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# Silencing diacylglycerol kinase-theta expression reduces steroid hormone biosynthesis and cholesterol metabolism in human adrenocortical cells☆

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## Abstract

Diacylglycerol kinase theta (DGKθ) plays a pivotal role in regulating adrenocortical steroidogenesis by synthesizing the ligand for the nuclear receptor steroidogenic factor 1 (SF1). In response to activation of the cAMP signaling cascade nuclear DGK activity is rapidly increased, facilitating PA-mediated, SF1-dependent transcription of genes required for cortisol and dehydroepiandrosterone (DHEA) biosynthesis. Based on our previous work identifying DGKθ as the enzyme that produces the agonist for SF1, we generated a tetracycline-inducible H295R stable cell line to express a short hairpin RNA (shRNA) against DGKθ and characterized the effect of silencing DGKθ on adrenocortical gene expression. Genome-wide DNA microarray analysis revealed that silencing DGKθ expression alters the expression of multiple genes, including steroidogenic genes, nuclear receptors and genes involved in sphingolipid, phospholipid and cholesterol metabolism. Interestingly, the expression of sterol regulatory element binding proteins (SREBPs) was also suppressed. Consistent with the suppression of SREBPs, we observed a down-regulation of multiple SREBP target genes, including 3-hydroxy-3-methylglutary coenzyme A reductase (HMG-CoA red) and CYP51, concomitant with a decrease in cellular cholesterol. DGKθ knockdown cells exhibited a reduced capacity to metabolize PA, with a down-regulation of lipin and phospholipase D (PLD) isoforms. In contrast, suppression of DGKθ increased the expression of several genes in the sphingolipid metabolic pathway, including acid ceramidase (ASAH1) and sphingosine kinases (SPHK). In summary, these data demonstrate that DGKθ plays an important role in steroid hormone production in human adrenocortical cells.

## Keywords

Diacylglycerol kinase theta; Phosphatidic acid; Cortisol; Adrenal cortex; cAMP

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## 1. Introduction

Steroid hormones are essential signaling molecules that regulate multiple physiological processes. In adrenal steroidogenesis, the synthesis of cortisol occurs in the zona fasciculata of the cortex where adrenocorticotropin (ACTH) binds to melanocortin 2 receptor (MC2R), thereby activating adenylyl cyclase leading to an increase of cAMP production. This action activates the cAMP-dependent protein kinase PKA which phosphorylates downstream targets, facilitating an increase in free cholesterol and in the transcription of genes required for glucocorticoid and adrenal androgen biosynthesis [1, 2]. We have identified roles for phospholipids and sphingolipids as transcriptional regulators of steroidogenic genes, where ACTH/cAMP signaling increases nuclear diacylglycerol kinase theta (DGKθ) activity, which produces phosphatidic acid (PA) a ligand for the nuclear receptor steroidogenic factor 1 (SF1) [3]. PA stimulates SF1-dependent transcription of CYP17A1 reporter plasmids, promotes coactivator recruitment to the CYP17A1 promoter, and induces the mRNA expression of CYP17A1 and several other steroidogenic genes. LXXLL motifs in DGKθ mediate a direct interaction of SF1 with the kinase and may facilitate binding of PA to the receptor. We have also shown that sphingosine (SPH) also binds to SF1, but in contrast to PA, SPH is an antagonist [4]. Consistent with the repressive role of SPH in inhibiting SF1-dependent gene expression, silencing acid ceramidase (ASAH1), the enzyme that produces SPH, results in an increase in steroidogenic gene expression and cortisol production [5]. Significantly, ASAH1 is recruited to the promoters of multiple steroidogenic genes and forms a complex with the receptor on DNA [6].

Mounting of evidence has shown that DGKs are the important regulators in cellular signaling and homeostasis [7–10]. DGKs modulate the concentrations of two lipid messengers: PA and diacylglycerol (DAG) through an ATP-dependent phosphorylation [11]. To date, there have been ten mammalian DGK isoforms identified, several of which are localized in the nucleus. All DGKs have at least two C-1 type motifs that are homologous to the protein kinase C (PKC) phorbol ester/DAG binding region [12]. In contrast to other DGKs, which contain two cysteine-rich domains (CRD) DGKθ has three CRDs, and a proline/ glycine-rich domain at its N-terminus, a pleckstrin homology domain, and a Ras-associating domain [13]. These functional domains enable the selective interaction with distinct effector proteins. For example, the binding of RhoA to the C-terminus of DGKθ inhibits catalytic activity [14]. DGKα [15], DGKδ [16], DGKθ [17] and DGKζ [18] are associated with PKC isoforms and are phosphorylated when complexed with select PKC isoforms. Similarly, DGKθ can be phosphorylated by PKCε and PKCη, and PKCε activation leads to DGKθ translocation to the plasma membrane [17].

DGKθ has been shown to be regulated by nerve growth factor in PC12 cells [19], by bile acids in hepatocytes [20], and by alpha-thrombin in fibroblasts [21]. We have recently shown that cAMP signaling induces DGKθ in H295R human adrenocortical cells via a pathway that requires SF1 and sterol regulatory element binding protein 1 (SREBP1) [22]. Moreover, we observed that cAMP-induced PA production is strongly associated with DGKθ gene expression. Based on our previous findings establishing a key role for DGKθ in glucocorticoid production, we sought to determine the role of the enzyme in regulating adrenocortical gene expression.

## 2. Materials and methods

### 2.1. Materials

Dibutyryl cAMP ($Bt_2$ cAMP) and tetracycline (tet) were obtained from Sigma-Aldrich (St. Louis, MO).

### 2.2. Cell culture

H295R adrenocortical cells [23,24] were generously donated by Dr. William E. Rainey (University of Michigan, Ann Arbor MA) and cultured in Dulbecco's modified Eagle's/F-12 (DME/F-12) medium (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Serum I (BD Bioscience, Palo Alto, CA), 1% ITS Plus (BD Bioscience, Palo Alto, CA), and antibiotics.

### 2.3. Generation of H295R DGKθ$^{kd}$ stable cell line

Tet-inducible DGKθ shRNA was generated followed by the instructions using the BLOCK-iT H1 RNAi Entry Vector kit (Invitrogen). H295R cells expressing tetracycline-inducible DGKθ shRNA were generated using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen) as previously described [5]. To construct an inducible vector for DGKθ shRNA, the following sequences were cloned into pENTR/H1/TO: 5′-ACC ***GCC CAG TAT TGA AGG CCT CAT CTT CA***C GAA TGA AGA TGA GGC CTT CAA TAC TGG G-3′ and 5′-AAA CCC AGT ATT GAA GGC CTC ATC TTC ATT CG***T GAA GAT GAG GCC TTC AAT ACT GGG C***-3′, and correspond to regions 2425–2443 of the coding region (NM_001347.3). H295R-TetR cells were stably transfected with the constructed pENTR/H1/TO-DGKθ shRNA expression vector or a vector containing a scrambled sequence using GeneJuice (EMD Biosciences), and cell clones were selected using 50 μg/ml zeocin. Clones were treated with 5 μg/ml tetracycline for 96 h and suppression of DGKθ protein levels in each clone was confirmed by western blotting using an anti-DGKθ antibody (HPA026797; Sigma).

### 2.4. DNA microarray

H295R wild type [24] or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were grown on 6 well plates and treated with 0.4 mM $Bt_2$cAMP for 24 h. Total RNA was isolated using the QIAGEN RNeasy kit (QIAGEN, Valencia, CA), and gene expression profiling was determined by Phalanx Biotech Group, Inc. (Palo Alto, CA) using the human Whole Genome One Array DNA Microarray.

### 2.5. RNA isolation and Real time RT-PCR

H295R WT or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were sub-cultured onto 12-well plates and 24 h later treated with 0.4 mM $Bt_2$cAMP for 24 h. Total RNA was extracted using Iso-RNA Lysis Reagent (5 Prime, Inc., Gaithersburg, MD) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA) and the primers listed in Table 1. DGKθ expression was normalized to β-actin content and calculated using delta-delta cycle threshold (ΔΔCT) method.

## 2.6. Western blotting

H295R WT or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were sub-cultured onto 6-well plates and treated with 0.4 mM Bt$_2$cAMP for 24 h to 72 h and harvested into radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 150 nM aprotinin, 1 mM leupeptin, 1 mM E-64, 500 mM 4-(2-aminoethyl)benzenesulfonylfluoride). Cells were then lysed by sonication (one 2 s burst) followed by incubation on ice for 30 min. Lysates were centrifuge for 10 min at 4 °C and the supernatant collected for analysis by SDS-PAGE. Protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay (Pierce). Aliquots of each sample (25 μg of protein) were run on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Blots were probed with the antibodies in Table 2. Blots were scanned on a VersaDoc 4000 (Bio-Rad, Hercules CA) and densitometric analyses were carried out using Quantity One software (Bio-Rad).

## 2.7. RNA interference (RNAi)

Transient silencing of DGKθ was performed by transfecting H295R cells with 100 nM of non specific small interfering RNA (siRNA) (D-001810-01-20, Thermo Scientific) or DGKθ siRNA (L005079-00-0010, Thermo Scientific) oligonucleotides using Lipofectamine RNAiMAX (Invitrogen). Cells were treated with 0.4 mM Bt$_2$cAMP and RNA or whole cell lysates isolated for qRT-PCR or western blotting, respectively.

## 2.8. Cortisol and DHEA assays

Cortisol and DHEA assay were carried out as in previous study [5]. In general, H295R WT or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were grown on 6-well plates and treated with 0.4 mM Bt$_2$cAMP for 48 h and then, the growth media and cell lysates were collected, respectively. The contents of DHEA and cortisol were determined using a 96-well plate EIA kit (Assay Designs, Inc., Ann Arbor, MI). Hormone concentrations were normalized to the total protein concentration.

## 2.9. PA assay

H295R WT or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were grown on 6-well plates and then treated with Bt$_2$cAMP from 24 h to 48 h and total lipid extract was harvested.PA content was quantified using a Total PA kit (Cayman Ann Arbor, MI) and a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA) at an excitation wavelength of 530–540 nM and an emission wavelength of 585–595 nm. Data was quantified using through SoftMax Pro Software (Molecular Devices, LLC, Sunnyvale, CA).

## 2.10. DAG assay

DAG assay was previously described [22]. Briefly, H295R WT cells or DGKθ$^{kd}$ cells (pretreated with 5 μg/ml tet for 96 h) were cultured onto 6-welled plates and treated with Bt$_2$cAMP from 72 h and lipids extracted. The content of DAG in each sample was determined using a Human DAG ELISA kit (MyBioSource, Inc., San Diego CA).

### 2.11. Cholesterol assay

H295R WT cells and DGKθ$^{kd}$ cells were cultured onto 6-well plates. After 48 h treatment with 0.4 mM $Bt_2cAMP$, the total cellular cholesterol was isolated and the level of cholesterol was determined by cholesterol/ cholesteryl ester detection kit (Abcam, Cambridge, MA).

### 2.12. Statistical analysis

One-way ANOVA and Tukey–Kramer multiple comparisons were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego CA). Significant difference value was set as $p < 0.05$.

## 3. Results

### 3.1. Generation of the H295R DGKθ$^{kd}$ cell line

To determine the significance of DGKθ gene expression in adrenocortical lipid metabolism, we generated an H295R cell line that stably expresses a tet-inducible DGKθ shRNA [5]. As shown in Fig. 1A, DGKθ mRNA levels were decreased by 68% in cells expressing the shRNA targeted against the lipid kinase. Consistent with our previous findings [22], dibutyryl cAMP ($Bt_2cAMP$) stimulation induced DGKθ mRNA expression by 2-fold in wild type H295R cells. Silencing DGKθ had no significant effect on the mRNA expression of the other DGK isoforms that are expressed in adrenocortical cells (data not shown). Western blot analysis revealed a 60% reduction DGKθ protein expression in tet-treated H295R cells (Fig. 1B and C).

Microarray studies were performed to assess the effect of reducing DGKθ expression on global gene expression, where 1726 genes differentially expressed (log2 ratio $\geq 1.0$ or $\leq -1.0$ and $p < 0.05$). Gene ontology (GO) analysis of differentially expressed genes revealed significant changes in gene clusters representing varied molecular functions, including phosphatase activity, chromatin binding, transcription factor binding, isomerase activity, and motor activity (Supplemental Fig. 1). GO analysis identified significant changes in several biological processes, notably transcription, kinase signaling, the cell cycle, and RNA metabolism.

### 3.2. Silencing of DGKθ suppressed the steroidogenic genes expression

As mentioned earlier, we have previously shown that cAMP signaling stimulates DGKθ-catalyzed nuclear PA production in H295R human adrenocortical cells [3]. Thus, we next determined the effect of silencing DGKθ on the expression of genes that are required for cortisol production (Fig. 2A). As shown in Fig. 2B, suppression of DGKθ reduced the expression of most genes that are involved in steroid hormone production. Real time RT-PCR analysis of RNA isolated from wild type and DGKθ knockdown (DGKθ$^{kd}$) cells revealed that silencing the lipid kinase reduced the basal expression of CYP17A1, CYP11A1 and 3βHSD type II (HSD3B2) by 54%, 58%, 45%, respectively, while the basal levels of CYP11B1 were not significantly changed (Fig. 2B). Suppressing DGKθ also abrogated cAMP-stimulated expression of these genes. In contrast, CYP21A2 mRNA expression was induced by 2.1-fold in the DGKθ$^{kd}$ cells, and Bt$_2$cAMP-stimulation further increased the mRNA expression.

Silencing DGKθ also reduced the mRNA expression of genes that are required for cholesterol mobilization, with the mRNA expression levels of steroidogenic acute regulatory protein (StAR), hormone sensitive lipase (HSL), scavenger receptor type B1 (SR-BI) and the low density lipoprotein receptor (LDLR) showing 74%, 67%, 51% and 38% decreases, respectively (Fig. 2C). Consistent with the changes in mRNA, the protein expression of all steroidogenic genes except CYP21A2 decreased in the DGKθ$^{kd}$ cell line (Fig. 2D). Quantification of cortisol released into the media revealed a 1.9-fold increase in wild type H295R cells in response to Bt$_2$cAMP (Fig. 2E). However, cortisol secretion was significantly increased in Bt$_2$cAMP-stimulated DGKθ$^{kd}$ cells. While activation of the cAMP signaling pathway increased DHEA production in the wild type cells, secretion of this steroid metabolite was attenuated in the DGKθ$^{kd}$ cell line. Moreover, silencing DGKθ reduced basal DHEA secretion by 45%.

Next we carried out rescue experiments to determine if exogenous administration of PA was sufficient to restore the expression of genes that were suppressed in the DGKθ$^{kd}$ cell line. These studies revealed that PA was able to induce the mRNA and protein expression of CYP17A1 and StAR (Fig. 3A and B). Consistent with our findings using the tet-inducible shRNA cell line, transient silencing of DGKθ using siRNA oligonucleotides resulted in similar decreases in the expression of CYP17A1 and StAR (Fig. 3C and D).

### 3.3. Silencing of DGKθ alters the expression of nuclear receptor genes

Accumulating evidence has demonstrated that several nuclear receptor actions in addition to SF1 regulate the transcription of steroidogenic genes in adrenocortical cells [25–28]. Our previous study has shown that DGK-produced PA is required for cAMP-dependent CYP17 transcription by binding to SF1 [3]. Because the silencing of DGKθ unregulated the gene expression of CYP21A2 (Fig. 2), we hypothesized that this effect might be mediated by an increase in the expression of another nuclear receptor. To test this hypothesis, we examined the expression of NR5A, NR0B1 and NR4A family members. As shown in Fig. 4A, reduced DGKθ expression did not significantly affect on the mRNA level of SF1 (NR5A1) and LRH1 (NR5A2). However, the constitutive mRNA expression of NR4A1 (Nur77, NGF1B), NR4A2 (NURR1), and NR4A3 (NOR1) was enhanced by 1.7-, 2.6-, and 2.5-fold induction, respectively. The mRNA expression of all three NR4A family members was induced in wild type cells treated with Bt$_2$cAMP, and the magnitude of induction was higher in the DGKθ$^{kd}$ cell line. Moreover, DGKθ$^{kd}$ cells showed an 85% decrease in NR0B1 (encodes dosage-sensitive sex reversal, adrenal hyperplasia critical region on chromosome X gene 1; DAX-1). Similar with induction of mRNA expression, the basal protein levels of NR4A nuclear receptors were also increased in cells with reduced DGKθ expression (Fig. 4B).

### 3.4. DGKθ deficiency affects sphingolipid gene expression

As discussed earlier, we have previously shown that SPH antagonizes the ability of SF1 to transactivate target genes [4–6]. Moreover, alterations in phospholipid metabolism can impinge on sphingolipid homeostasis (Fig. 5A). Therefore we determined the effect of silencing DGKθ on the expression of genes involved in sphingolipid metabolism. Microarray analysis revealed no significant changes in the expression of most enzymes in the sphingolipid biosynthetic pathway. However, ASAH1, sphingosine kinase (SPHK) 1 and SPHK2 were increased in expression in the DGKθ$^{kd}$ cell line. qRT-PCR revealed that DGKθ depletion increased the basal mRNA expression of ASAH1, SPHK1 and SPHK2 by 2.0-, 2.6-and 4.6-fold, respectively (Fig. 5B). cAMP stimulation leads to a further in crease in the mRNA expression of ASAH1, SPHK1, and SPHK2 in the DGKθ$^{kd}$ cell line. As with our previous studies, cAMP signaling increased the expression of ASAH1 in wild type H295R cells [29]. In contrast, DGKθ suppression did not affect the mRNA expression of ASAH2 (encodes neutral ceramidase), but resulted in a 72% decrease in the expression of alkaline ceramidase 3 (ACER3). Concordant with the induction of mRNA expression in the DGKθ$^{kd}$ cell line, the basal and Bt$_2$cAMP-stimulated protein expression of ASAH1, SPHK1, and SPHK2 was increased (Fig. 5C). Despite the marked decrease in ACER3 mRNA expression, no significant change was observed in the protein expression of this lipid hydrolase, which is consistent with our previous studies where silencing ASAH1 in the H295R cell line induced ACER3 mRNA, but had no effect on the protein expression of the enzyme [5].

### 3.5. Depletion of DGKθ reduces PA content

We have recently shown that DGKθ plays a predominant role in cAMP-stimulated PA production in human adrenocortical cells [22]. Therefore, we sought to determine if DGKθ suppression changed the ex pression of other genes that are involved in PA metabolism. First we confirmed that silencing DGKθ abolished the ability of Bt$_2$cAMP to de crease DAG (Fig. 6A) and increase PA (Fig. 6B). Next we examined the expression of other gene families that contribute to cellular PA in wild type and DGKθ$^{kd}$ cells. PA can also be generated by phospholipase D (PLD) via the hydrolysis of phosphatidylcholine [30]. Notably, the mRNA expression of PLD1 and PLD2 was reduced by 71% and 88%, respectively (Fig. 6C) and the protein expression of PLD1/2 was decreased by 58% (Fig. 6E) in the DGKθ$^{kd}$ cell line. Another family of enzymes that regulate cellular PA contents is lipins, which harbor phosphatidate phosphatase activity and convert PA to DAG [31, 32]. Silencing DGKθ leads to species-specific changes in the expression of each lipin isoform, with the mRNA (Fig. 6D) and protein (Fig. 6E) expression of lipin2 (LPIN2) being reduced by 70% and 55%, respectively. As shown in Fig. 6D, basal lipin1 (LPIN1) mRNA expression was decreased by 30% in the DGKθ$^{kd}$ cell line. In contrast, expression level of lipin3 (LPIN3) was not significantly changed by DGKθ silencing. Additionally, we observed no effect of silencing DGKθ on the expression of lysophosphatidic acid acyltransferases or GPAT (glycerol-3-phosphate acyltransferase) isoforms (data not shown).

### 3.6. DGKθ$^{kd}$ decreases cholesterol levels

Because we observed decrease in the expression of genes involved in cholesterol uptake and trafficking (Fig. 2C), we sought to determine if silencing DGKθ affected de novo cholesterol biosynthesis. As shown in Fig. 7A cellular cholesterol concentrations were reduced by 38% in DGKθ$^{kd}$ cells. Consistent with this finding, the mRNA and protein expression of multiple genes in the cholesterol biosynthetic pathway, including lanosterol 14α-demethylase (CYP51) and 3-hydroxy-3-methylglutary coenzyme A reductase (HMG-

CoA red), was significantly decreased (Fig. 7B and D) in the DGKθ$^{kd}$ cell line. In contrast, silencing DGKθ increased the mRNA expression of lanosterol synthase [33] by 3-fold. Since most of cholesterologenic genes are regulated by the sterol regulatory element binding proteins (SREBPs) [34–36], we also assessed the expression of these transcription factors in DGKθ$^{kd}$ cells and found that the mRNA (Fig. 7C) and protein (Fig. 8D) expressions of both SREBP1 and SREBP2 were significantly repressed. Finally, the mRNA expression of insulin-induced gene (INSIG) 1 and INSIG2 which regulate the ability of SREBPs cleaved and imported into the nucleus were decreased by 68% and 48%, respectively (Fig. 7C). While silencing DGKθ repressed the protein levels of INSIG2, the decrease in INSIG1 that was observed at the mRNA level (Fig. 7C) was not seen in the protein expression (Fig. 7D).

## 4. Discussion

In the human adrenal cortex, cortisol and adrenal androgen biosynthesis is tightly regulated by the ACTH peptide hormone, which activates a cAMP-dependent pathway [2]. ACTH/cAMP signaling activates multiple cellular processes, including the increased mobilization of cholesterol and the induction of gene expression. One of the transcription factors that plays a key role not only in the cAMP-dependent transcription of steroidogenic genes, but also in the development of the adrenal gland is the nuclear receptor SF1. The ability of SF1 to activate target genes is regulated by various mechanisms, such as post-translational modification [37–41] and ligand binding [3, 4, 42–44]. We have previously shown that cAMP signaling increases PA, a ligand for SF1, concentrations in the nucleus of adrenocortical cells via a mechanism that required DGK activity, where chemical inhibition of DGK activity using R59949 decreased SF1-dependent activation of a CYP17A1 reporter gene [3]. Here in, we demonstrate that silencing DGKθ expression reduces the expression of most genes required for steroidogenesis. DGKθ knockdown repressed the expression of most enzymes required for the conversion of cholesterol to cortisol and DHEA (Fig. 2). We have previously demonstrated that DGKθ activity is required for SF1/cAMP-stimulated CYP17A1 transcription [3]. Consistent with these published studies, our data show that suppression of DGKθ repressed both the basal and cAMP-dependent mRNA and protein expression of CYP17A1 (Fig. 2B and D). While similar findings were observed with the expression of CYP11A1, CYP11B, and 3β-HSD in the DGKθ$^{kd}$ cells, silencing DGKθ increased the expression of CYP21A2. Given that PA activates SF1-dependent transcription [3] and that SF1 regulates CYP21A2 expression [45], these findings are un expected. However, CYP21A2 is regulated by other transcription factors. Members of the NR4A subfamily of nuclear receptors, all of which are induced in the DGKθ$^{kd}$ cell line (Fig. 4), positively regulate CYP21A2 expression [27, 46]. Notably, although SF1 levels is unaltered in NGFI-B knockout mice, targeted disruption of the receptor abolished ACTH-stimulated CYP21A2 expression [46], demonstrating the role of multiple nuclear receptors in conferring optimal steroidogenic capacity. Moreover, NGFI-B can bind to SF1 sites and activate target gene transcription [28]. In addition to regulating ACTH-stimulated steroidogenesis, members of the NR4A family also play key roles in the production of aldosterone, particularly by regulating the induction of CYP11B2 and 3β-HSD [25, 26, 47, 48]. Interestingly, although silencing DGKθ elicits opposite effects on steroidogenic gene expression (e.g. CYP17A1, StAR, CYP11A1, CYP11B) as silencing ASAH1 [5], suppression of both lipid enzymes results in marked increases in the expression of NR4A family members. The underlying mechanism by which NR4A family members are uniquely sensitive to the changes in the expression of ASAH1 and DGKθ is unclear, particularly since we have not observed an increase in NR4A members when other sphingolipid and PA metabolizing en zymes are silenced (Kai and Sewer, unpublished observations, 2013). It is also plausible that alterations in the expression of other transcription factors in the DGKθ$^{kd}$ cell line may lead to changes in the expression of steroidogenic genes. While the most significantly altered transcription factor in the DGKθ$^{kd}$ cell line was SREBP1, microarray studies detected changes in the expression of other transcription factors, such as liver X receptor, CCAAT/enhancer binding protein gamma, or STAT1 (signal transducer and activator of transcription 1) that may regulate steroidogenic gene transcription. Finally, though our findings are unexpected when comparing the effect of silencing ASAH1 to DGKθ, on steroidogenic capacity, we find it noteworthy that the ratio of cortisol to DHEA is markedly different in these two cell lines. Silencing ASAH1 resulted in a ratio of cortisol:DHEA of 1:4 [5], whereas this ratio was 3.8:1 in the DGKθ$^{kd}$ cell line. Thus, although similarities exist with regard to the increase in cortisol when the concentrations of SF1 ligands PA (DGKθ-produced agonist) and SPH (ASAH1 produced antagonist) are reduced, differences in the ratio of cortisol:DHEA suggest distinct roles for these two enzymes in regulating the overall balance of these two steroid metabolites.

We show that silencing DGKθ results in an increase in select genes that are involved in sphingolipid metabolism (Fig. 5). Notably, we only observed an increase in the expression of enzymes that are involved in ceramide hydrolysis and sphingosine phosphorylation (Fig. 5B and C). Microarray analysis of wild type and DGKθ$^{kd}$ RNA revealed no significant change in the expression of enzymes that are required for de novo sphingolipid biosynthesis, nor did we observe a change in the expression of ceramide synthases or the sphingomyelin synthases (data not shown). These findings are in contrast to our previous studies where we found that silencing ASAH1 leads to significant changes in the expression of most of the enzymes that are involved in sphingolipid metabolism [5]. Notably, the enzymes that exhibited increased expression in the DGKθ$^{kd}$ cell line (ASAH1, SPHK1, SPHK2; Fig. 5) were enzymes that have been shown to be expressed in the nucleus. SPHK2 regulates gene transcription [49, 50] and DNA synthesis [51] by catalyzing the production of sphingosine-1-phosphate in the nucleus. We have shown that ASAH1 is expressed in the nucleus of adrenocortical cells, where it functions as a transcriptional coregulatory protein by binding to SF1 when the receptor is bound to DNA [6]. Moreover, we have preliminary data indicating a role for the nuclear import of SPHK1 in adrenocortical steroidogenesis (Li and Sewer, unpublished observations, 2013). Given that DGKθ is also expressed in the nucleus of adrenocortical cells [22], it is likely that silencing DGKθ selectively alters nuclear sphingolipid and phospholipid concentrations. Additionally, SPH has been reported to increase both DGK-dependent PA production [52, 53], suggesting interplay between these two bioactive lipids. However, further studies are required to determine the molecular mechanism that underlies the increase in ASAH1, SPHK1, and SPHK2 expression in DGKθ$^{kd}$ cells.

In addition to serving as a ligand for SF-1, PA plays a key intermediate role in glycerophospholipid and triacylglycerol synthesis, as well as in the regulation of cell signaling [54]. PA metabolism can be achieved by the action of lipins. Of note, lipin1 and lipin2 are expressed in the nucleus of H295R cells (Betancourt-Torres and Sewer, unpublished observations, 2013). Herein, silencing DGKθ expression attenuated the cAMP-induced reduction of DAG (Fig. 6A). However, in contrast to our predictions, DAG did not accumulate in DGKθ$^{kd}$ cells, which may be due to the decreased expression of lipin1 and/or lipin2 (Fig. 6D and E). ACTH/cAMP signaling rapidly increases PA in adrenocortical cells [3, 55]. Silencing of DGKθ completely abolished cAMP-stimulated increased in cellular PA (Fig. 6B), suggesting that DGKθ plays a predominant role in generating PA in response to cAMP signaling. However, since PA is generated by multiple sources, including PLD this enzyme family has the capacity to contribute to the increase in PA, particularly since the mRNA and protein expression of PLD1 is up-regulated by Bt$_2$cAMP in wild type H295R adrenocortical cells (Fig. 6C and D). However, silencing DGKθ decreased the basal and cAMP-stimulated mRNA and protein levels of PLD1 and PLD2 (Fig. 6). Studies are ongoing to identify all of the enzymes that regulate nuclear PA concentrations and to determine their relative roles in cAMP-stimulated adrenocortical steroid hormone biosynthesis.

Silencing DGKθ significantly decreases the expression of multiple genes in the de novo cholesterol biosynthetic pathway (Fig. 7). The repression of cholesterologenic genes is likely due to the effect of DGKθ knockdown on the expression of the SREBP transcription factors, particularly SREBP2. SREBP2 plays a critical role in maintaining cholesterol homeostasis by activating the transcription of cholesterol biosynthetic enzymes in response to low cellular cholesterol [56]. Consistent with the role of this transcription factor, cholesterol levels in the DGKθ$^{kd}$ cells were reduced (Fig. 7A). Notably, in addition to the genes examined in Fig. 7, microarray analysis of the DGKθ$^{kd}$ cell line revealed that most genes in the cholesterol biosynthetic pathway, including 24-dehydrocholesterol reductase, squalene epoxidase, mevalonate kinase, and HMG CoA-synthase, were all reduced at the level of mRNA expression (data not shown). Although the mechanism by which DGKθ may contribute to regulating cellular cholesterol concentrations and cholesterol biosynthetic gene expression is unclear, our previous studies have shown that silencing SF1 also abrogates the expression of SREBP isoforms in adrenocortical cells [22]. Thus, DGKθ, possibly via PA-dependent activation of SF-1 may play a role in regulating the expression of genes in the de novo cholesterol biosynthetic pathway. In conclusion, we show that silencing DGKθ expression alters not only steroidogenic gene expression and hormone output, but also the expression of genes required for de novo cholesterol biosynthesis, sphingolipid metabolism, and phospholipid homeostasis. Our findings reveal novel roles for this nuclear kinase in regulating the flux through multiple lipid metabolic pathways.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **DGK** | diacylglycerol kinase |
| **PA** | phosphatidic acid |
| **SF1** | steroidogenic factor 1 |
| **CYP** | cytochrome P450 |
| **DAG** | diacylglycerol |
| **DHEA** | dehydroepiandrosterone |
| **StAR** | steroidogenic acute regulatory protein |
| **ASAH1** | acid ceramidase |
| **SPHK** | sphingosine kinase |
| **PLD** | phospholipase D |
| **LPIN** | lipin |
| **SREBP** | sterol regulatory element binding protein |
| **LDLR** | low density lipoprotein receptor |

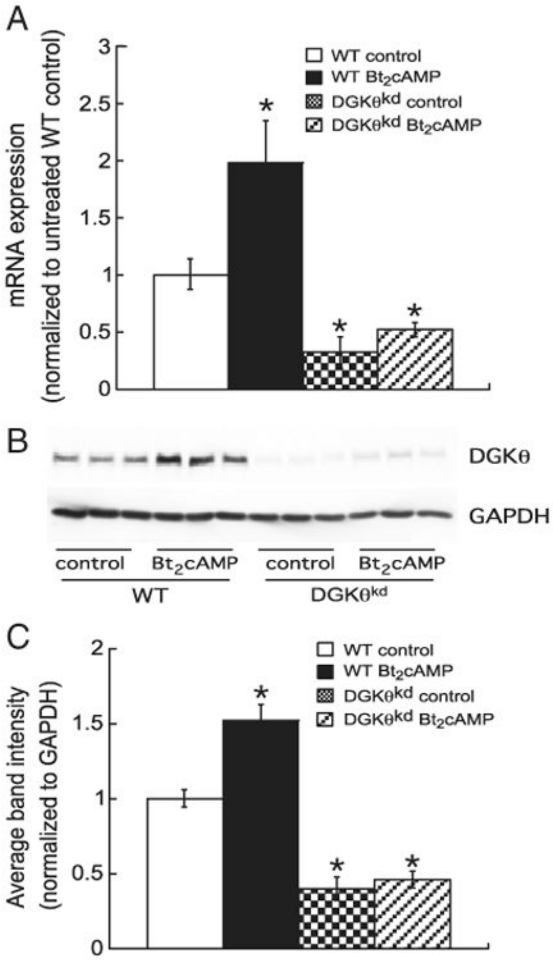

**Fig. 1.**

Characterization of H295R DGKθ$^{kd}$ cell line. Wild type (WT) and DGKθ$^{kd}$ cells were plated and the DGKθ$^{kd}$ cells treated with 5 μg/ml tet per day for 96 h. Cells were incubated with 0.4 mM Bt$_2$cAMP for 24 h (A) or48 h (B and C). (A) Total RNA was isolated from WT and DGKθ$^{kd}$ cells and the expression of DGKθ measured by real time RT-PCR. Data are graphed as fold change in DGKθ mRNA expression, normalized to the mRNA expression of β-actin, and represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisk (*) denotes statistically significant difference from untreated WT control group, $p < 0.05$. (B) Representative western blot of lysates isolated from untreated and Bt$_2$cAMP-treated WT and DGKθ$^{kd}$ cells. Protein was separated by SDS-PAGE and DGKθ and GAPDH assessed by western blotting. (C) Densitometric analysis of DGKθ and GAPDH protein expression in WT and DGKθ$^{kd}$ untreated and Bt$_2$cAMP-stimulated cells. Data graphed represent the mean ± SEM of three separate experiments, each carried out in triplicate. Asterisks indicate a statistically significant difference compared to untreated WT controls ($p < 0.05$).

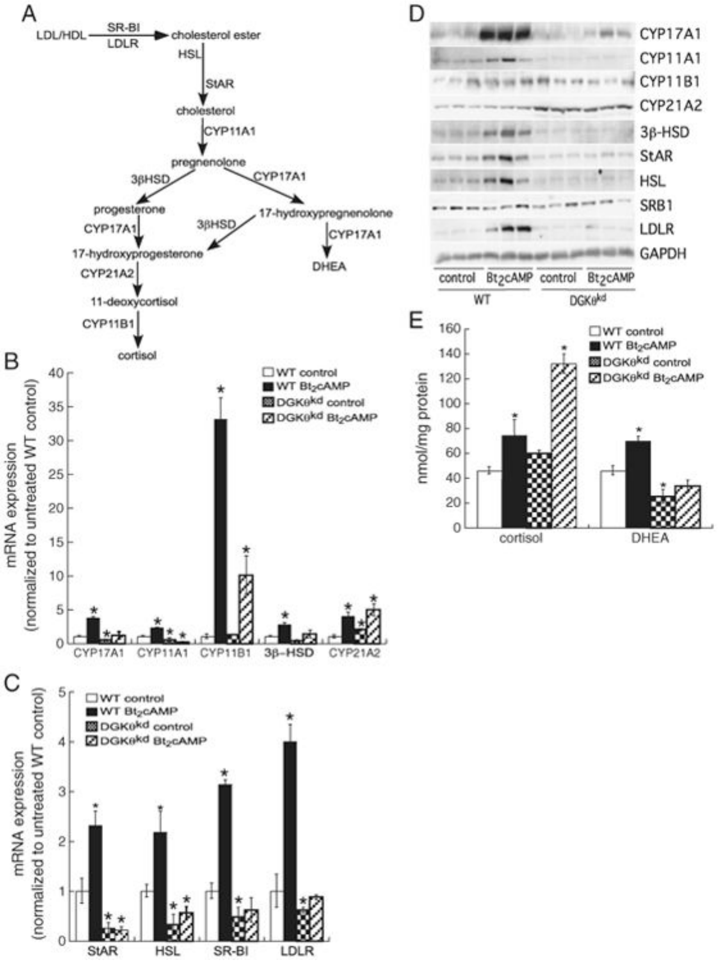

**Fig. 2.**

Silencing DGKθ abrogates basal and cAMP-stimulated steroidogenic gene expression. (A) Diagram of the adrenocortical steroid hormone biosynthetic pathway. WT and DGKθ$^{kd}$ (96 h tet pretreated) cells were treated with 0.4 mM Bt$_2$cAMP for 24 h (B) (C) or 48 h (D). (B) and (C) Total RNA was isolated and steroidogenic gene expression quantified by real time RT-PCR and normalized to mRNA expression of β-actin. Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) compared to the untreated WT. (D) Total cell lysates from WT and DGKθ$^{kd}$ cells were separated by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with antibodies against CYP17A1, CYP11A1, CYP11B, CYP21A2, 3β-HSD, StAR, HSL, SR-BI, LDLR and GAPDH. (E) Growth media from untreated and Bt$_2$cAMP-stimulated (48 h) WT and DGKθ$^{kd}$ cells was collected and the amounts of cortisol and DHEA quantified by ELISA. Steroid hormone amounts were normalized to the cellular protein concentration. Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) compared to the untreated WT.

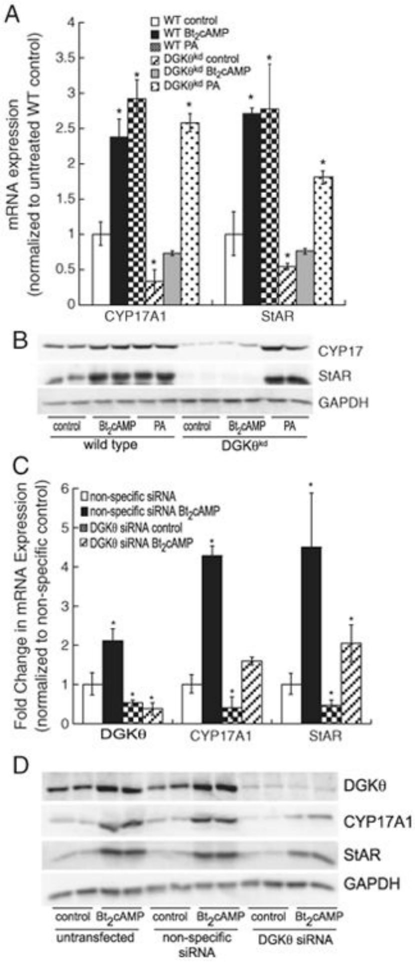

**Fig. 3.**

PA rescues steroidogenic gene expression. H295R WT and DGKθ$^{kd}$ cells were incubated with 0.4 mM Bt$_2$cAMP or 10 μM DLPA for 24 h (A) or48 h (B). (A) Total RNA was isolated and CYP17/StAR gene expression quantified by real time RT-PCR and normalized to mRNA expression of β-actin. Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) compared to the untreated WT. (B) Lysates from WT and DGKθ$^{kd}$ H295R cells were separated by SDS-PAGE and protein expression assessed by western blotting. (C and D) H295R cells were sub-cultured into 12 well plates and transfected with 100 nM nonspecific or DGK6 siRNA oligonucleotides followed by treatment with 0.4 mM Bt$_2$cAMP for 24 h (C) or 48 h (D). The mRNA and protein expression of CYP17 and StAR were quantified by qRT-PCR and western blot analysis, as described above.

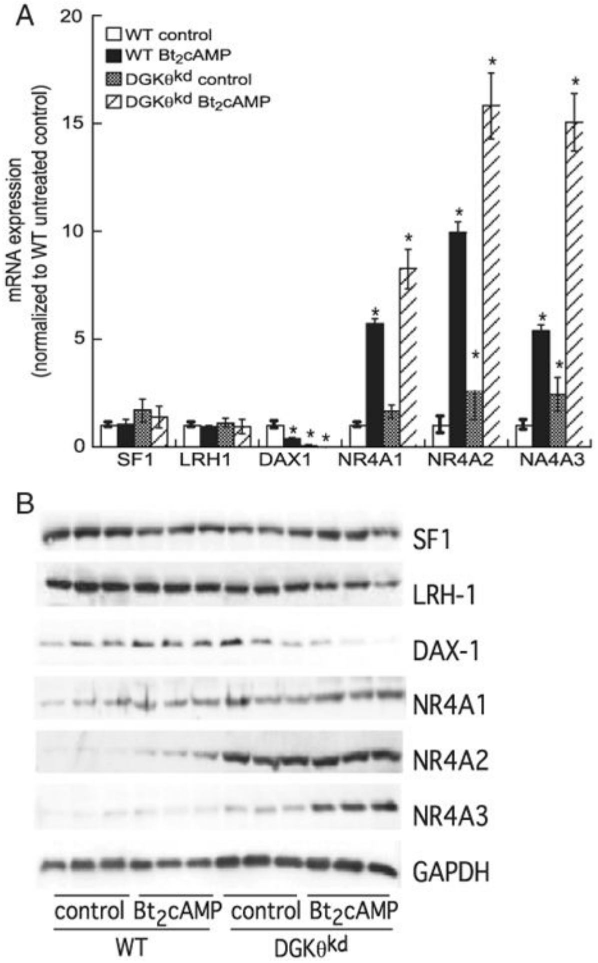

**Fig. 4.**

Nuclear receptor expression is altered in DGKθ$^{kd}$ cells. WT and 96 h tet pretreated-DGKθ$^{kd}$ H295R cells were treated with 0.4 mM $Bt_2cAMP$ for 24 h (A) or 48 h (B). (A) Real time RT-PCR was performed on RNA that was isolated from untreated or $Bt_2cAMP$-stimulated WT and DGKθ$^{kd}$ H295R cells. Data are graphed as fold change over untreated WT and the expression of each nuclear receptor is normalized to the mRNA expression of β-actin. Data represent the mean ± SEM of three separate experiments, each per formed in triplicate. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) from the untreated WT group. (B) Lysates from WT and DGKθ$^{kd}$ H295R cells were separated by SDS-PAGE and nuclear receptor expression assessed by western blotting.

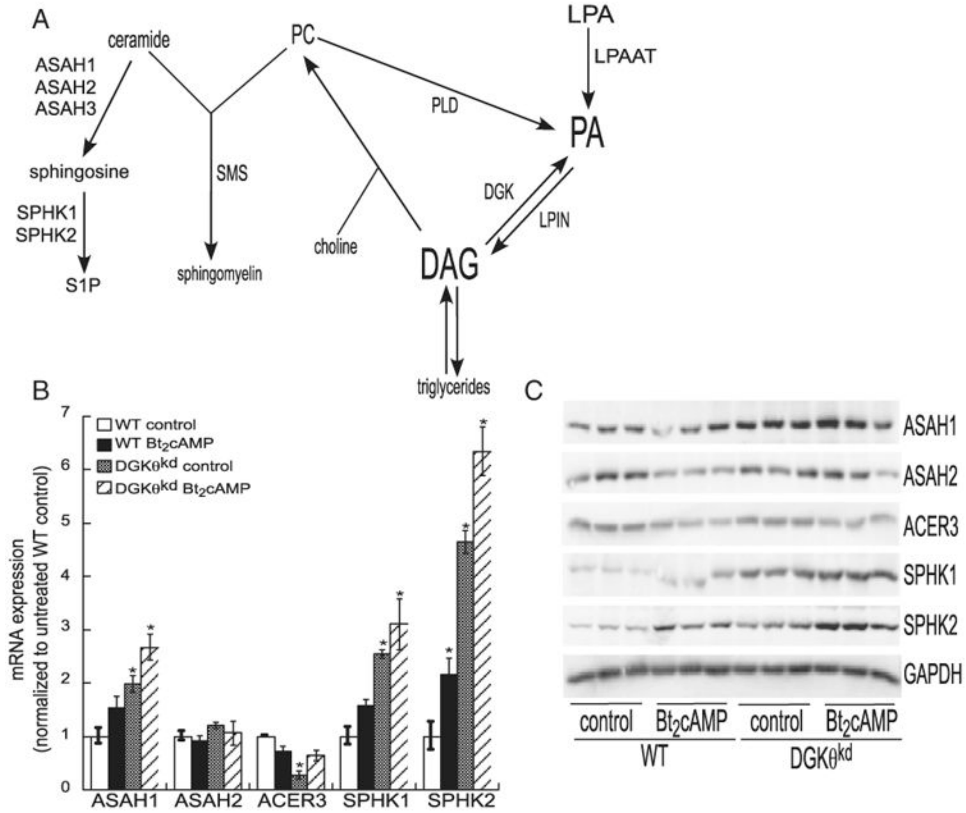

**Fig. 5.**

Silencing of DGKθ induces the expression of sphingolipid metabolic enzymes. (A) Overview of sphingolipid and phospholipid metabolic pathway. PA is synthesized from lysophosphatidic acid (LPA) by lysoPA-acyltransferase (LPAAT), phosphatidylcholine (PC) by phospholipase D (PLD), and DAG by DGKs. Ceramide and phosphatidylcholine are converted to sphingomyelin and DAG by sphingomyelin synthase. Ceramide is hydrolyzed by ceramidase (ASAH) generated sphingosine, which is further phosphorylated to form S1P by SPHK1 and SPHK2. (B) RNA was isolated from WT and tet-pretreated DGKθ$^{kd}$ cells that were stimulated with 0.4 mM Bt$_2$cAMP for 24 h. Real time RT-PCR was performed as described in the Materials and methods using the primers listed in Table 1. Data graphed represent the mean ± SEM of three separate experiments that were performed in triplicate and the expression of ASAH1, ASAH2, ACER3, SPHK1, and SPHK2 normalized to β-actin mRNA content. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) from the WT control group. (C) Western blotting was performed on lysates that were isolated from WT and DGKθ$^{kd}$ cells that were incubated with Bt$_2$cAMP for 48 h with antibodies against ASAH1, ASAH2, ACER3, SPHK1, SPHK2, and GAPDH. Shown are representative blots from protein expression studies that were carried out on three individual occasions, each time in triplicate.

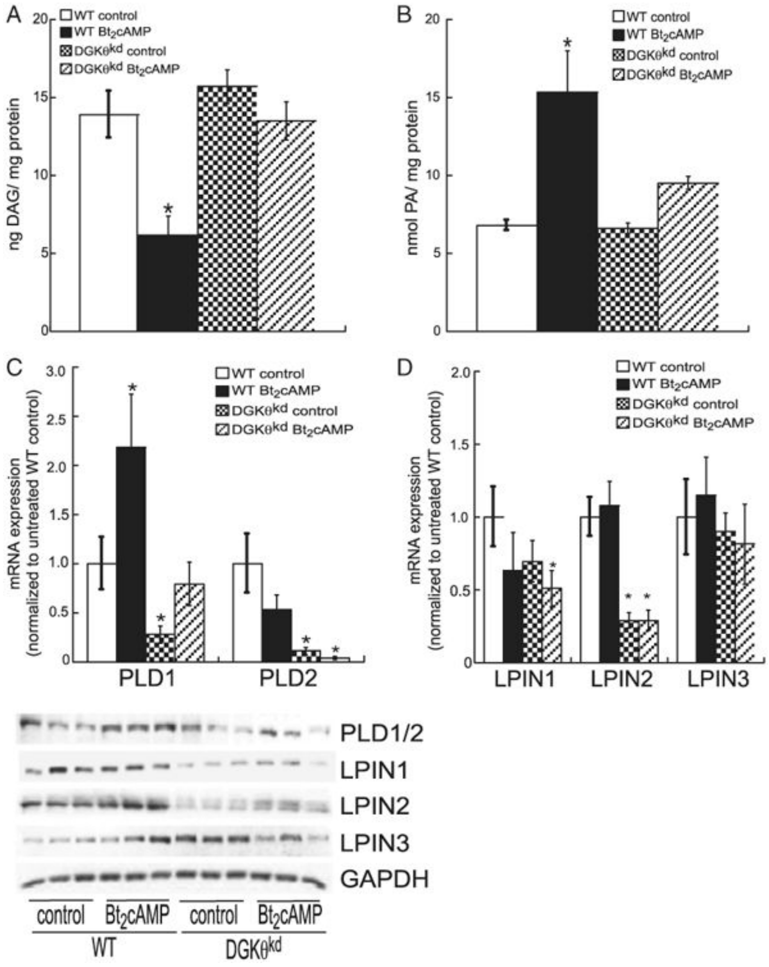

**Fig.6.**

Effect of DGKθ silencing on PA and DAG metabolism. (A) and (B) WT or DGKθ$^{kd}$ H295R cells were cultured on 6-welled plates and treated with 0.4 mM Bt$_2$cAMP for 72 h. The cellular content of DAG (A) and PA (B) assessed as described in the Materials and methods section. Total lipid content was normalized to the cellular protein concentration and graphed data represent the mean ± SEM of three experiments (n = 3 per experiment). (C) and (D) RNA was isolated from WT and DGKθ$^{kd}$ cells that were treated with Bt2cAMP for 24 h and real time RT-PCR performed for PLD1 and PLD2 (C) or LPIN1, LPIN2, and LPIN3 (D). Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) indicate a statistically significant difference from untreated group, $p < 0.05$. (E) Western blotting was performed on protein lysates that were isolated from untreated and Bt$_2$-cAMP-stimulated (48 h) WT and DGKθ$^{kd}$ cells with antibodies against PLD1/2, LPIN1, LPIN2, LPIN3, and GAPDH. Shown are representative blots from protein expression studies that were carried out on three individual occasions, each time in triplicate.

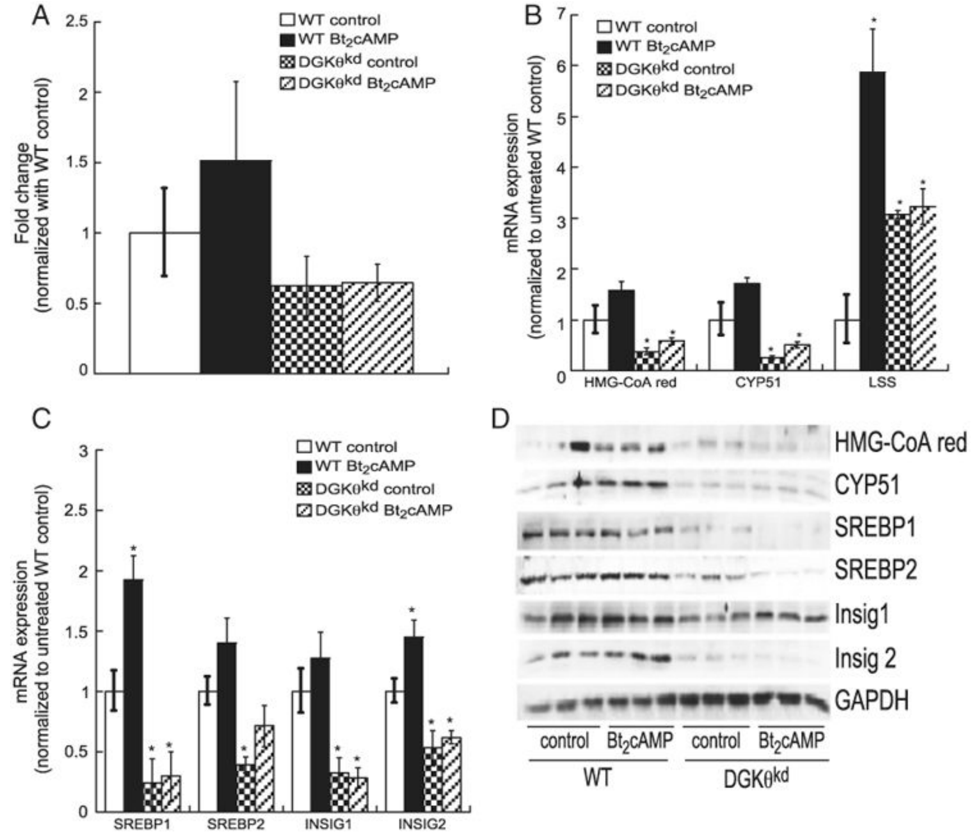

**Fig. 7.**

Silencing DGKθ represses de novo cholesterol biosynthesis. (A) The amount of cholesterol in WT and tet-pretreated DGKθ$^{kd}$ cells was determined as described in the Materials and methods section and normalized to the cellular protein concentration. Data graphed represent the mean ± STD of three experiments (n = 3 per experiment). (B) and (C) RNA from WT and DGKθ$^{kd}$ cells that were treated for 24 h with 0.4 mM $Bt_2cAMP$ was subjected to real time RT-PCR and the expression of HMG-CoA red, CYP51 and LSS(B) or SREBP1, SREBP2, Insig1 and Insig2 (C) quantified using the primers listed in Table 1. Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate and the mRNA expression of cholesterologenic genes normalized to β-actin mRNA expression. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) compared to the untreated WT. (D) SDS-PAGE and western blotting were performed on protein lysates that were isolated from untreated and $Bt_2cAMP$-stimulated (48 h) WT DGKθ$^{kd}$ H295R cells. Shown are representative blots from protein expression studies that were carried out on three individual occasions, each time in triplicate.

## Table 1

Primer sets used in RT-PCR.

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| β-Actin | ACGGCTCCGGCATGTGCAAG | TGACGATGCCGTGCTGCATG |
| DGKθ | CGTTCTCCGTACTGCTGTC | GTCTGCCGTGTCGTTCTC |
| CYP17A1 | CTCTTGCTGCTTCACCTA | TCAAGGAGATGACATTGGTT |
| CYP11A1 | CGTGGAGTCGGTTTATGTC | CTCTGGTAATACTGGTGATAGG |
| CYP11B1/2 | ACGGCGACAACTGTATCC | AGAGCGTCATCAGCAAGG |
| 3β-HSD | CCAGTAGCATAGAGGTAGCC | TCAGATTCCACCCGTTAGC |
| CYP21A2 | TGTGGAACTGGTGGAAGC | GGTGGAGCCTGTAGATGG |
| StAR | GCTCTCTACTCGGTTCTC | GCTGACTCTCCTTCTTCC |
| HSL | CACTACAAACGCAACGAGAC | CCAGAGACGATAGCACTTCC |
| SR-BI | CCATCCTCACTTCCTCAAC | CCACAGGCTCAATCTTCC |
| LDLR | ACGGTGGAGATAGTGACAATG | AGACGAGGAGCACGATGG |
| NR5A1 | GGAGTTTGTCTGCCTCAAGTTCA | CGTCTTTCACCAGGATGTGGTT |
| NR5A2 | TACCGACAAGTGGTACATGGAA | CGGCTTGTGATGCTATTATGGA |
| NR0B1 | CCAAATGCTGGAGTCTGAACATC | CCCACTGGAGTCCCTGAATGTA |
| NR4A1 | GGACAACGCTTCATGCCAGCAT | CCTTGTTAGCCAGGCAGATGTAC |
| NR4A2 | AAACTGCCCAGTGGACAAGCGT | GCTCTTCGGTTTCGAGGGCAAA |
| NR4A3 | ACTGCCCAGTAGACAAGAGACG | GTTTGGAAGGCAGACGACCTCT |
| NR0B1 | CCAAATGCTGGAGTCTGAACATC | CCCACTGGAGTCCCTGAATGTA |
| NR0B2 | TGCCTGAAAGGGACCATCCTCT | GTTCCAGGACTTCACACAGCAC |
| ASAH1 | GCACAAGTTATGAAGGAAGCCAAG | TCCAATGATTCCTTTCTGTCTCG |
| ASAH2 | GCATCAACACAGGAGAGTC | GGAGGCAGAGGCATAGAG |
| ACER3 | ATCCGCCTGGTCTTCATC | CTCCTTATTGCTGGTCTTCC |
| SK1 | CTGGCAGCTTCCTTGAACCAT | TGTGCAGAGACAGCAGGTTCA |
| SK2 | CCAGTGTTGGAGAGCTGAAGGT | GTCCATTCATCTGCTGGTCCTC |
| PLD1 | GCCTATGGAAGGTGGGACGAC | GGAGTACCTGTCAATGAAATCAGC |
| PLD2 | GAACAGGGGCAGTGTTTCCGA | CGCTGTTTCTTGCCACAGCTG |
| Lipin1 | AACACCACAATCAAGGAG | GACATTAGGCAGAAGAGG |
| Lipin2 | AACAAGTCATCGTATCACAGG | CTCGCCAGTAGCAGAAGG |
| Lipin3 | TGAGCAGTGATGACGATG | CTTCTTGTAGGTAGGAGTGG |
| HMG-CoA red | GACGTGAACCTATGCTGGTCAG | GGTATCTGTTTCAGCCACTAAGG |
| CYP51 | GCAGATTTGGATGGAGGTTTC | TCCTTGATTTCCCGATGAGC |
| LSS | GACGACCGATTCACCAAGAGCA | AGACATGCTCCTGGAAGGCAGT |
| SREBP1 | ACTTCTGGAGGCATCGCAAGCA | AGGTTCCAGAGGAGGCTACAAG |
| SREBP2 | CCCTGGGAGACATCGACGA | CGTTGCACTGAAGGGTCCA |
| Insig1 | TTGTTGGCATTAACCACGCC | GTCGTCCTATGTTCCCCACC |
| Insig2 | GACTCGTTGGTGGAATGCCT | AAGAGGTGCTGGTTGCCATT |

## Table 2

Antibodies used for western blotting.

| Target protein | Catalog no. and vendor |
|---|---|
| DGKθ | HPA026797, Sigma-Aldrich (St. Louis, MO) |
| GAPDH | sc-25778, Santa Cruz Biotechnology, Inc. |
| CYP17A1 | sc-66849, Santa Cruz Biotechnology, Inc. |
| CYP11A1 | Dr. Michael R. Waterman, Vanderbilt University School of Medicine |
| CYP11B | sc-28205, Santa Cruz Biotechnology, Inc. |
| 3β-HSD | sc-28206, Santa Cruz Biotechnology, Inc. |
| StAR | sc-25806, Santa Cruz Biotechnology, Inc. |
| HSL | sc-25843, Santa Cruz Biotechnology, Inc. |
| SR-BI | SC-67098, Santa Cruz Biotechnology, Inc. |
| LDLR | ab30532, Abcam |
| SF-1 | 07-618, Millipore |
| LRH-1 | sc-25389, Santa Cruz Biotechnology, Inc. |
| DAX-1 | ab60144, Abcam |
| NR4A1 | SAB2101650, Sigma-Aldrich |
| NR4A2 | N6538, Sigma-Aldrich |
| NR4A3 | sc-100906, Santa Cruz Biotechnology, Inc. |
| ASAH1 | HPA005468, Sigma-Aldrich (St. Louis, MO) |
| ASAH2 | PRS4743, Sigma-Aldrich |
| ACER3 | sc-18822, Santa Cruz Biotechnology, Inc. |
| SK1 | ab71700, Abcam |
| SK2 | ab11948, Abcam |
| PLD1/2 | 05-608, Millipore |
| Lipin1 | ab70138, Abcam |
| Lipin2 | ab63928, Abcam |
| Lipin3 | ab70159, Abcam |
| HMG-CoA red | sc-33827, Santa Cruz Biotechnology, Inc. |
| CYP51 | ab135723, Abcam |
| SREBP1 | sc-8984, Santa Cruz Biotechnology, Inc. |
| SREBP2 | sc-5603, Santa Cruz Biotechnology, Inc. |
| Insig1 | ab70784, Abcam |
| Insig2 | sc-66936, Santa Cruz Biotechnology, Inc. |