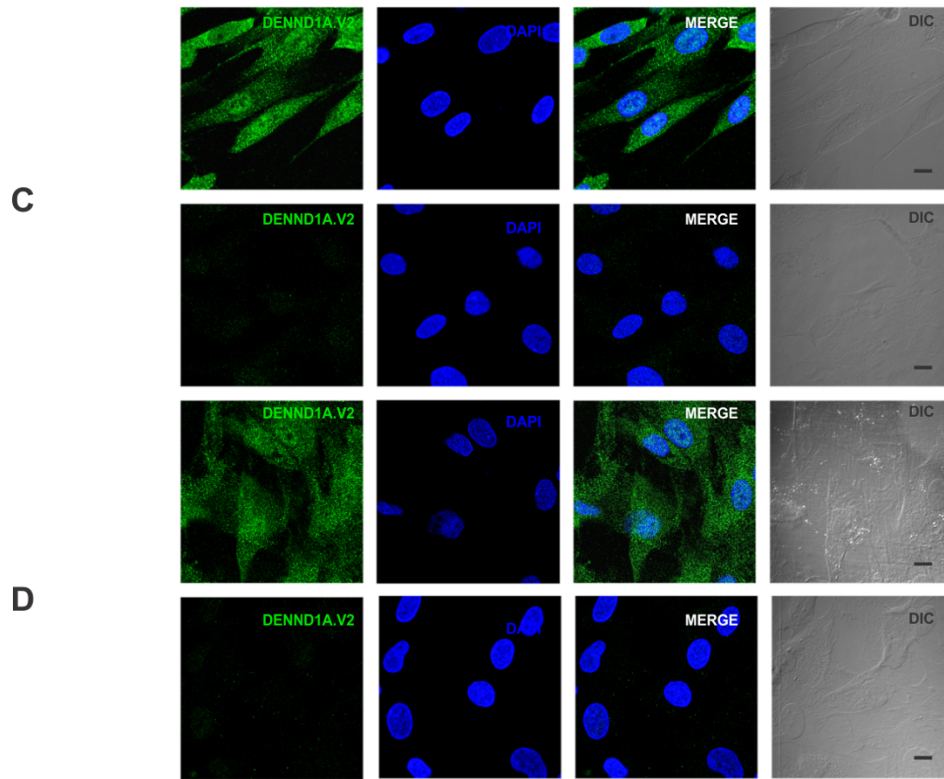


Figure 3. Characterization of the polyclonal antibody used to detect DENND1A.V2.

Representative full-length western blot of extracts of CHO cells transfected with a plasmid (pCMV6-XL5) expressing human DENND1A.V2 (+) or empty plasmid vector transfected cells (-). β -actin was used as a protein loading control. B. Top Panel: IF detection of DENND1A.V2 in transfected CHO cells. Bottom Panel: Suppression of IF in studies with neutralized polyclonal anti-DENND1A.V2 antibody. C. Top Panel: IF detection of DENND1A.V2 in normal theca cells. Bottom Panel: The absence of signal when antibody was neutralized with immunogenic peptide. D. Top Panel: IF detection of DENND1A.V2 in PCOS theca cells. Bottom Panel: The absence of signal when antibody was neutralized

with immunogenic peptide. Size bars =10 μm . Representative images, from n number of experiments, where n=3.

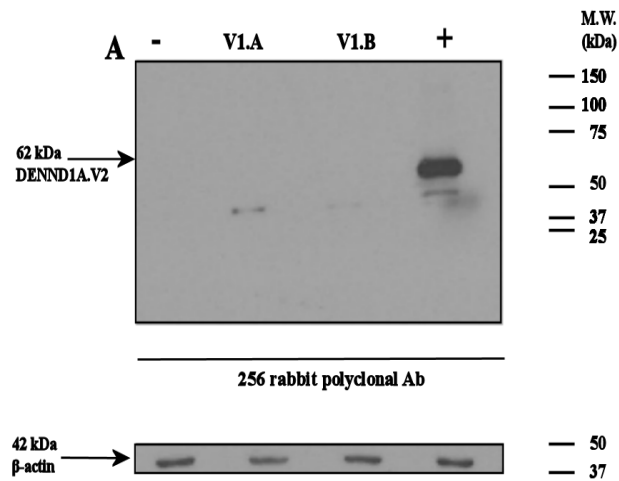


Figure 4. DENND1A.V2 antibody is specific for V2 and do not react with V1. Antibody specificity was evaluated by western blotting using total proteins extracted from CHO cells transfected to express DENND1A.V1 and DENND1B.V1. A, full-length blotting using 256 rabbit antibody. Representative images, from n number of experiments, where n=3.

Localization of DENND1A.V2 and LHCGR in transfected CHO cells.

CHO cells were co-transfected with the human DENND1A.V2 expression plasmid and pGFP2-N2-myc-hLHCGR. After 48 h, the cells were fixed with 4% formalin and then treated with the primary 256 DENND1A.V2 polyclonal antibody. The cells were later

incubated with secondary Cy3-labelled anti-rabbit antibody. GFP tagged LHCGR was visualized directly. In the presence of LHCGR, DENND1A.V2 appears to move towards the plasma membrane and colocalize with LHCGR. (Fig. 5C). However, the GFP tagged LHCGR maintains the same localization in presence or absence of DENND1A.V2 (Fig. 5 B and C).

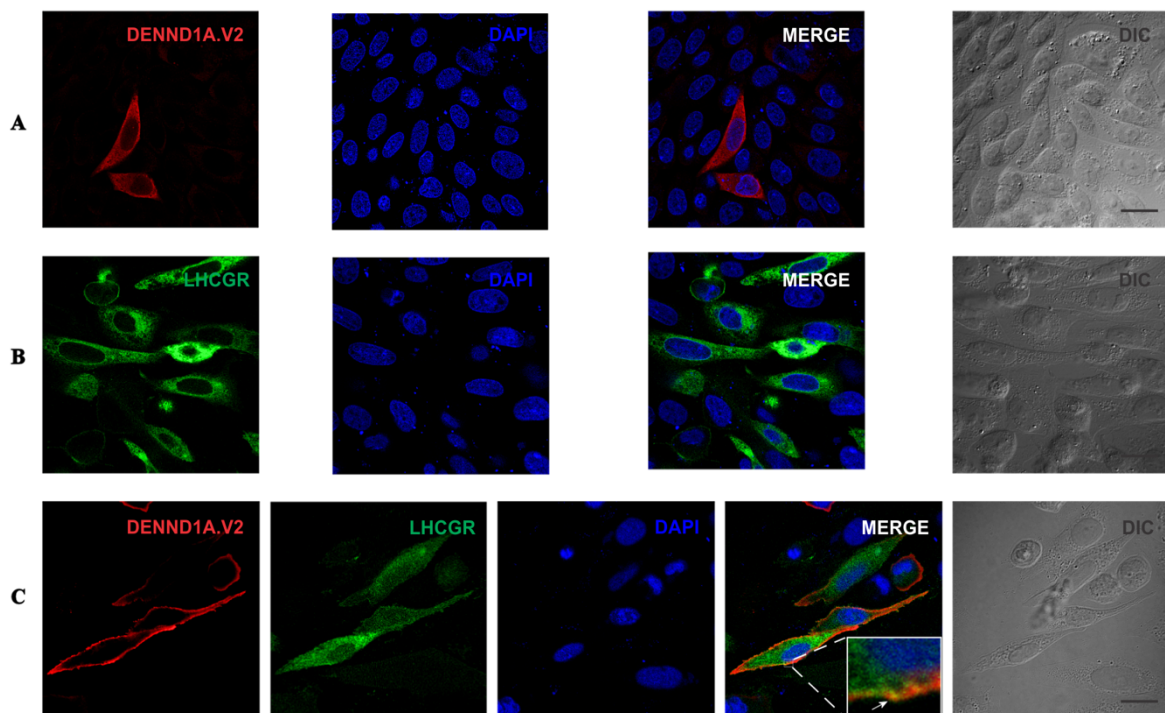


Figure 5. Localization of DENND1A.V2 and LHCGR in co-transfected CHO cells. A, CHO cells transfected to express DENND1A.V2, detected by polyclonal antibody. B, CHO cells transfected with GFP tagged human LHCGR. C, CHO cells co-transfected with DENND1A.V2 and LHCGR. Insert shows colocalization of DENND1A.V2 and LHCGR at the plasma membrane. Size bar=10 μ m. Representative images, from n number of experiments, where n=3.

Co-localization of LHCGR and DENND1A.V2 in transfected CHO cells and effect of hCG treatment.

CHO cells were transfected with DENND1A.V2 expression plasmid, pGFP2-N2-myc-hLHCGR, or both together. After 48 h, the cells were washed with DMEM media with serum to remove the transfection medium. Then the cells were treated with hCG at a concentration of 1 IU/ml for the time points 0, 10, 30, 60, 90, 120, and 150 min, and fixed after several washes. There was no appreciable change in localization of DENND1A.V2, in presence of hCG in the cells transfected with DENND1A.V2 alone (Fig. 6), whereas LHCGR was seen to move towards the peri-nuclear (presumptive Golgi) area with an increasing incubation time with hCG (Fig. 7). However, in presence of hCG in cells transfected with both DENND1A.V2 and LHCGR, DENND1A.V2 appeared to move into the cytoplasm, with less staining in the plasma membrane, and colocalization with LHCGR in the peri-nuclear area after increasing time of hCG treatment (Fig. 8). At later time points of hCG treatment, there appeared to be some nuclear punctate DENND1A.V2 signals.

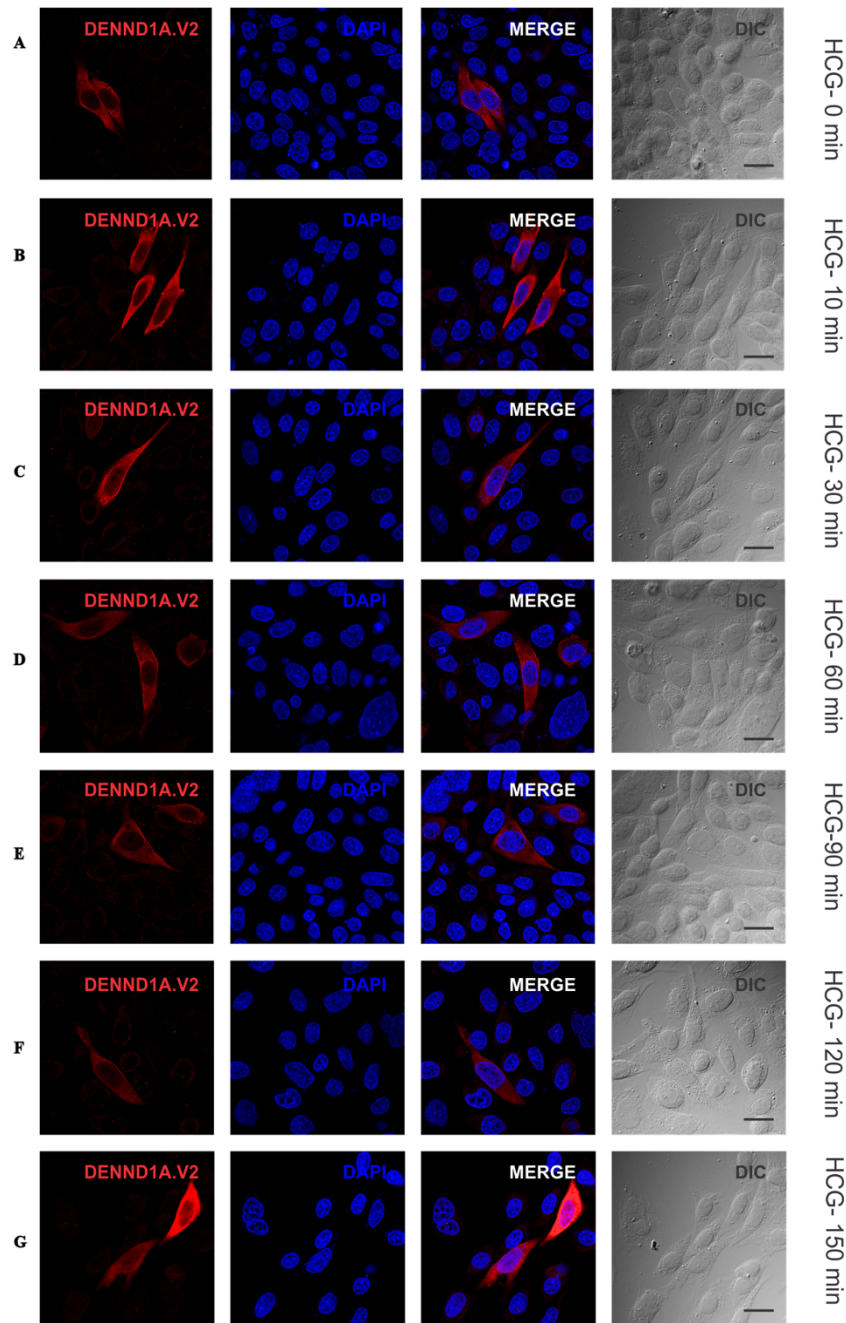


Figure 6. Time-course of hCG treatment on the localization of DENND1A.V2 in transfected CHO cells. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. Size bars= 10 μ m. Representative images, from n number of experiments, where n=3.

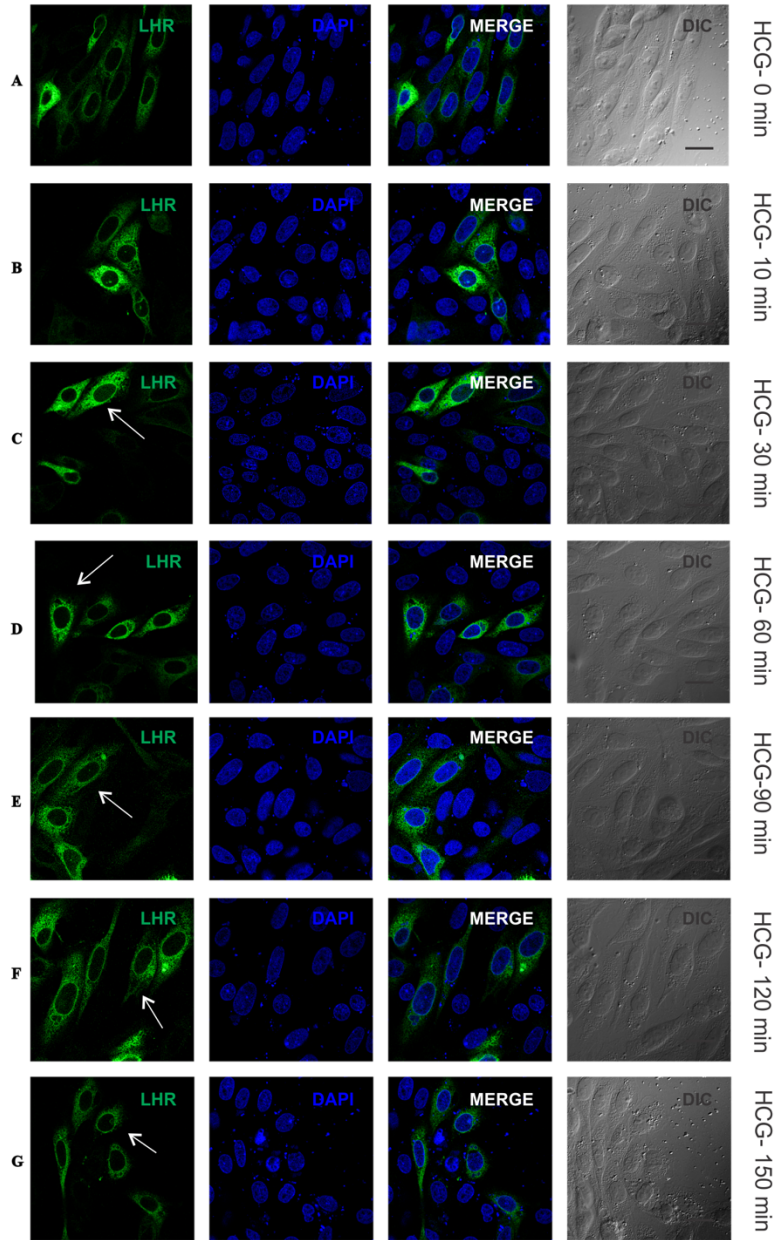


Figure 7. Time-course of hCG treatment influences the localization of human LHCGR in transfected CHO cells. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150

min. White arrows point to the peri-nuclear, presumptive Golgi, area. Size bars= 10 μ m.

Representative images, from n number of experiments, where n=3.

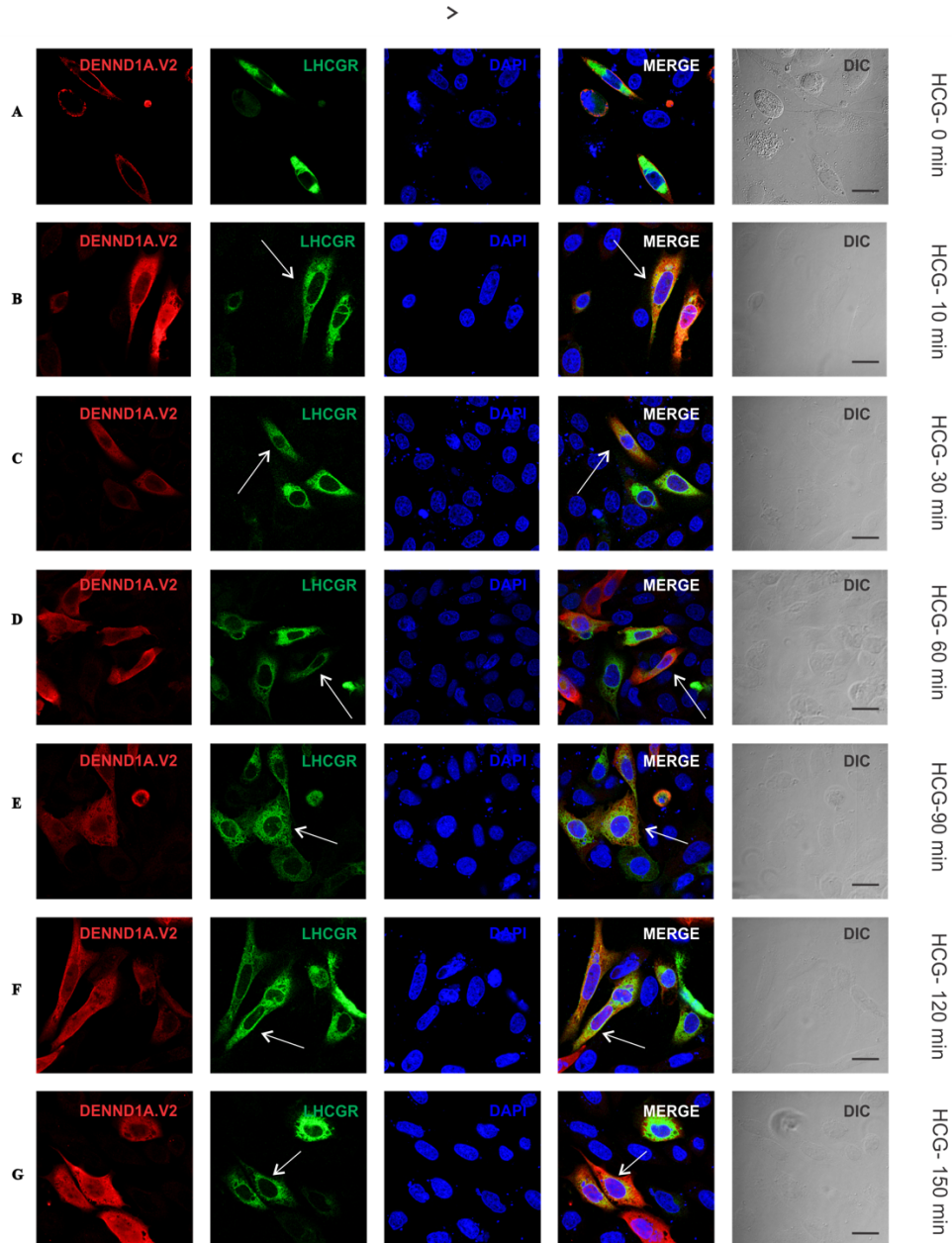


Figure 8. Time-course of hCG treatment on the localization of DENND1A.V2 and LHCGR in transfected CHO cells. CHO cells were transfected to express DENND1A.V2

and GFP tagged LHCGR, and treated with 1 IU/ml hCG for the time points of : A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. White arrows point to the peri-nuclear, presumptive Golgi, area. Size bars= 10 μ m. Representative images, from n number of experiments, where n=3.

Co-localization of DENND1A.V2 and RAB5B in human theca cells.

As noted previously, the guanine nucleotide exchange factor DENND1A is reported to interact with the RAB GTPase, RAB35. Given that RAB5B was identified as a PCOS GWAS candidate, we hypothesized that RAB5B might also interact with DENND1A, particularly DENND1A.V2. Thus, studies were performed to explore whether DENND1A.V2 co-localizes with RAB5B in normal and/or PCOS theca cells. Figure 9A and C shows that some DENND1A.V2 co-localizes with RAB5B in both normal and PCOS theca cells. High-resolution images were captured and analyzed with an N-SIM microscope. Figure 9E shows co-localization of DENND1A.V2 and RAB5B in vesicle-like structures, with a high colocalization co-efficient of about 80% for RAB5B and around 50% for DENND1A.V2 (Fig. 9F).

Figure 10 shows the 3D and orthogonal images of theca cells stained using anti-DENND1A.V2 polyclonal antibody and goat polyclonal anti-RAB5B. Greater nuclear staining for DENND1A.V2 and RAB5B was detected in PCOS theca cells as compared to normal theca cells, as indicated by the orthogonal z-stack (Fig 10).

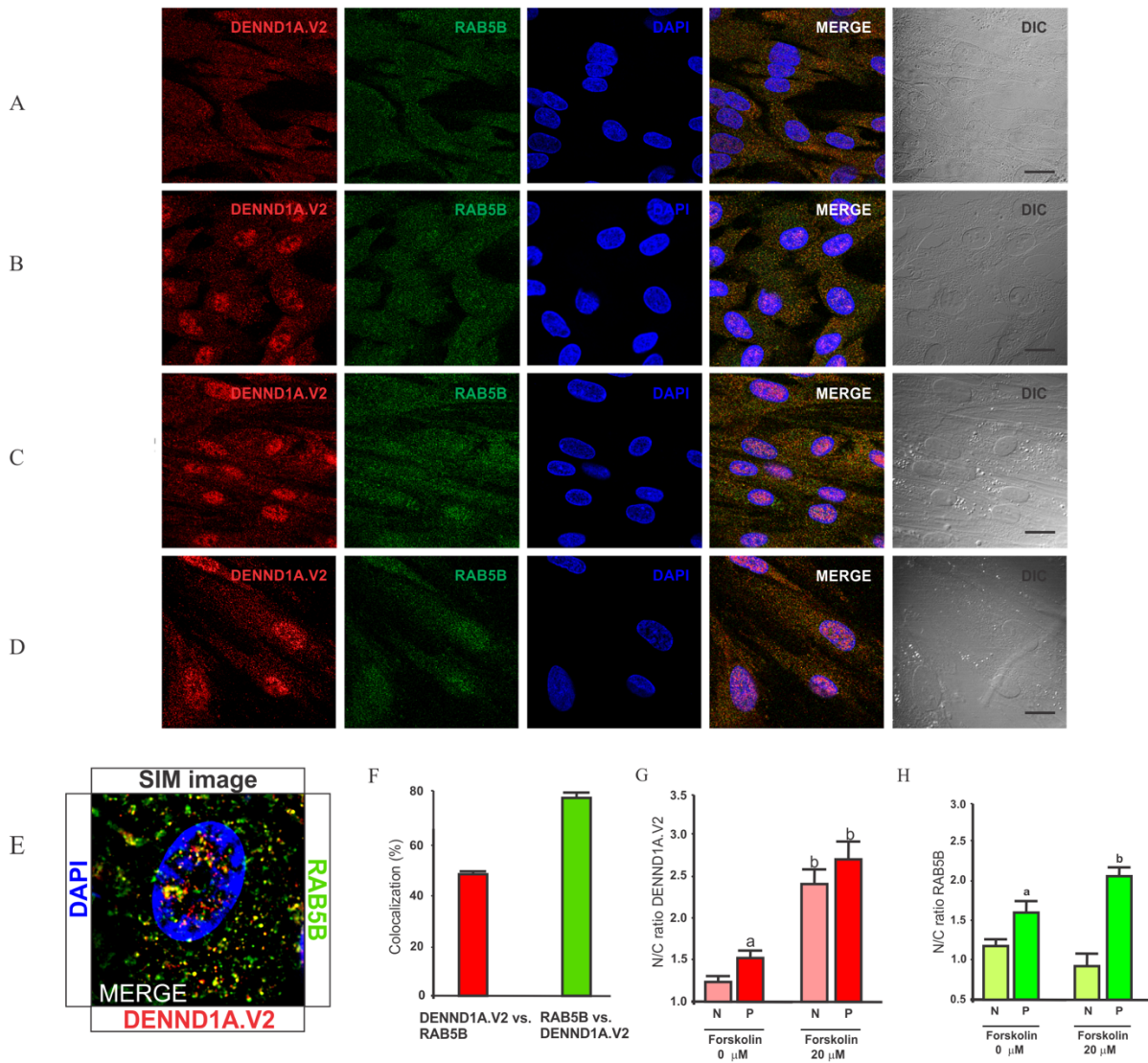
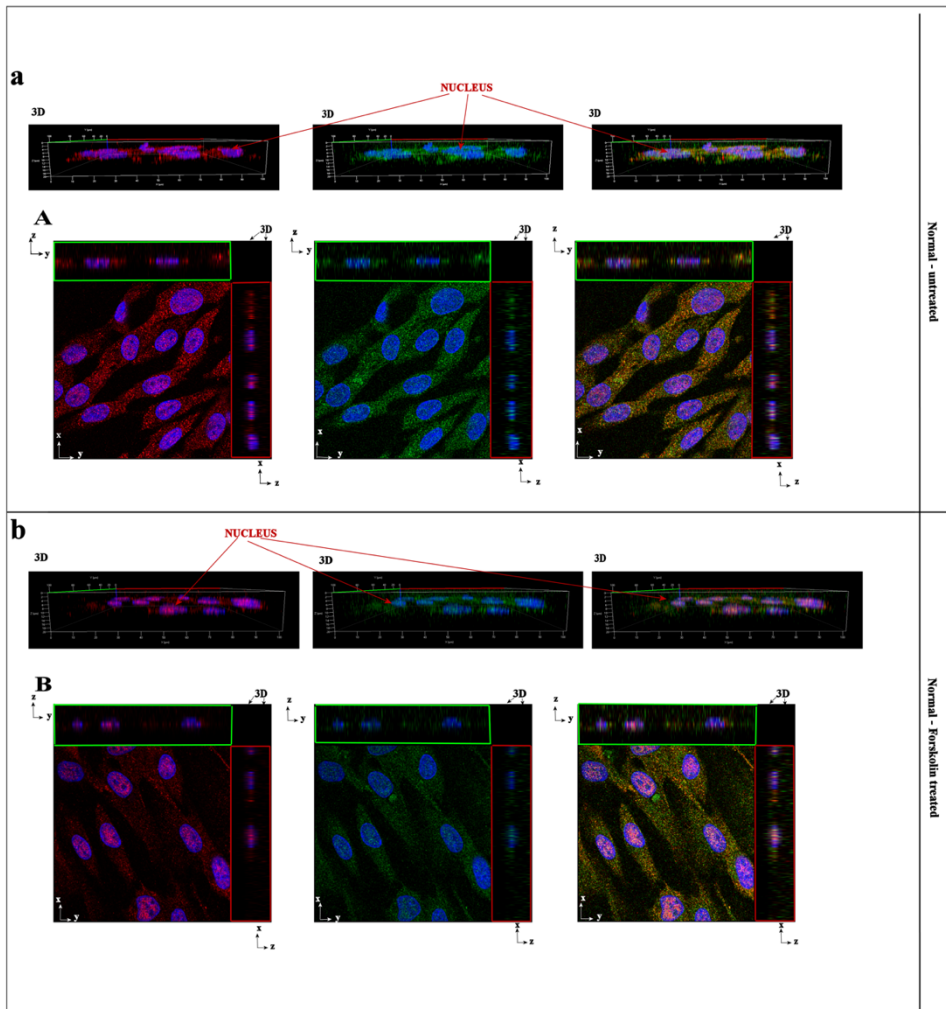


Figure 9. DENND1A.V2 co-localizes with the endosome protein RAB5B in normal and PCOS theca cells. A, Normal untreated theca cells. B, Normal Forskolin treated theca cells. Size bars= 10 μ m. C, PCOS untreated theca cells. D, PCOS theca cells treated with 20 μ M forskolin. E, A representative image captured by using SIM microscope, showing colocalization at higher magnification. F, A graph showing percent

of colocalization between DENND1A.V2 and RAB5B. G, Quantification of nucleus/cytoplasm ratio for DENND1A.V2 expression. ^a Statistically significant differences between P vs N without forskolin treatment, $p= 0.006$. ^b Statistically significant differences between 0 μ M vs 20 μ M forskolin treatment, $p=0.0001$ (One way ANOVA and Newman-Keuls as post-hoc test). H, Quantification of nucleus/cytoplasm ratio for RAB5B expression. ^a Statistically significant differences between P vs N without forskolin treatment, $p=0.0001$. ^b Statistically significant differences between 0 μ M vs 20 μ M forskolin treatment, $p=0.0001$ (One way ANOVA and Newman-Keuls as post-hoc test). Representative images, from n number of experiments, where $n=3$.



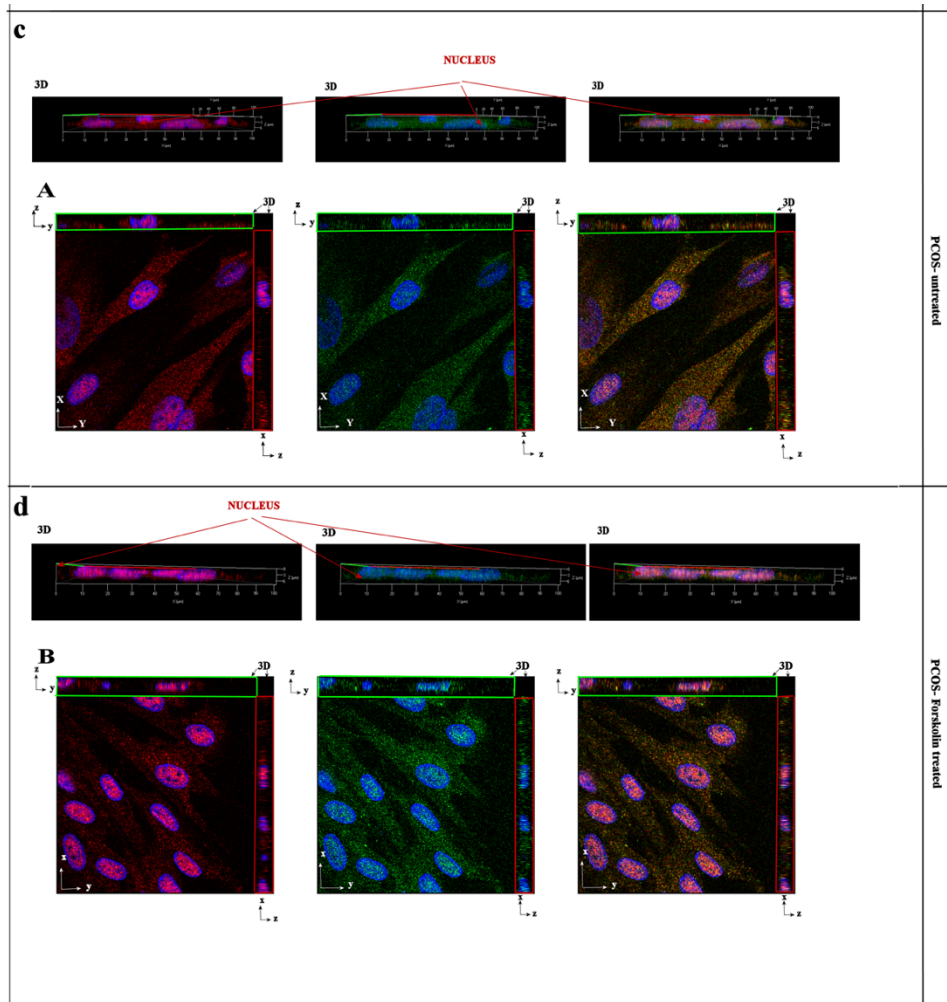


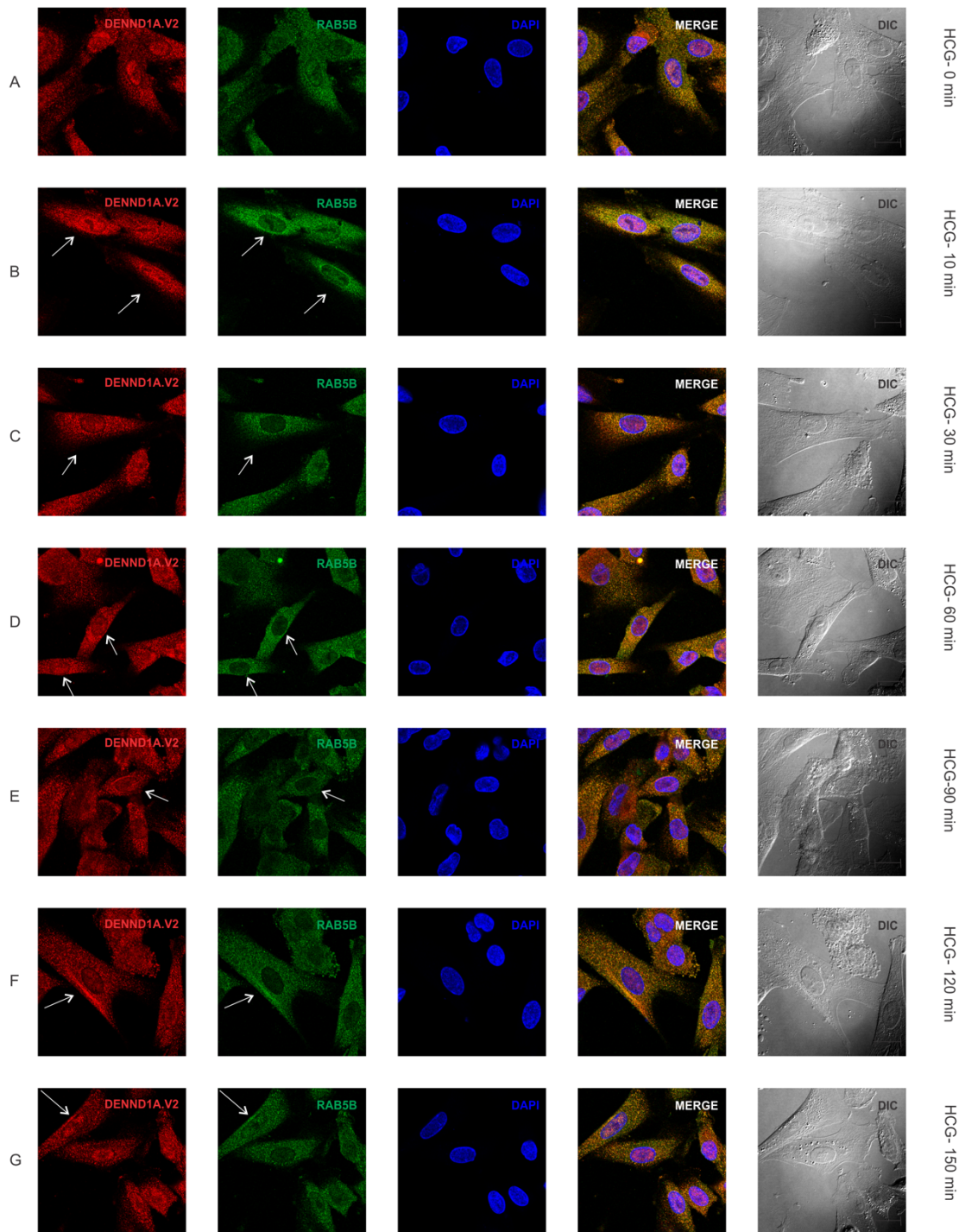
Figure 10. 3D reconstruction image showing colocalization of DENND1A.V2 and RAB5B in the cytoplasm as well as the nucleus of normal and PCOS theca cells.

Top panels show 3D side view and bottom panels show 2D front and z stack view for x and y coordinates. A normal untreated cells; C, PCOS theca cells. Representative images, from n number of experiments, where n=3.

The effect of hCG treatment on DENND1A.V2, RAB5B and LHCGR localization in normal and PCOS theca cells.

To determine if there was any change in localization of DENND1A.V2 RAB5B and LHCGR in normal and PCOS theca cells treated with hCG at various time points. Normal and PCOS theca cells were treated with hCG at a concentration of 1 IU/ml for 0, 10, 30, 60, 90, 120, and 150 min, and were fixed at the appropriate time points. In normal theca cells, was observed that DENND1A.V2 and RAB5B colocalize, but the percentage of colocalization was unaffected in response to hCG treatment (Fig. 11H and I). However, DENND1.V2 and LHCGR showed increases in the percentage of colocalization at 150 min of hCG treatment (Fig.13H). In addition, hCG treatment seems to have influenced translocation of DENND1A.V2, RAB5B and LHCGR towards the Golgi area, with increasing time of hCG treatment (indicated by arrows in figures 11 and 13). Similar results were observed with PCOS theca cells. However, DENND1A.V2, RAB5B and LHCGR movement was delayed to a later time point than that observed in normal theca cells (Fig. 12 and 14). The percentage of colocalization of DENND1A.V2 and LHCGR was significantly lower at 90 and 120 minutes of hCG treatment (Fig. 14H), perhaps reflecting different kinetics of protein/vesicle compartment movement.

Z-stack analysis was performed to determine whether LHCGR entered into the nucleus and no nuclear receptor was detected (Fig.15). In order to verify that DENND1A.V2, RAB5B and LHCGR were localizing in the Golgi, cells were stained with Cy3-labeled lectin specific for Golgi and Golgi vesicles. Figure 16 shows some colocalization of all three proteins with a lectin Golgi vesicle marker.



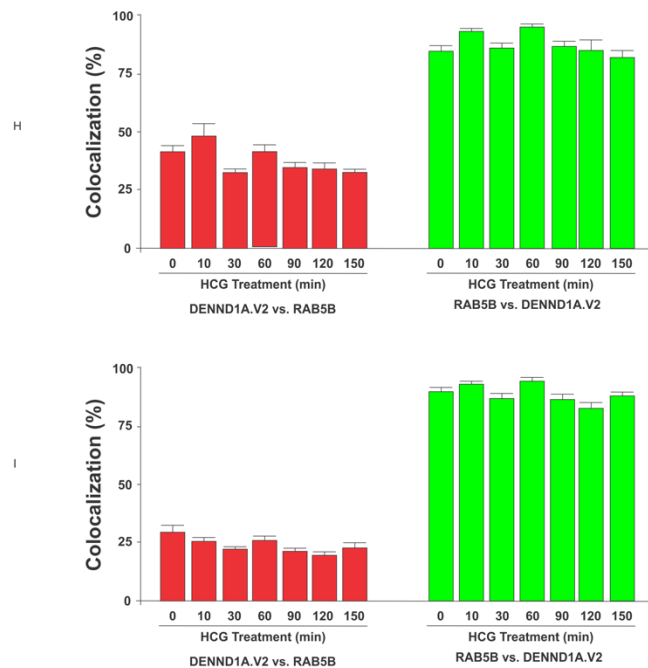
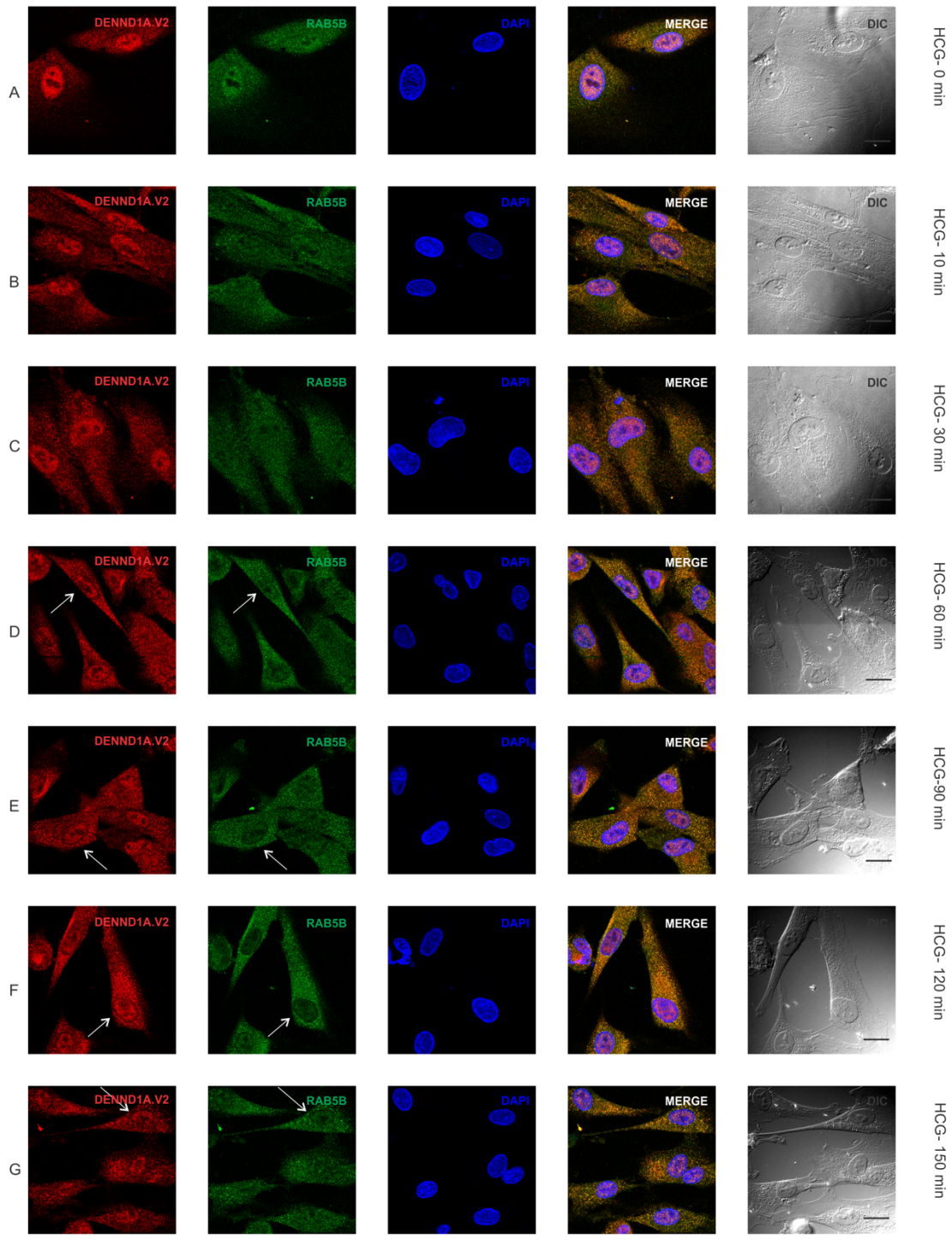


Figure 11: Time-course of hCG treatment of normal theca cells on the localization of DENND1A.V2 and RAB5B. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. White arrows point to peri-nuclear areas. Size bars = 10 μ m. H, Graph showing high colocalization co-efficient of about 90% for RAB5B with DENND1A.V2 and around 45% for DENND1A.V2 with RAB5B in Golgi area. I, Graph showing high colocalization co-efficient of about 90% for RAB5B with DENND1A.V2 and around 30% for DENND1A.V2 with RAB5B in nucleus. Representative images, from n number of experiments, where n=3.



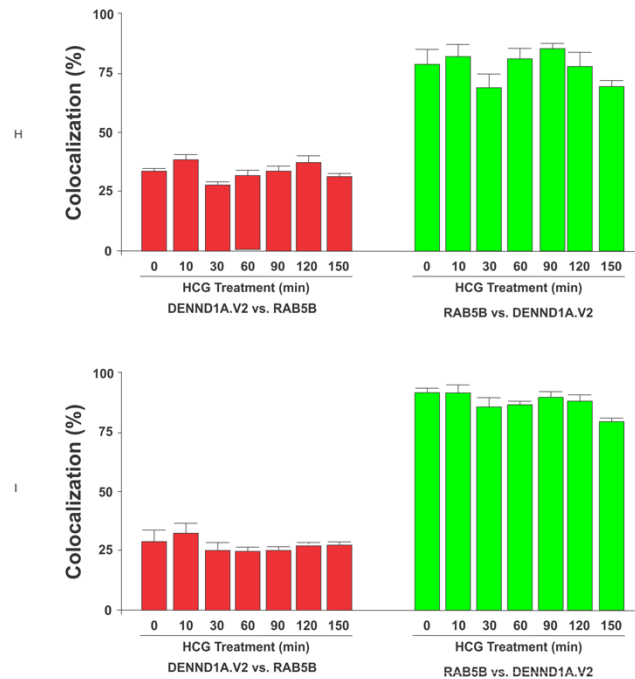
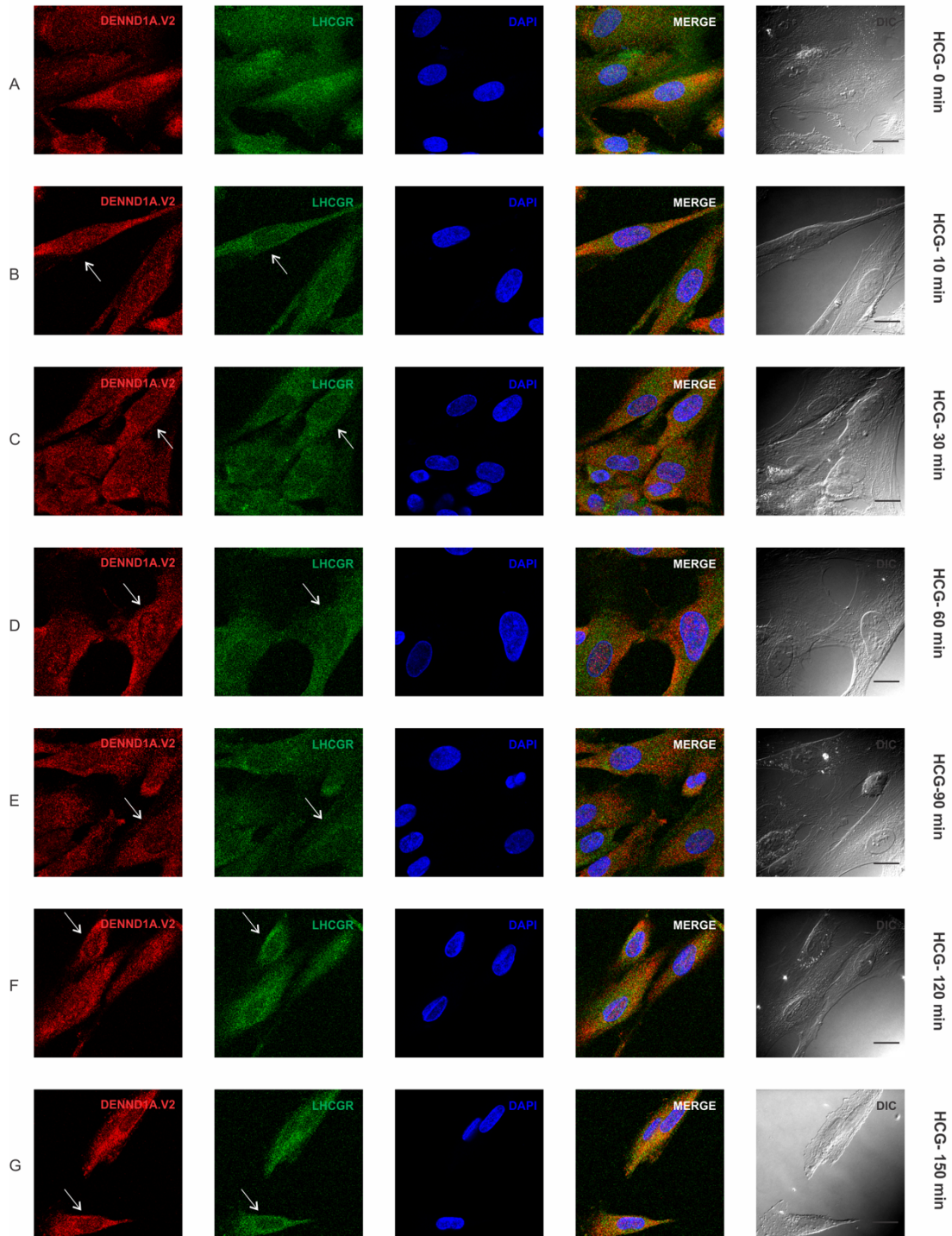


Figure 12. Time-course of hCG treatment of PCOS theca cells and the localization of DENND1A.V2 and RAB5B. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. White arrows point to peri-nuclear areas. Size bars =10 μ m. H, Graph showing high colocalization co-efficient of about 80% for RAB5B with DENND1A.V2 and around 30% for DENND1A.V2 with RAB5B in Golgi area. I, Graph showing high colocalization co-efficient of about 80% for RAB5B with DENND1A.V2 and around 25% for DENND1A.V2 with RAB5B in nucleus. Representative images, from n number of experiments, where n=3.



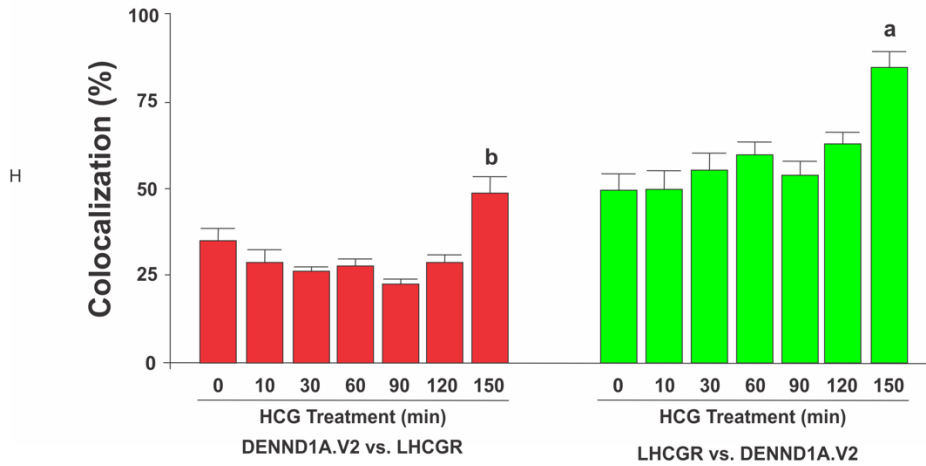
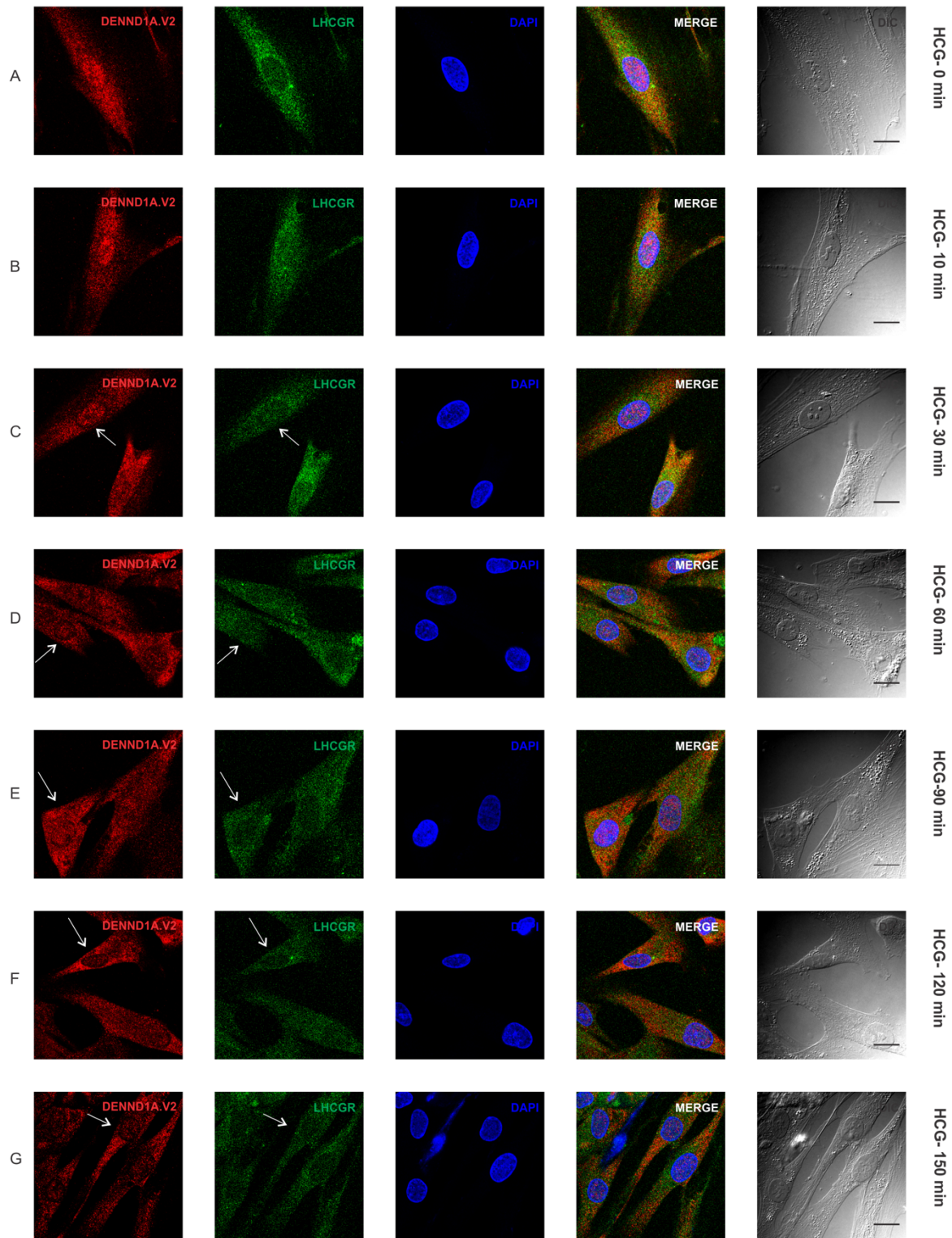


Figure 13. Time-course of hCG treatment of normal theca cells on localization of DENND1A.V2 and LHCGR. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. White arrows point to peri-nuclear areas. Size bars =10 μ m. H, Graph showing high colocalization co-efficient of about 70% for LHCGR with DENND1A.V2 and around 35% for DENND1A.V2 with RAB5B in peri-nuclear area. Representative images, from n number of experiments, where n=3.



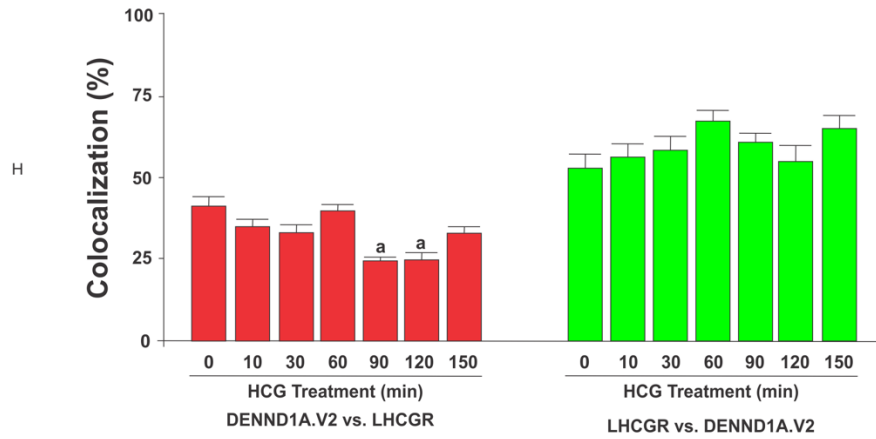


Figure 14. Time-course of hCG treatment of PCOS theca cells and the localization of DENND1A.V2 and LHCGR. Cells were treated with 1 IU/ml hCG for the time points of: A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 90 min; F, 120 min; and G, 150 min. Increasing concentrations of hCG drives DENND1A.V2 and LHCGR movement towards the Golgi area (white arrows) but at a latter time point than that with normal theca cells. H, Graph showing high colocalization co-efficient of about 70% for LHCGR with DENND1A.V2 and around 40% for DENND1A.V2 with RAB5B in peri-nuclear area. Representative images, from n number of experiments, where n=3.

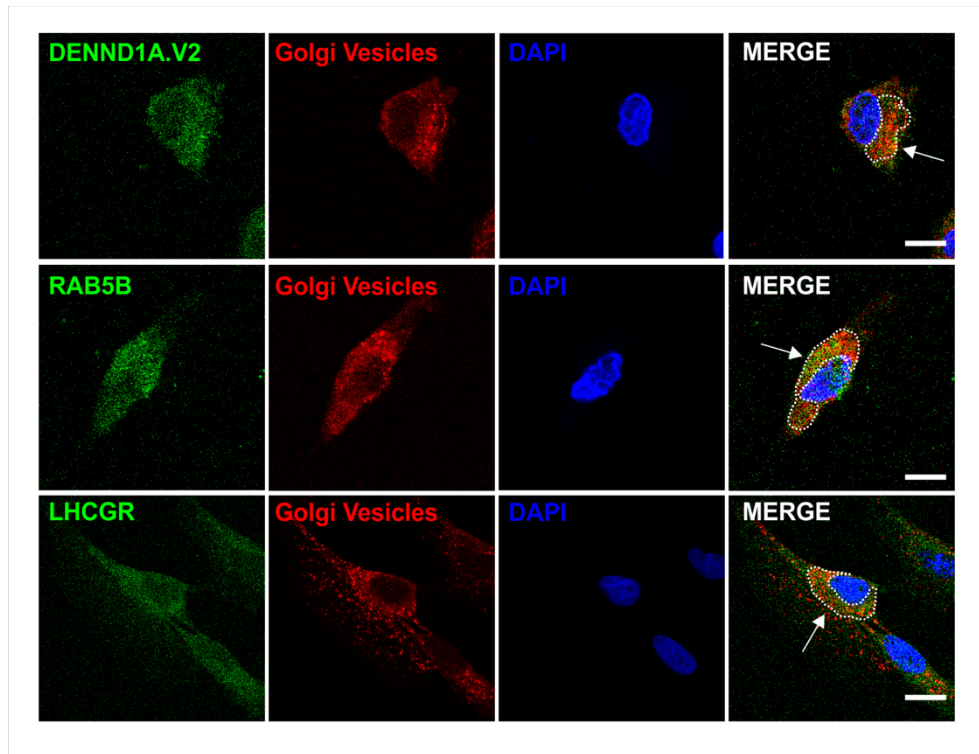


Figure 15: Representative figures of DENND1A.V2, RAB5B and LHCGR localizing in the Golgi. A, DENND1A.V2 with Golgi vesicles. B, RAB5B with Golgi vesicles. C LHCGR with Golgi vesicles.

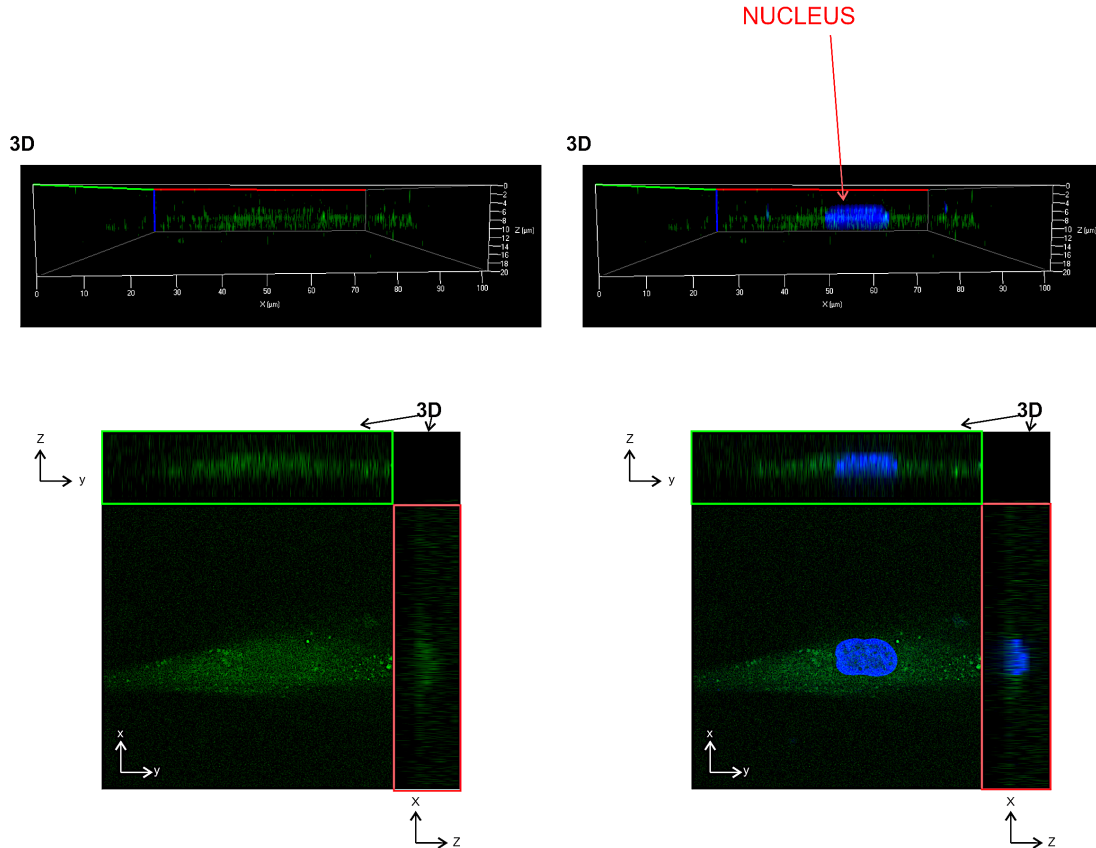


Figure 16. Representative 3D reconstruction image showing LHCGR does not enter nucleus. Top panels show 3D side view and bottom panels show 2D front and z stack view for x and y coordinates for untreated PCOS cells.

Effect of forskolin treatment on localization of DENND1A.V2 and RAB5B in human theca cells.

The hCG-triggered movement of DENND1A.V2 and the increased nuclear localization of DENND1A.V2 in the nuclei of PCOS theca cells raised the possibility that the nuclear accumulation of DENND1A.V2 might be the result of altered signal transduction involving cAMP, which is known to stimulate thecal androgen production. To evaluate this

possibility, normal and PCOS theca cells were treated with or without 20 μ M forskolin for 24 h prior to fixation to determine if there is difference in localization of DENND1A.V2 and RAB5B (Fig. 9B and D). It has been previously shown that forskolin treatment increases theca cell androgen biosynthesis and expression of genes encoding enzymes involved in androgen production (7). In the presence of forskolin, the PCOS theca cells showed significantly more nuclear staining for DENND1A.V2 and RAB5B (higher N/C ratio) (Figure 9G and H). However, there was no significant change in normal theca cells in the case of RAB5B.

PART B: Monoclonal Antibodies

Specificity of the monoclonal anti-DENND1A.V2 antibody and its use in immunofluorescence studies (IF).

Specificity of anti-DENND1A.V2 antibodies was evaluated by western blot using the whole cell lysates of CHO cells transfected with a vector expressing DENND1A.V2. It was observed all the two antibodies correctly detected the protein of interest (Fig. 17). Additionally, these antibodies were also tested in CHO cells transfected with DENND1A.V1 or DENND1B.V1 expressing vectors. None of these two antibodies recognized V1 protein suggesting they are specific to DENND1A.V2 protein (Fig. 18). The localization of DENND1A.V2 by IF was examined in CHO cells transfected with a plasmid expressing human DENND1A.V2 (pCMV6-XL5). Some DENND1A.V2 was localized to the plasma membrane, but the majority was in the cytoplasm of transfected cells using monoclonal anti-DENND1A.V2 antibodies. The signals were completely suppressed when IF was carried out with peptide-neutralized antibodies (Fig. 19). In transfected CHO cells, there was no nuclear staining, verified when z-stack images were obtained (Fig. 20).

The two mouse monoclonal antibodies detected DENND1A.V2 in patterns identical to the rabbit polyclonal antibody in transfected CHO cells, but the monoclonal antibodies gave no signal in IF staining of human theca cells (data not shown). These disparities suggest that there may be structural differences in the DENND1A.V2 C-terminus in theca cells (e.g., post-translational modification), or antigen masking due to protein-protein interaction, which prevent interaction with the monoclonal antibodies. Another possibility

could be that the monoclonal antibodies are more specific but less sensitive than the polyclonal antibody.

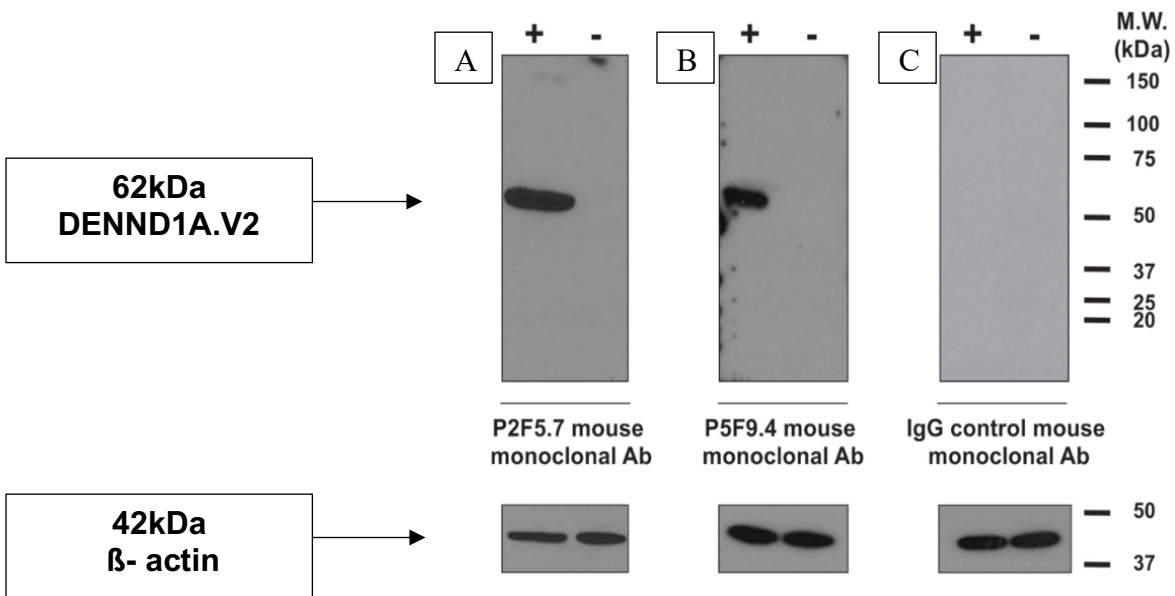


Figure 17: Characterization of antibodies used to detect DENND1A.V2.

Representative full-length western blottings of CHO cells transfected to express DENND1A.V2. All the antibodies used specifically recognize the 62kDa DENND1A.V2 protein. Representative β -Actin for each blotting are shown in the lower panels, A, P2F5.7 mouse monoclonal antibody. B, P5F9.4 mouse monoclonal antibody. C, IgG control mouse monoclonal antibody. +CHO cells transfected with pCMV6-XL5 plasmid to express DENND1A.V2 as the positive control. -CHO cells transfected with empty vector pCMV-BAM, as a negative control.

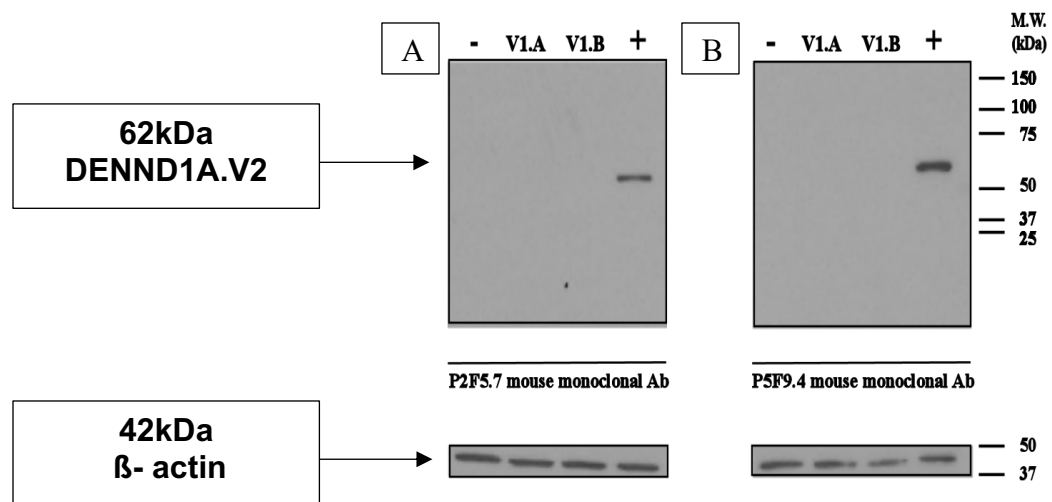


Figure 18: DENND1A.V2 monoclonal antibodies are specific for DENND1A.V2 and do not react with DENND1A.V1. Antibody specificity was evaluated by western blotting using total proteins extracted from CHO cells transfected to express DENND1A.V1 and DENND1B.V1. A, using P2F5.7 mouse monoclonal antibody. B, P5F9.4 mouse monoclonal antibody. - Empty vector pCMV-BAM, as a negative control. DENND1A.V1 using pCMV Tag2B plasmid. DENND1B.V1 using human pCDNA3-FLAG. + DENND1A.V2 using pCMV6-XL5 plasmid as positive control.

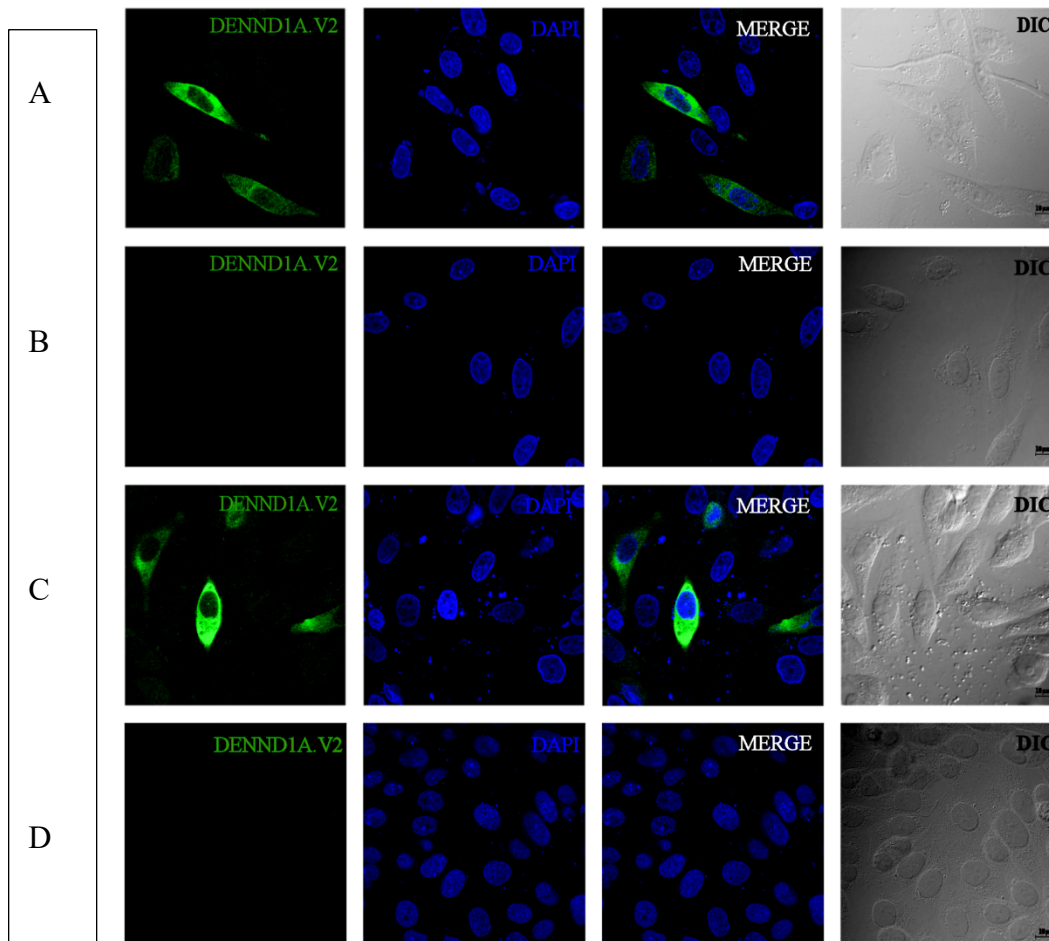


Figure 19: DENND1A.V2 localizes in the cytoplasm of transfected CHO cells.

CHO cells were transfected with pCMV6-XL5 plasmid to characterize the subcellular localization of DENND1A.V2 . A, DENND1A.V2 detection by P2F5.7 mouse monoclonal antibody. B, Peptide neutralization assay for P2F5.7 mouse monoclonal antibody. C, DENND1A.V2 detection by P5F9.4 mouse monoclonal antibody. D, Peptide neutralization assay for P5F9.4 mouse monoclonal antibody. Representative images, from n number of experiments, where n=3.

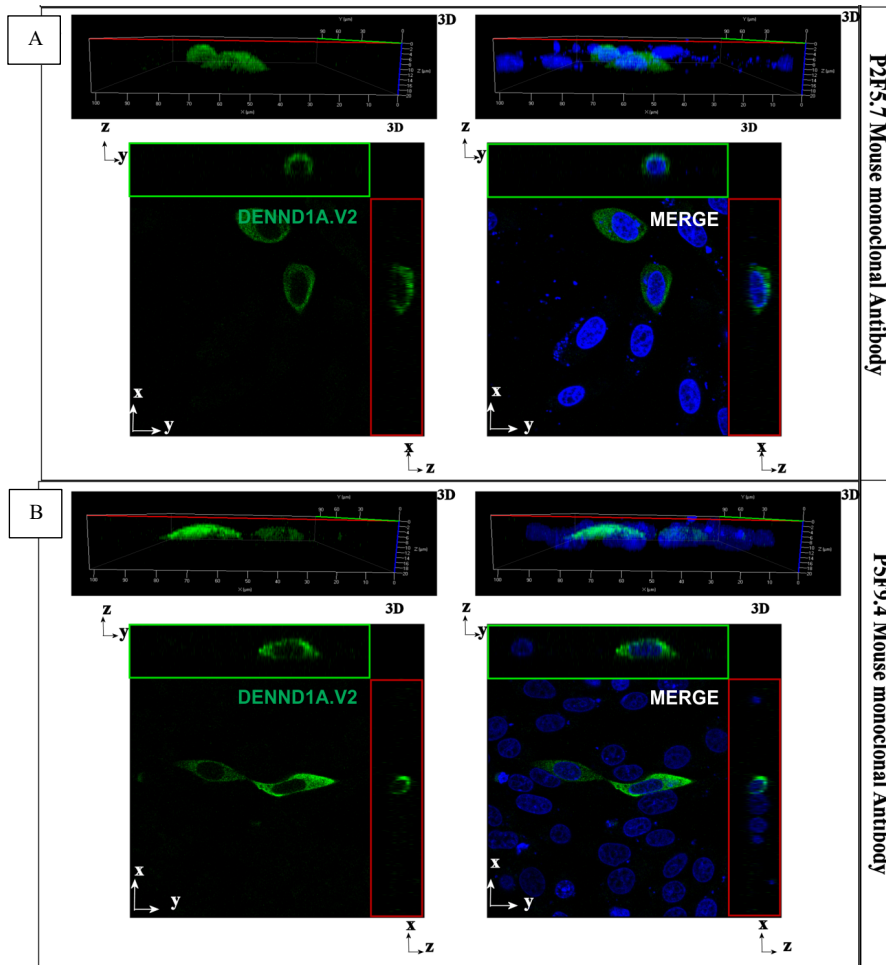


Figure 20. 3D reconstruction image showing that DENND1A.V2 localizes only in the cytosolic portion of transfected CHO cells. Top panels show 3D side view and bottom panels show 2D front and z stack view for x and y coordinates. A, CHO cells expressing DENND1A.V2, detected by P2F5.7 mouse monoclonal antibody. B, detection by P5F9.4 mouse monoclonal antibody. Representative images, from n number of experiments, where n=3.

CHAPTER 4

RESULTS SUMMARY

1. In CHO cells, DENND1A.V2 was localized in the cytoplasm of transfected cells with was no nuclear staining
2. In human theca, DENND1A.V2 was detected in a puncta pattern in the cytosol and nucleus, with significantly more DENND1A.V2 nuclear staining in untreated PCOS theca cells
3. Co-transfection of DENND1A.V2 and LHCGR in CHO cells, translocates DENND1A.V2 to plasma membrane
4. With increasing time-points of hCG in CHO cells transfected with DENND1A.V2 and LHCGR , DENND1A.V2 seems to be more in the entire cytoplasm and less in the membrane whereas LHCGR was seen to move towards the perinuclear area
5. With increasing time-points of hCG in theca cells, DENND1A.V2, RAB5B and LHCGR seems to to move towards the perinuclear area
6. After treatment with forskolin, significant translocation was observed for DENND1A.V2 in both Normal and PCOS theca cells, and RAB5B in only PCOS theca cells

CHAPTER 5

DISCUSSION

Over the past decade convincing evidence has accumulated regarding genetic factors that contribute to PCOS. The studies of genetic and molecular factors involved in the pathophysiology of PCOS accelerated after milestone GWAS studies identified PCOS candidate loci including those near *DENND1A*, *INSR*, *YAP1*, *C9orf3*, *RAB5B*, *HMGA2*, *TOX3*, *SUMO1P1/ZNF217*, *THADA* and *LHCGR* (14–18). Truncated splice variant of PCOS GWAS candidate gene *DENND1A*, termed DENND1A.V2, was shown to increase the expression in PCOS theca cells (4,7). DENND1A has been described as a clathrin binding protein, that most likely is related to gonadotropin receptor signaling as part of the clustering and endocytosis of plasma membrane receptors via the involvement of RAB5 proteins (45,46).

The functional importance of DENND1A.V2 in the PCOS phenotype of human theca cells was established by demonstrating that increased DENND1A.V2 stimulates androgen synthesis by normal theca cells and knock-down of DENND1A.V2 diminishes androgen production in PCOS theca cells (7). Although these studies provided evidence for a direct role for DENND1A.V2 in production of the ovarian PCOS phenotype, the mechanisms of DENND1A.V2 action and the relationship of DENND1A.V2 to other PCOS candidate genes was unknown. Moreover, little was known about DENND1A.V2 and its cellular location in normal and PCOS theca cells until the present studies.

Based on the available information on the function of DENND1A, its clathrin-binding domain, the role of the DENN domain as a guanine nucleotide exchange factor, the critical

role of LH action in theca cell androgen production, as well as the existing literature on the role of RAB5 proteins in gonadotropin receptor function; it is possible to propose that LHCGR, DENND1A.V2 and RAB5B function in a network to create the ovarian theca cell PCOS phenotype (26). Consistent with this hypothesis, this thesis provides the first cytological evidence to demonstrate interactions of cellular compartments containing LHCGR, DENND1A.V2 and RAB5B using transfected CHO cells, and well characterized cultured theca cells obtained from the ovaries of normal cycling and PCOS women. Based on these combined observations, this thesis demonstrated that LHCGR and DENND1A.V2 are co-localized on the plasma membrane, and that DENND1A.V2 is also co-localized with RAB5B in the endocytic vesicle, and peri-nuclear area. Furthermore, following hCG/forskolin stimulation, DENND1A.V2 translocates to the peri-nuclear region and nucleus to a larger extent in PCOS theca cells as compared to normal cells and may subsequently mediate increased expression of *CYP17A1* and *CYP11A*, leading to hyperandrogenism in PCOS theca cells. These studies also suggest that the nuclear localization of DENND1A.V2 and RAB5B may mediate changes in signaling in PCOS theca cells that may contribute to alterations in gene expression.

Relatively little is known about the role of RAB5B in gonadotropin receptor trafficking, as most studies have focused on the highly related RAB5A protein, which is thought to participate in both FSHR and LHCGR endocytosis and signaling (45). However, it is known that RAB5B is involved in phosphatidylinositol 3 kinase (PI3K), protein kinase B (PKB/AKT), and mitogen activated protein kinase (MAPK/ERK) signaling pathways, which could play important roles in LHCGR stimulation of steroidogenesis (47).

The most unexpected observation was the detection of DENND1A.V2 in the nuclei of theca cells, with PCOS theca cells showing a greater nuclear accumulation than normal theca cells. The nuclear translocation of DENND1A.V2 was stimulated by forskolin, suggesting that the translocation process is cAMP-dependent. RAB5B was co-localized with DENND1A.V2 in the nucleus of PCOS theca cells, with greater accumulation in the nuclei of forskolin-stimulated cells. It is noteworthy that in earlier studies using immunoperoxidase staining of sections of normal and PCOS ovaries both cytoplasmic and nuclear DENND1A.V2 staining in theca interna were detected (7), which is consistent with IF studies on cultured theca cells showed here.

The entry of DENND1A.V2 and RAB5B into the nucleus suggests that these proteins may participate in the regulation of steroidogenic enzyme genes beyond their roles in the plasma membrane and cytoplasmic endocytic vesicles, perhaps in part explaining the increased transcription of *CYP11A1* and *CYP17A1* associated with the elevated level of DENND1A.V2 protein in PCOS theca cells and the effects of forced DENND1A.V2 expression in normal theca cells. Future studies should explore the molecules that interact with DENND1A.V2 and RAB5B in the nucleus of PCOS theca cells and their relationship to chromatin. One possible mechanism of gene regulation involves APPL1, a multifunctional endosomal adaptor protein interacts with RAB5 molecules. APPL1 is translocated into the nucleus, where it has been shown to modulate histone deacetylases, participate in nucleosome remodeling and gene transcription (48).

Thus, in future some immunoprecipitation would help to understand what DENND1A.V2 and RAB5B are doing inside the nucleus, and how are they interacting with other molecules and are associated with increasing the expression of *CYP11A1* and *CYP17A1*.

The discovery that DENND1A.V2 and RAB5B both enter the nucleus, with more prominent nuclear localization in PCOS theca cells, raises the possibility that LHCGR signaling also occurs at multiple locations in theca cells, and that quantitative differences in DENND1A.V2 and its nuclear translocation explain phenotypic differences between normal and PCOS theca cells. These findings also raise many unanswered questions regarding the translocation including the nature of the nuclear localization signal and how the movement of DENND1A.V2 is increased by cAMP.

In summary, we have identified a network involving genes encoded by three different loci associated with PCOS. Figure 20 presents a schematic of how these three proteins and/or their compartments might be interacting with each other. DENND1A.V2 and RAB5B are both involved in the early endocytic pathways. Thus, their proximity to each other followed by the observation of their movement into the nucleus after treatment by forskolin suggests that they may be working together to increase the androgen production inside the cells. LHCGR which is a receptor for both LH and hCG, and is known to activate the cAMP pathway, in presence of hCG to increase the androgen production in the cells. Thus, this identified network involves proteins including LHCGR, DENND1A.V2 and RAB5B that are plausible determinants of the theca cell phenotype of excess androgen production. It should be noted that this model is based on static images and cannot provide details regarding the specific itineraries that each protein follows. It is clear however, that both DENND1A.V2 and RAB5B enter into the nucleus, and that represents an unexpected destination. It remains to be determined whether DENND1A.V2 and RAB5B play roles in signaling through other plasma membrane receptors including FSHR

and INSR, which have also been implicated in GWAS and other genetic studies to be involved in the pathophysiology of PCOS.

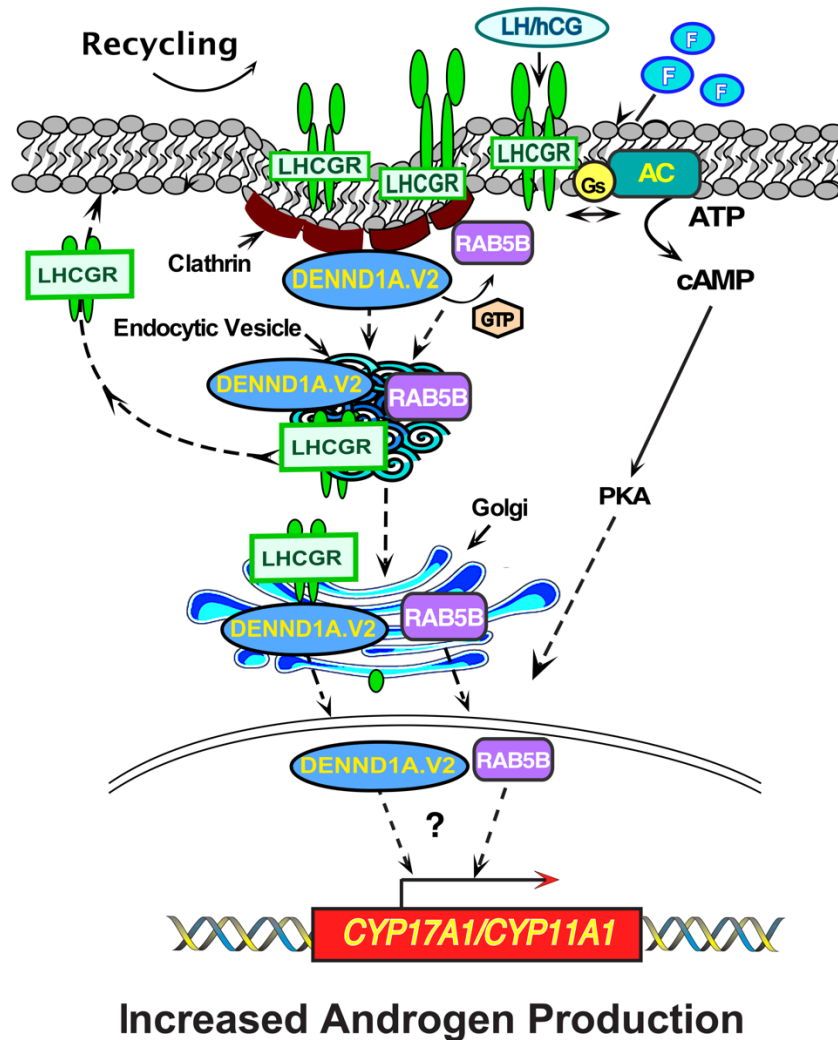


Figure 20. Schematic showing the proposed network involving dynamic changes in the interaction and trafficking of LHCGR, DENND1A.V2, and RAB5B through various cellular compartments, resulting in the hyperandrogenism associated with PCOS. As shown, LH/hCG binding to the LH-receptor (LHCGR), or forskolin-stimulation (F), results in the interaction between the GEF, DENND1A.V2 in the clathrin coated pit of

the lipid bilayer with the GTPase, RAB5B, resulting in movement of LHCGR, DENND1A.V2 and RAB5B to the early endocytic vesicles. LHCGR is subsequently recycled to the cellular membrane from the endocytic vesicles. This is followed by successive trafficking of LHCGR, DENND1A.V2 and RAB5B into the Golgi, and movement of DENND1A.V2 and RAB5B into the nucleus, resulting in increased transcription of the *CY17A1* and *CYP11A1*, along with the cAMP signaling pathway, and augmented androgen production.

CHAPTER 6

REFERENCES

1. **Rodgers R, Avery J, Moore V, Davies M, Azziz R, Stener-Victorin E, Moran L, Robertson S, Stepto N, Norman R, Teede HJ.** Complex diseases and co-morbidities: polycystic ovary syndrome and type 2 diabetes mellitus. *Endocr. Connect.* 2019 8(3): R71–R75.
2. **Cooney LG, Dokras A.** Beyond fertility: polycystic ovary syndrome and long-term health. *Fertil. Steril.* 2018;110(5):794–809.
3. **Azziz R, Carmina E, Chen Z, Dunaif A, Laven JSE, Legro RS, Lizneva D, Natterson-Horowitz B, Teede HJ, Yildiz BO.** Polycystic ovary syndrome. *Nat. Rev. Dis. Prim.* 2016;2:16057.
4. **Tee MK, Speek M, Legeza B, Modi B, Teves ME, McAllister JM, Strauss JF, Miller WL.** Alternative splicing of DENND1A, a PCOS candidate gene, generates variant 2. *Mol. Cell. Endocrinol.* 2016;434:25–35.
5. **Balen A, Homburg R, Franks S.** Defining polycystic ovary syndrome. *BMJ* 2009;338. doi:10.1136/bmj.a2968.
6. **Dunaif A.** Genes, aging and sleep apnea in polycystic ovary syndrome. *Nat. Rev. Endocrinol.* 2011;8:72.
7. **McAllister JM, Modi B, Miller BA, Biegler J, Bruggeman R, Legro RS, Strauss JF.** Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype. *Proc. Natl. Acad. Sci.* 2014;111(15):E1519–E1527.
8. **Bani Mohammad M, Majdi Seghinsara A.** Polycystic Ovary Syndrome (PCOS),

- Diagnostic Criteria, and AMH. *Asian Pac. J. Cancer Prev.* 2017;18(1):17–21.
9. **Homburg R.** The management of infertility associated with polycystic ovary syndrome. *Reprod. Biol. Endocrinol.* 2003;1:1–9.
 10. **Januszewski M, Issat T, Jakimiuk AA.** Metabolic and hormonal effects of a combined Myo-inositol and D-chiro-inositol therapy on patients with polycystic ovary syndrome (PCOS). 2019;90(1):7–10.
 11. **Bo-Jeong P, Yang H, Sohn E, Yi Yu S, Lee D, Jung DH, Ko BS, Lee HW.** Tetragonia tetragonioides (pall.) kuntze regulates androgen production in a letrozole-induced polycystic ovary syndrome model. *Molecules* 2018;23(5). doi:10.3390/molecules23051173.
 12. **Legro RS, Driscoll D, Strauss JF 3rd, Fox J, Dunaif A.** Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 1998;95(25):14956–14960.
 13. **Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI.** Heritability of polycystic ovary syndrome in a Dutch twin-family study. *J. Clin. Endocrinol. Metab.* 2006;91(6):2100–2104.
 14. **Liu H, Zhao H, Chen ZJ.** Genome-Wide Association Studies for Polycystic Ovary Syndrome. *Semin. Reprod. Med.* 2016;34(4):224–229.
 15. **Chen Z-J, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, Liang X, Zhao X, Zhao J, Sun Y, Zhang B, Jiang H, Zhao D, Bian Y, Gao X, Geng L, Li Y, Zhu D, Sun X, Xu J, Hao C, Ren C, Zhang Y, Chen S, Zhang W, Yang A, Yan J, Li Y, Ma J, Zhao Y.** Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21

and 9q33.3. *Nat. Genet.* 2010;43:55.

16. **Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, Zhang B, Liang X, Li T, Chen J, Shen J, Zhao J, You L, Gao X, Zhu D, Zhao X, Yan Y, Qin Y, Li W, Yan J, Wang Q, Zhao J, Geng L, Ma J, Zhao Y, He G, Zhang A, Zou S, Yang A, Liu J, Li W, Li B, Wan C, Qin Y, Shi J, Yang J, Jiang H, Xu JE, Qi X, Sun Y, Zhang Y, Hao C, Ju X, Zhao D, Ren CE, Li X, Zhang W, Zhang Y, Zhang J, Wu D, Zhang C, He L, Chen ZJ.** Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. *Nat. Genet.* 2012;44(9):1020–1025.
17. **Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, Karaderi T, Barber TM, McCarthy MI, Franks S, Lindgren CM, Welt CK, Diamanti-Kandarakis E, Panidis D, Goodarzi MO, Azziz R, Zhang Y, James RG, Olivier M, Kissebah AH, Stener-Victorin E, Legro RS, Dunaif A, Alvero R, Barnhart HX, Baker V, Barnhart KT, Bates GW, Brzyski RG, Carr BR, Carson SA, Casson P, Cataldo NA, Christman G, Coutifaris C, Diamond MP, Eisenberg E, Gosman GG, Giudice LC, Haisenleder DJ, Huang H, Krawetz SA, Lucidi S, McGovern PG, Myers ER, Nestler JE, Ohl D, Santoro N, Schlaff WD, Snyder P, Steinkampf MP, Trussell JC, Usadi R, Yan Q, Zhang H.** Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. *Nat. Commun.* 2015;6(May 2015):1–12.
18. **Chen LI, Hu L, Wang Y, Yang H, Huang X.** Genome-wide association study for SNPs associated with PCOS in human patients. 2017;(14):4896–4900.
19. **Goodarzi MO, Jones MR, Li X, Chua AK, Garcia O, Chen DI, Krauss RM,**

- Rotter JI, Ankener W, Legro RS, Azziz R, Iii JFS, Dunaif A, Urbanek M.** with Polycystic Ovary Syndrome in European Cohorts. 2013;49(2):90–95.
20. **Louwers Y V., Stolk L, Uitterlinden AG, Laven JSE.** Cross-Ethnic meta-Analysis of genetic variants for polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* 2013;98(12):2006–2012.
21. **Marat AL, McPherson PS.** The connecdenn family, Rab35 guanine nucleotide exchange factors interfacing with the clathrin machinery. *J. Biol. Chem.* 2010;285(14):10627–10637.
22. **Kulasekaran G, Nossova N, Marat AL, Lund I, Cremer C, Ioannou MS, McPherson PS.** Phosphorylation-dependent regulation of connecdenn/DENND1 guanine nucleotide exchange factors. *J. Biol. Chem.* 2015;290(29):17999–18008.
23. **Ye B, Duan B, Deng W, Wang Y, Chen Y, Cui J, Sun S, Zhang Y, Du J, Gu L, Lin L, Tang Y.** EGF stimulates Rab35 activation and gastric cancer cell migration by regulating DENND1A-Grb2 complex formation. *Front. Pharmacol.* 2018;9(NOV):1–12.
24. **Chiariello M, Bruni CB, Bucci C, Nazionale C, Pansini VS.** The small GTPases Rab5a , Rab5b and Rab5c are differentially phosphorylated in vitro. *FEBS Letters.* 1999;453:20–24.
25. **Sun Y, Yuan Y, Yang H, Li J, Feng T, Ouyang Y, Jin T, Liu M.** Association between Common Genetic Variants and Polycystic Ovary Syndrome Risk in a Chinese Han Population. [J Clin Res Pediatr Endocrinol](#). 2016;8(4):405–410.
26. **Mcallister JM, Legro RS, Modi BP, State P.** HHS Public Access. 2016;26(3):118–124.

27. **Liu N, Ma Y, Wang S, Zhang X, Zhang Q, Zhang X, Fu L, Qiao J.** Association of the genetic variants of luteinizing hormone, luteinizing hormone receptor and polycystic ovary syndrome. *Reprod. Biol. Endocrinol.* 2012;10:1–7.
28. **Zhou Y-C, Fu Q-G, Zhao R-Q, Ni Y-D, Chen J.** [Expression of mRNAs for GHR, IGF-IR, FSHR and LHR in granulosa and theca layers of ovarian follicles of Shaoxing ducks]. *Yi Chuan Xue Bao* 2003;30(9):840—846.
29. **Choi J, Smitz J.** Luteinizing hormone and human chorionic gonadotropin: Origins of difference. *Mol. Cell. Endocrinol.* 2014;383(1–2):203–213.
30. **Zhang M, Guan R, Segaloff DL.** Revisiting and Questioning Functional Rescue between Dimerized LH Receptor Mutants. *Mol. Endocrinol.* 2012;26(4):655–668.
31. **Wickenheisser JK, Biegler JM, Nelson-DeGrave VL, Legro RS, Strauss JF, McAllister JM.** Cholesterol Side-Chain Cleavage Gene Expression in Theca Cells: Augmented Transcriptional Regulation and mRNA Stability in Polycystic Ovary Syndrome. *PLoS One* 2012;7(11). doi:10.1371/journal.pone.0048963.
32. **Nelson-DeGrave VL, Wickenheisser JK, Hendricks KL, Asano T, Fujishiro M, Legro RS, Kimball SR, Strauss JF, McAllister JM.** Alterations in Mitogen-Activated Protein Kinase Kinase and Extracellular Regulated Kinase Signaling in Theca Cells Contribute to Excessive Androgen Production in Polycystic Ovary Syndrome. *Mol. Endocrinol.* 2005;19(2):379–390.
33. **Nelson VL, Legro RS, Iii JFS, Mcallister JM.** a Stable Steroidogenic Phenotype of Propagated Theca Cells from Polycystic Ovaries. :946–957.
34. **Nelson VL, Qin KN, Rosenfield RL, Wood JR, Penning TM, Legro RS, Strauss JF, McAllister JM.** The biochemical basis for increased Testosterone

- production in theca cells propagated from patients with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* 2001;86(12):5925–5933.
35. **Wickenheisser JK, Quinn PG, Nelson VL, Legro RS, Iii JFS, McAllister JANM.** Differential Activity of the Cytochrome P450 17 α -Hydroxylase and Steroidogenic Acute Regulatory Protein Gene Promoters in Normal and Polycystic Ovary Syndrome Theca Cells *. 2000;85(6):2304–2311.
36. **Strauss JF.** Some new thoughts on the pathophysiology and genetics of polycystic ovary syndrome. *Ann. N. Y. Acad. Sci.* 2003;997:42–48.
37. **Wickenheisser JK, Nelson-DeGrave VL, Quinn PG, McAllister JM.** Increased Cytochrome P450 17 α -Hydroxylase Promoter Function in Theca Cells Isolated from Patients with Polycystic Ovary Syndrome Involves Nuclear Factor-1. *Mol. Endocrinol.* 2004;18(3):588–605.
38. **Wood JR, Ho CKM, Nelson-DeGrave VL, McAllister JM, Strauss JF.** The molecular signature of polycystic ovary syndrome (PCOS) theca cells defined by gene expression profiling. *J. Reprod. Immunol.* 2004;63(1):51–60.
39. **Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M, McAllister JM, Mosselman S, Strauss JF.** The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. *J. Biol. Chem.* 2003;278(29):26380–26390.
40. **Nelson-DeGrave VL, Wickenheisser JK, Cockrell JE, Wood JR, Legro RS, Strauss JF, McAllister JM.** Valproate Potentiates Androgen Biosynthesis in Human Ovarian Theca Cells. *Endocrinology* 2004;145(2):799–808.
41. **Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R,**

- Welt CK.** Diagnosis and treatment of polycystic ovary syndrome: An endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 2013;98(12):4565–4592.
42. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A.** Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 2012;9(7):676–682.
43. **Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM.** CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 2006;7(10):R100.
44. **Balzarini M, Gonzalez L, Tablada M, Casanoves F, Di Rienzo J, Robledo C.** *Infostat: manual del usuario.*; 2008.
45. **Gulappa T, Clouser CL, Menon KMJ.** The role of Rab5a GTPase in endocytosis and post-endocytic trafficking of the hCG-human luteinizing hormone receptor complex. *Cell. Mol. Life Sci.* 2011;68(16):2785–2795.
46. **Bowersox S, Chen S, Beard G, Jean-Alphonse F, Hanyaloglu AC, Puthenveedu MA.** Spatially Restricted G Protein-coupled Receptor Activity via Divergent Endocytic Compartments. *J. Biol. Chem.* 2013;289(7):3960–3977.
47. **Wilson DB, Wilson MP.** Identification and subcellular localization of human rab5b , a new member of the ras-related superfamily of GTPases . [J Clin Invest](#) . 1992;89(3):996–1005.
48. **Rashid S, Pilecka I, Torun A, Olchowik M, Bielinska B, Miaczynska M.**

Endosomal adaptor proteins APPL1 and APPL2 are novel activators of beta-catenin/TCF-mediated transcription. *J. Biol. Chem.* 2009;284(27):18115–28.

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