Functional Genomics of PCOS: From GWAS to Molecular Mechanisms

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Abstract

Polycystic ovary syndrome (PCOS) is a common endocrinopathy characterized by increased ovarian androgen biosynthesis, anovulation, and infertility. PCOS has a strong heritable component based on familial clustering and twin studies. Genome-wide association studies (GWAS) identified several PCOS candidate loci including, *DENND1A, LHCGR, FSHR, ZNF217, YAP1, INSR, RAB5B, and C9orf3*. Here, we review the functional roles of strong PCOS candidate loci focusing on *FSHR, LHCGR, INSR* and *DENND1A*. We propose that these candidates comprise a hierarchical signaling network by which *DENND1A, LHCGR, INSR, RAB5B*, adapter proteins, and associated downstream signaling cascades converge to regulate theca cell androgen biosynthesis. Future elucidation of the functional gene networks predicted by the PCOS GWAS will result in new diagnostic and therapeutic approaches for women with PCOS.

Keywords

PCOS; hyperandrogenism; theca; genomics; GWAS; signaling

Hyperandrogenemia and Polycystic ovary syndrome (PCOS)

PCOS is a common disorder that is reported to affect 5–7% women of reproductive age. The incidence of PCOS appears to be similar across racial/ethnic groups. Although there has been debate about the diagnostic criteria for PCOS, an observed hyperandrogenemia/hyperandrogenism that cannot be explained by other causes, is a hallmark of the disorder, and it is included as an essential element in all "consensus" diagnosis schemes [1, 2]. The hyperandrogenemia is widely believed to be primarily of ovarian theca cell origin, although the adrenal zona reticularis contributes androgens, mainly dehydroepiandrosterone (DHEA), in approximately 25% of cases. The interconnectedness between the two androgen-secreting organs in PCOS is supported by the suppression of both adrenal and ovarian androgens by

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oral contraception [3] or insulin sensitizing agents [4, 5]. This review focuses on the genetic component underlying androgen excess in PCOS.

The phenotype of human theca cells from normal and PCOS ovaries

Studies on freshly isolated thecal tissue from normal and PCOS ovaries, or cultures of human theca cells derived from normal and PCOS women, have demonstrated that PCOS theca secretes greater amounts of androgen than theca tissue or cells from regularly ovulating women [6–12]. Recent successes in developing conditions to propagate human theca cells isolated from individual, size-matched follicles from ovaries of normal cycling women and women with PCOS provided evidence of increased expression of the rate limiting enzyme in androgen biosynthesis, P450 17α-hydroxylase/17,20 lyase, encoded by the *CYP17A1* gene. In PCOS theca cells augmented *CYP17A1* expression results in the increased metabolism of progestin precursors into androgens, among other steroids [10, 13–15]. Previous molecular characterization of PCOS theca cells and normal theca cells from multiple individuals by microarray analysis and quantitative PCR established that PCOS theca cells have molecular signatures that are distinct from those in normal theca cells [16, 17]. In PCOS, this molecular signature is associated with increased expression of certain steroidogenic enzymes involved in androgen biosynthesis including *CYP17A1*, P450 cholesterol side chain cleavage (*CYP11A*), 3β-hydroxysteroid type II (*HSD2B*), 20α-hyroxysteroid (*AKR1C1*), but not steroidogenic acute regulatory factor (*STARD1*) [8, 9]. In addition, an array of structural proteins, cell cycle proteins, and transcription factors [16, 17], including the transcription factor GATA-6 show increased expression. GATA-6 was subsequently shown to be a transcriptional activator of the main P450 steroidogenic enzymes involved in androgen biosynthesis including *CYP17A1* and *CYP11A1* [18].

The genetics of PCOS

PCOS is a heterogeneous disorder with strong evidence for a genetic component [19, 20]. Although there is some evidence for autosomal dominant inheritance, most investigators believe that an oligogenic/polygenic model is most likely, a belief reinforced by the results of recent genome-wide association studies (GWAS) that have identified multiple loci associated with PCOS [20]. Incomplete penetrance, epigenetic modifications, and environmental contributions have complicated attempts to clarify the underlying mode of inheritance. Despite advances in genetic technologies, very few PCOS susceptibility genes have been validated. Numerous candidate gene association studies have been conducted, but few have yielded statistically significant associations that have been consistently replicated [20]. The publication of GWAS with large populations of Han Chinese individuals identified eleven PCOS candidate loci: *DENND1A, INSR, YAP1, C9orf3, RAB5B, HMGA2, TOX3, SUMO1P1/ZNF217, THADA, FSHR and LHCGR* [21, 22]. The initial study encompassed a total of 4082 cases (defined by the "Rotterdam Criteria") and 6678 controls, and identified three loci [21] and a follow-up study involving additional patients added confirmed the three loci previously reported, and identified eight new candidate loci [22]. Four studies in European populations published soon thereafter confirmed the association of several of these loci, including the *FSHR/LHCGR, DENND1A* loci, as well as *RAB5B* and *THADA* [23–26].

However, it is important to recognize that different diagnostic criteria (e.g. NIH Consensus vs Rotterdam) have been used in these reported studies, which may confound interpretation.

GWAS permits discovery of unexpected loci contributing to disease risk, including those that confer only modest risk. While loci in or near the genes encoding the FSH receptor (*FSHR*), LH receptor (*LHCGR*), and insulin receptor (*INSR*) might have been anticipated based on the known roles of FSH, LH and insulin in controlling follicular growth, differentiation and steroidogenesis [23], pathophysiological links of the other GWAS loci to reproduction, ovarian function, steroidogenesis or the metabolic phenotype of PCOS were not readily apparent. Moreover, it is not known whether each individual gene or [27] multiple genes are involved in the PCOS phenotype, what the "causal" variants in or near these genes might be, or how they might contribute to the disease phenotype. Here, we review the functional roles of strong candidate PCOS candidate loci identified in the GWAS studies noted above, and subsequently replicated in other populations, focusing on *FHSR, LHCGR, INSR* and *DENND1A*.

Coding Sequence Variants in FSHR, LHCGR and INSR associated with PCOS and their functional significance

Follicle stimulating hormone (FSH), luteinizing hormone (LH) and insulin, all have gonadotropic functions. Although FSH acts on granulosa cells, its regulation of granulosa cell function influences the production of paracrine factors that alter theca cell activity. Both LH and insulin have direct actions on theca cells and increase their steroidogenic activity [27]. Consequently, genetic association of the cognate receptors for these gonadotropins and PCOS is not unanticipated.

In addition to the GWAS and replication studies noted above that implicated the *FSHR/LHCGR* and *INSR* loci in PCOS, a number of family-based association studies and subsequent candidate gene studies were carried out in different populations for common single nucleotide polymorphisms (SNPs) in these genes [28–33]. Several of these SNPs result in amino acid substitutions and, therefore, might affect protein expression or function [29, 34]. The associations have not always been found, perhaps due to population differences and a limited cohort size and a limited number of genetic variants examined [30, 35].

The FSH receptor (FSHR)

Previous studies have shown that FSHR is associated with the ovarian response to FSH, thereby making it a compelling PCOS candidate gene [37]. Inactivating mutations in the FSHR gene leads to hypergonadotropic hypogonadism, and in the absence of a functional FSHR follicular development is generally halted at the preantral stage. Conversely, mutations in the transmembrane helices and the extracellular domain of *FSHR* are associated with spontaneous ovarian hyperstimulation syndrome [36, 37]. Several *FSHR* variants have been studied in association with PCOS (for e.g. two variants in exon 10: rs6165 (T307A) and rs6166 (N680S)) [28, 29, 34, 35, 38, 39]. The SNP rs6166 has been reported to be in association with PCOS in Dutch and Japanese women. Other studies have reported variants

in association with circulating FSH levels [33, 35] and *in vivo* responses to exogenous gonadotropins and clomiphene citrate [40], suggesting that some of these variants might have functional significance in a PCOS context. However, when studied in model cell systems, the variants encoded by the major and minor SNP alleles behave identically with respect to FSH binding and signal transduction [41, 42]. Therefore, there is no clear explanation for the correlation between genotype of the *FSHR* variants and FSH levels or response to gonadotropins and by extension how exactly these variants contribute to PCOS is not clear.

The LH/CG receptor (LHCGR)

The *LHCGR* gene codes for a G-protein coupled receptor for LH and human chorionic gonadotropin (hCG). In the ovary, induction of the *LHCGR* during granulosa cell differentiation is responsible for the pre-ovulatory follicle to respond to the mid-cycle LH surge resulting in ovulation. In women, inactivating mutations of *LHCGR* are associated with increased LH levels, enlarged ovaries and oligomenorrhea. Women with inactivating *LHCGR* mutations have no obvious reproductive phenotype as opposed to the precocious puberty observed in males [43]. *LHCGR* variants have been found to be associated with PCOS in some studies [33, 44–46]. A variant in exon 10 (rs12470652) resulting in an amino acid substitution (N291S) that affects glycosylation and slightly reduces the EC50 for hCG, suggesting it might be more active, has been reported to be associated with PCOS in one small study, but not in another [46]. Another nearby SNP in exon 10 (rs2293275) that causes an amino acid substitution, S312N, has been associated with PCOS in another study [47]. Its functional significance is unknown in that it does not appear to affect glycosylation. The fact that known activating mutations of the *LHCGR* that cause precocious puberty in males because of premature Leydig cell activation and testosterone production are not associated with hyperandrogenemia of ovarian origin in women calls into question the notion that "hyper-responsive" LHCGR isoforms are related to theca cell dysfunction [45].

The insulin receptor (INSR)

The INSR plays a significant role in insulin metabolism and can play a critical role in PCOS pathogenesis via insulin resistance [21]. The importance of insulin signaling in PCOS is evident through the HAIR-AN syndrome (hyperandrogenism, insulin resistance acanthosis nigricans), which is a sub-phenotype of PCOS characterized by severe insulin resistance [48]. Mutations in the insulin receptor gene, especially in the tyrosine kinase domain have been implicated in HAIR-AN syndrome [49]. An early attempt to identify PCOS genes using a candidate approach of genotyping variable number tandem repeats (VNTRs) identified a statistically significant association with a marker (D19S884, allele 8) selected for its proximity to the *INSR* gene on chromosome 19p13.2 [50]. This association has been replicated in some but not all studies [51–54]. D19S884 was subsequently found to be in an intron of the fibrillin 3 (*FBN3*) gene and its relationship to *INSR* gene expression or function has not been established [54, 55]

A silent SNP (rs1799817) in exon 17, which encodes the tyrosine kinase domain of the *INSR*, has been associated with PCOS in some studies, but not others [32, 56–62]. Its functional significance (e.g., translational efficiency) in theca cell biology or insulin

resistance is unknown. Other SNPs in the *INSR* associated with PCOS include rs225673, which resides in intron 11, its impact on *INSR* gene expression or its relationship to a causal genetic variation are unknown [63]. The same is true for SNPs around exon 9 that have been associated with PCOS (rs8107575, rs2245648, rs2245649, rs2963, rs2245655, and rs2962) [64].

Collectively, candidate gene association studies provide some additional support for the notion that the *FSHR, LHCGR*, and *INSR* loci are associated with PCOS, consistent with the GWAS and replication studies noted above. However, there has yet to be convincing evidence produced that the variants identified in these genes are functionally significant and contribute to the PCOS theca cell phenotype. Together, these studies also suggest an intersection between the genetic basis of PCOS and T2D in addition to the epidemiological evidence. Genes involved in gonadotropin secretion and insulin signaling may act in a common pathway or network leading to the PCOS theca cell phenotype.

DENND1A - A novel starting point for dissecting the molecular mechanisms underlying PCOS

DENND1A, also termed connecdenn 1, is a member of the conndecdenn family of proteins, which are encoded by 18 genes in humans. The domains of DENND1A are tripartite, consisting of an upstream, core, and downstream DENN domains. DENND1A functions as a guanine nucleotide exchange factor that interacts with members of the Rab family of small GTPases [65]. DENND1A is thought to be involved in clathrin-mediated endocytosis, facilitating internalization of proteins and lipids, receptor recycling, and membrane trafficking [65]. DENND1A has also been associated with phosphoinositol-3-phosphate, and endocytosis/endosome proteins [20, 65].

The *DENND1A* gene yields two principal transcripts via alternative splicing. DENND1A variant 1 (DENND1A.V1) which encodes a 1009 amino acid (AA) protein with a large C-terminal proline-rich domain; and DENND1A.V2, encodes a 559 AA protein that lacks the proline-rich domain, and includes a distinct C-terminal 33 AA sequence that differs from DENND1A.V1. Both DENND1A.V1 and V2 contain clathrin-binding domains, which may interact differentially with membrane adaptor proteins as a consequence of differences in their distinct C-terminal domains. Notably, as described below, DENND1A is highly expressed in ovarian theca cells and the adrenal zona reticularis, both androgen-producing tissues. Up until recently, little was known about *DENND1A* expression in cells and tissues related to reproduction [20].

DENND1A.V2 in PCOS theca cells

DENND1A.V2 expression is increased in PCOS theca cells

Studies using theca cells isolated from normal cycling and PCOS women provided the first evidence to demonstrate a functional relationship between augmented DENND1A.V2 and *CYP17A1* expression and increased androgen biosynthesis in PCOS theca cells [66]. Immunohistochemical localization studies showed that DENND1A.V2 was increased in PCOS theca cells as compared to normal theca cells, and was localized in the cytosol and

nuclei of both PCOS granulosa and theca [66]. In contrast, DENND1A.V1 protein levels are decreased in PCOS theca cells, and the ratio of DENND1A.V2 to DENND1A.V1 is increased in PCOS theca cells. DENND1A.2 mRNA is similarly increased in PCOS theca cells, and precedes the augmented induction of CYP17 mRNA and androgen biosynthesis. In subsequent studies, forced expression of DENND1A.V2 in normal cells converted the cells to a PCOS phenotype of increased *CYP17A1* and *CYP11A1* gene transcription and androgen biosynthesis. In contrast, knockdown DENND1A.V2 expression, or ablation of DENND1A.V2 function in PCOS theca cells utilizing DENND1A.V2 specific antibodies converts the cells to a normal phenotype.

These data provide strong evidence that increased DENND1A.V2 expression mediates augmented *CYP17A1* gene expression and androgen biosynthesis in PCOS theca cells [66]. While the studies summarized above suggest that DENND1A.V2, when over expressed, can produce a theca cell PCOS phenotype, these findings do not exclude the possibility that DENND1A.V2 acts in concert with genetic variants of *FSHR, LHCGR* and *INSR* to modulate signal transduction pathways that promote thecal androgen synthesis. However, these data suggest that DENND1A.V2 is a novel therapeutic and diagnostic target for PCOS.

DENND1A.V2: a potential biomarker for PCOS?

Exosomes are small nucleic acid rich vesicles shed into blood and urine, which provide a source of RNA that can be conveniently utilized for diagnostic implications. Studies comparing DENNDD1.V2 mRNA in urine exosomes isolated from normal cycling and PCOS women demonstrated that DENND1A.V2 RNA is increased in PCOS compared to normally cycling women. These experiments provided the first evidence to suggest that increased urine exosomal DENND1A.V2 could be diagnostic for PCOS [66]. A diagnostic method based on urine exosomal RNA would have value in settings when non-invasive diagnostics are needed such as in prepubertal and adolescent females. However, this test requires extensive validation to assess its sensitivity, specificity and positive and negative predictive values.

Functional consequences of DENND1A.V2 overexpression in PCOS

To date, a genetic explanation for increased expression of DENND1A.V2 has not been identified. The *DENND1A* SNPs identified by GWAS are located in introns and it is not known whether they have functional roles. Moreover, whole exome sequencing has not identified *DENND1A* coding region variants in PCOS [25]. Possible mechanisms for overexpression include copy number variation (CNV), increased promoter activity, the influence of microRNAs on protein expression, or splicing variation favoring the production of DENND1A.V2. To date, CNVs and proximal promoter variation that could explain the increased expression of DENND1A.V2 have not been reported. At this juncture, genetic variations affecting splicing mechanisms that differentiate DENND1A.V2 from DENND1A.V1 appears to be the most likely explanation.

The mechanism(s) by which DENND1A.V2 alters theca cell function is not known. However, the structural features of the DENND1A.V2 protein and its cellular localization offer some important clues. First, DENND1A.V2 could modify signaling via gonadotropin

or insulin receptors; perhaps biasing activation of signal transduction cascades, leading to increased expression of steroidogenic enzymes (Fig. 1). DENND1A.V2 could have direct effects in this regard, or it could modify or interfere with the action of DENND1A.V1. Since cyclic AMP-stimulated *CYP17A1* and *CYP11A1* gene expression and steroidogenesis is amplified by increased DENND1A.V2 expression, DENND1A.V2 appears to have a boosting effect that augments cAMP signaling. Alternatively, DENND1A.V2 localized in the nucleus could influence gene transcription, either through the transport of ligand and/or receptor into the nucleus with the activation of intra-nuclear signal transduction, or DENND1A.V2 might play a role as a scaffold for transcription factors that regulate steroidogenic enzyme gene expression (Fig. 1).

Could DENND1A be a "unifying gene" in explaining multiple PCOS phenotypes?

As noted above, PCOS is associated with excess adrenal androgen levels in approximately 25% of women, consistent with an adrenal steroidogenic abnormality [19, 67–70]. The localization of DENND1A.V2 in the human adrenal zona reticularis provides evidence that alterations in expression of this isoform may contribute to the adrenal steroidogenic abnormality in addition to the thecal derangement in androgen production. *DENND1A* SNPs have been reported to be associated with diverse phenotypes found in PCOS women, including elevated insulin levels and endometrioid carcinoma [71, 72]. Additionally, quantitative trait analyses have linked the *DENND1A* locus with glucose and insulin levels and body weight in humans [73]. Thus, DENND1A.V2 could possibly contribute directly to or modify a number of PCOS phenotypes, which heretofore have not been tied to a single genetic locus.

A PCOS genetic network incorporating DENND1A

Among the loci associated with PCOS in Han Chinese, several reside in or near genes that potentially define a network. While loci in or near the genes encoding the FSH receptor, LH receptor, and insulin receptor are plausible PCOS candidates; the pathophysiological links of the other GWAS loci to reproduction, ovarian function, steroidogenesis or the metabolic phenotype of PCOS have not been readily apparent [20, 22, 74]. It is currently unknown whether each individual gene or multiple genes are involved in the PCOS phenotype, or what the functional variants in or near these genes might be, or how they might contribute to the disease phenotype.

Kyoto Encyclopedia of Genes and Genomes (KEGG) network pathway analysis [75–77] from RNA-Seq expression profiles of the GWAS candidates in normal and PCOS theca cells revealed that *DENND1A, ZNF217, YAP1*, and *HMGA2* are associated in an inter-related pathway. A review of the literature has also revealed that the GWAS candidates, LHCGR, INSR, DENND1A, RAB5B, mediate overlapping intracellular signaling pathways, including the PI3K, PKB, and/or MAPK signaling cascades [14, 78–82]. Comparison of the activation states of the AKT/PKB and MAPK/MEK/ERK signaling pathways in normal and PCOS theca cells has provided evidence that alteration of these signaling cascades may underlie increased androgen production and *CYP17A1* gene expression in PCOS [14, 78].

Thus, it is conceivable that various GWAS candidates comprise a hierarchical signaling network by which *DENND1A, LHCGR, INSR, RAB5B*, adapter proteins, and their associated downstream signaling cascades converge to regulate *CYP17A1* expression and androgen biosynthesis. Thus, *DENND1A* and other PCOS GWAS genes might function in a signaling pathway beginning with cell surface receptors, leading to receptor coupling/recycling, and then to downstream molecules that ultimately regulate gene transcription, either of steroidogenic genes directly, or possibly through the up-regulation of other transcription factors that directly influence steroidogenic gene promoter function (e.g., GATA6) (Fig. 1) [16, 18].

Potential Clinical Utility of DENND1A

The current diagnostic criteria for PCOS are expert based, and accordingly there are a profusion of society based and country based criteria, also proposals to split PCOS into a reproductive and a metabolic disorder [83]. The recent NIH sponsored Evidence Based Methodology Workshop on PCOS endorsed the expert based Rotterdam criteria, but stressed the need for scientifically justified criteria [84]. Having a molecular marker that may identify a pediatric female population who are just developing the disorder at adrenarche or pubarche would allow earlier intervention. Diagnosing PCOS in this population is particularly problematic lacking even expert consensus [85].

Concluding Remarks

An additional area of further exploration is the role of DENND1A variants in predicting response to common treatments for women with PCOS. Hyperandrogenism is clearly associated with the development of hirsutism in women with PCOS, and further is an important predictor of anovulation and failure to respond to ovulation induction. Thus this may be a particularly useful molecular marker for pharmacogenomics studies. Recent large multi-center studies of ovulation induction methods in women with PCOS and anovulatory infertility will certainly aid these studies.

Acknowledgments

Glossary

DENN/MADD domain containing 1A (DENND1A)	is a member of the connecdenn family of proteins that function as a guanine nucleotide exchange factor (GEF) regulating clathrin-mediated endocytosis through RAB35 activation. DENND1A promotes the exchange of GDP to GTP, converting inactive GDP-bound RAB35 into its active GTP-bound form.
Follicle-stimulating hormone receptor (FSHR)	is a transmembrane, G-protein coupled receptor found in the ovary, testis and uterus. It is required for the functioning of the Follicle Stimulating Hormone (FSH)

Hyperandrogenemia	is a medical condition characterized by excessive levels of androgens (supraphysiologic levels of free testosterone) in the body, and one of the primary symptoms of PCOS.
Insulin receptor (INSR)	belongs to the tyrosine kinase receptor family and is mainly involved in regulation of glucose homeostasis and plays a critical role in PCOS pathogenesis via insulin resistance.
Luteinizing hormone/ choriogonadotropin receptor (LHCGR)	is a G-protein coupled receptor predominantly expressed in the ovary and the testis and is involved in the signaling via luteinizing hormone (LH) and the human chorionic gonadotropin (hCG). In the ovary, LHCGR is involved in the follicular maturation and ovulation.
Ovarian theca cells	are endocrine cells associated with ovarian follicles that play a key role in fertility. They produce the androgen substrate required for ovarian estrogen biosynthesis. Excessive proliferation of theca cells and ovarian hyperandrogenism is a hallmark of the most common endocrine cause of infertility.
Zona reticularis	is the inner most layer of the adult human adrenal cortex and the source of the adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS).

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Highlights

- GWAS identified *FHSR, LHCGR, INSR* and *DENND1A* as PCOS candidate genes.

- Overexpression of DENND1A Variant 2 increases androgen biosynthesis in theca cells from normal cycling women, converting them to a PCOS phenotype.

- *DENND1A, LHCGR, INSR, RAB5B* form a hierarchal signaling network that can influence androgen synthesis.

- DENND1A.V2 is a new diagnostic and therapeutic target for PCOS.
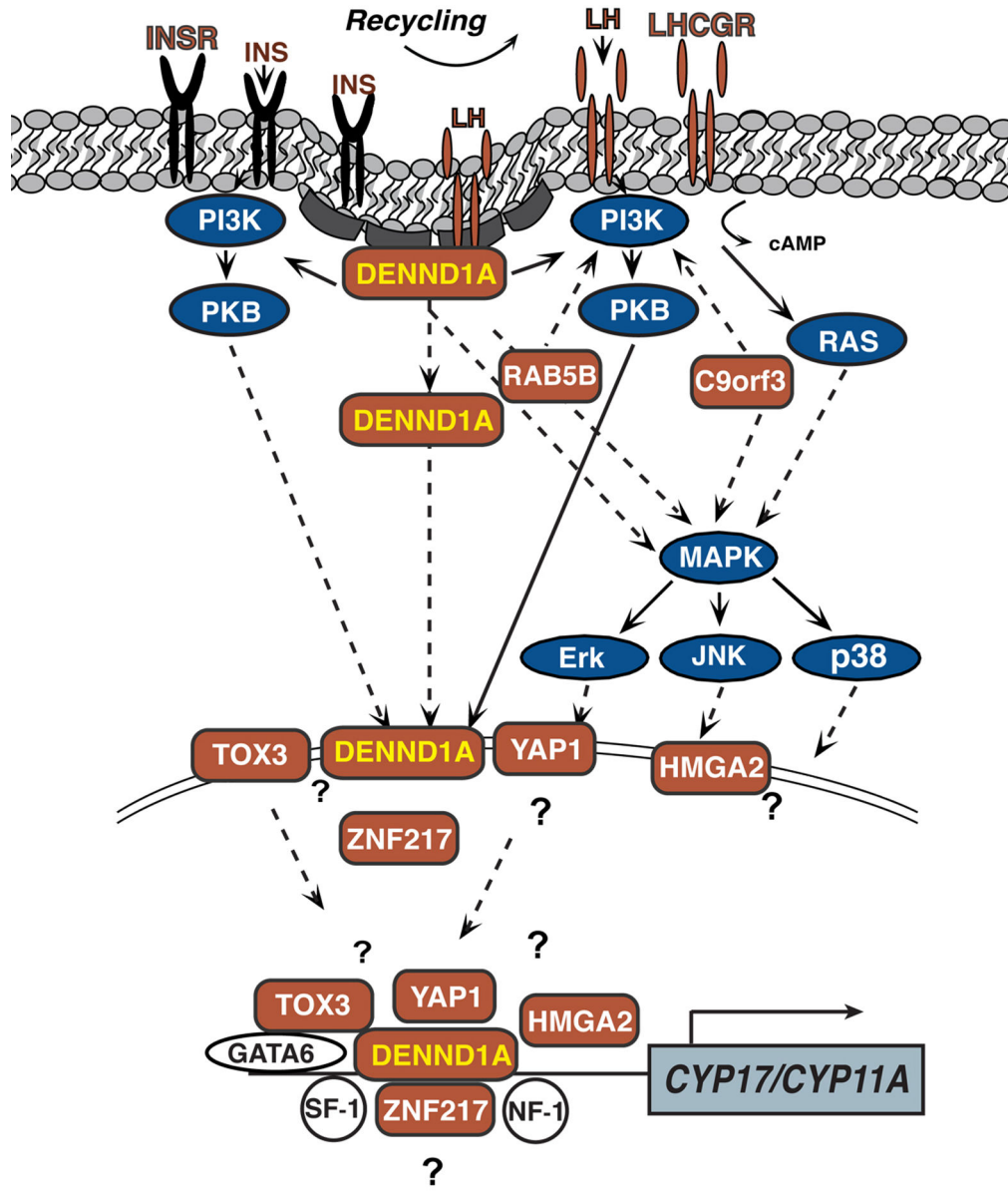
Figure 1. Hypothetical model of a GWAS Signaling Cascades involved in PCOS
The schematic shows a putative network of genes postulated to contribute to the
hyperandrogenimia associated with PCOS, based on the loci identified in published GWAS
and replication studies (GWAS candidates are labeled in rust). The GWAS loci associated
with PCOS include: 1) plasma membrane receptors (*LHCGR, FSHR INSR*); 2) proteins
associated with protein trafficking, endocytotic processes, and receptor recycling
(*DENND1A, RAB5B*); and 3) transcription factors (*ZNF217, YAP1, TOX3, HMG*). The
LH/hCG receptor, FSH-receptor, and insulin receptor are all plasma membrane receptors,

which are internalized/recycled by clathrin coated pits [20]. *DENND1A* encodes the plasma membrane protein connecdenn 1, which is involved in clathrin-binding, endocytic processes and receptor recycling [65, 74, 86]. Ras related protein 5B (RAB5B) is a Rab-GTPase, and is also involved in membrane trafficking, endocytosis and receptor recycling [87, 88]. Yes-associated protein (YAP1), transcriptional coactivator of the p300/CBP-mediated transcription complex (TOX3), high mobility group AT-hook 2 (HMGA2), and zinc finger protein 217 (ZNF217) have all be shown to be involved in transcriptional activation/repression [75–77, 89].

Abbreviations: PCOS, polycystic ovary syndrome; **GWAS**, Genome Wide Association Study; *DENND1A*, connecdenn 1; **DENND1A.V2**, DENND1A variant 2; *LHCGR*, LH/CG receptor; *YAP1*, Yes-associated protein; *TOX3*, transcriptional coactivator of the p300/CBP-mediated transcription complex; *HMGA2*, high mobility group AT-hook 2; *ZNF217*, zinc finger protein 217; *RAB5B*, ras related protein 5B; *INSR*, insulin receptor; *CYP17A1*, cytochrome P450 17α-hydroxylase *CYP11A1*, cytochrome P450 cholesterol side chain cleavage.