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Dkk3 is a component of the genetic circuitry regulating aldosterone biosynthesis in the adrenal cortex

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Primary aldosteronism (PA, autonomous aldosterone production from the adrenal cortex) causes the most common form of secondary arterial hypertension (HT), which is also the most common curable form of HT. Recent studies have highlighted an important role of mutations in genes encoding potassium channels in the pathogenesis of PA, both in human disease and in animal models. Here, we have exploited the unique features of the hyperaldosteronemic phenotype of *Kcnk3* null mice, which is dependent on sexual hormones, to identify genes whose expression is modulated in the adrenal gland according to the dynamic hyperaldosteronemic phenotype of those animals. Genetic inactivation of one of the genes identified by our strategy, *dickkopf-3* (*Dkk3*), whose expression is increased by calcium influx into adrenocortical cells, in the *Kcnk3* null background results in the extension of the low-renin, potassium-rich diet insensitive hyperaldosteronemic phenotype to the male sex. Compound *Kcnk3/Dkk3* animals display an increased expression of Cyp11b2, the rate-limiting enzyme for aldosterone biosyntheis in the adrenal *zona glomerulosa* (ZG). Our data show that *Dkk3* can act as a modifier gene in a mouse model for altered potassium channel function and suggest its potential involvement in human PA syndromes.

INTRODUCTION

Primary aldosteronism (PA) is a common form of endocrine hypertension (HT) suspected in the presence of arterial hypertension, hypokalemia and low plasma renin levels. PA is now recognized as the most common form of secondary HT, with a prevalence estimated of ~10% in referred patients and 4% in primary care (1), and the most common curable form of HT (2). Early detection of PA has enormous impact on the clinical outcome and survival, given the severe adverse effects of aldosterone excess independent of high blood pressure levels. Patients with PA have been reported to exhibit more severe left ventricular hypertrophy and diastolic dysfunction than patients with essential HT (3) and a high prevalence of myocardial infarction, stroke and atrial fibrillation (4).

While for a long time the genetic causes of PA have remained unknown, recent findings have revealed an important role for an altered function of potassium channels in the

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pathogenesis of this disease. The basis for the unique sensitivity of adrenal *zona glomerulosa* (ZG) cells for plasma potassium concentration is a very high membrane background potassium conductance, which is dependent on the expression at high levels of 2-pore domain (K2P) potassium channels, TWIK-related acid-sensitive K+ channel 1 (TASK1) (KCNK3) and Task3 (KCNK9) (reviewed in 5). Recent studies have shown that mice lacking *Kcnk3* (6), *Kcnk9* (7,8) or both *Kcnk3* and *Kcnk9* (9) have PA, which is determined by different mechanisms. In *Kcnk3* null mice, hyperaldosteronism is present in young animals of either sex, but is corrected in males after puberty. Strikingly, in these animals, hyperaldosteronism associates with abnormal adrenal cortex functional zonation, since *Cyp11b2,* the rate-limiting enzyme for aldosterone production, is expressed in the inner region of the adrenal cortex and not in the glomerulosa (6). Remarkably, hyperaldosteronism can be suppressed by dexamethasone administration in these animals. This phenotype is under the control of sex hormones, as shown by the fact that castration of male *Kcnk3* null animals prevents normal *Cyp11b2* distribution, while testosterone injection in female *Kcnk3* null mice restores the expression of *Cyp11b2* in ZG cells. Conversely, *Kcnk9* null mice display salt-sensitive HT, an elevated aldosterone/renin ratio (ARR), hypersensitivity to angiotensin II and failure to suppress aldosterone production with dietary salt loading, similarly to human subjects with low-renin HT (7,8). Moreover, compound *Kcnk3/Kcnk9* null male mice fed either a low- or a high-sodium diet present milder PA, compared with female *Kcnk3* null mice, which is not sensitive to AT1 receptor blockade. In these animals, TASK-like background currents are absent in ZG cells and their membrane potential is significantly depolarized (9). No sign of tumor formation has been described in the adrenal cortex of any of these mutant mice. In human Conn's adenomas, which represent the most important cause of clinical PA, recurrent somatic mutations of the *KCNJ5* gene, encoding a potassium channel highly expressed in the human adrenal cortex, are found in more than one-third of the patients (10,11). Those mutations alter the selectivity filter of the channel and produce increased sodium conductance and cell depolarization causing calcium entry. This has been hypothesized to produce increased aldosterone production and induce cell proliferation. Remarkably, a germline mutation in the same gene was also found in a familial form of severe PA with massive bilateral adrenal hyperplasia (10).

We took advantage of the unique characteristics of the *Kcnk3* null mouse model to search for genes that can modify their phenotype of PA and adrenocortical functional zonation. Using gene expression profiling in the adrenal glands of *Kcnk3* null mice and exploiting the possibility of modulation of their phenotype by sexual hormones, we identified a cluster of genes closely associated with hyperaldosteronism in a sex- and hormone-dependent dynamic fashion. Among these genes, we focused our attention on *Dkk3* (*dickkopf3*), encoding a peculiar member of the *dickkopf* family of Wnt signaling modulators (reviewed in 12) because of its close association with aldosterone-producing cells in humans (13). Inactivation of *Dkk3* in the *Kcnk3* null background causes extension of the hyperaldosteronemic phenotype and increased expression of *Cyp11b2* in the adrenal gland to the male sex, without affecting functional zonation. These data indicate that *Dkk3* is a component of the genetic circuitry regulating expression of *Cyp11b2* and suggest that it may be implicated in the pathogenesis of low-renin hyperaldosteronism in humans.

RESULTS

Adrenocortical morphological zonation is not altered in Kcnk3 null mice

The distribution of Cyp11b2 expression in the adrenal cortex of *Kcnk3* null mice is sexually dimorphic, with females displaying aberrant expression in the *zona fasciculata*, while males retain expression in the ZG (6). Remarkably, the adrenal gland of female *Kcnk3* null mice has a morphologically normal architecture (Fig. 1A) and the ZG marker Dab2 (14) is expressed in the outer part of the gland, similarly to wild-type (WT) animals (Fig. 1B). These data show that lack of *Kcnk3* expression in female mice is sufficient to profoundly modify the domain of expression of *Cyp11b2* in the adrenal cortex, but not morphological zonation of the gland and molecular differentiation of the ZG.

Expression of a set of genes is altered in the adrenals of Kcnk3 null mice according to altered functional zonation

With the purpose of identifying the molecular pathways modulating Cyp11b2 expression in the adrenal glands of *Kcnk3* null mice, we exploited the unique properties of hormonal regulation of their phenotype (6). We studied whole adrenal gene expression profiles of male and female *Kcnk3* null mice under basal conditions or after hormonal treatment (castration for males and testosterone injection for females), producing reversal of their sex-dependent adrenal phenotype, respectively (Fig. 2A). For comparison, WT animals of both sexes were also subjected to the same treatment. Microarray analysis of gene expression profiles revealed that the expression of only 68 transcripts significantly differed according to the normal or aberrant expression of *Cyp11b2* in the adrenal gland in a fashion modulated by genotype and hormonal treatments (Fig. 2B and Supplementary Material, Table S1). Gene ontology analysis showed that most of these transcripts are involved in signaling cascades (Supplementary Material, Table S2). This set of genes was further divided into subgroups depending on their modulations following castration or testosterone injection, reflecting rather a basal (group 1) or dynamic (group 2) zonation defect (Fig. 2C and Supplementary Material, Table S1). We selected 26 genes (group 3) that were significantly modulated under all conditions and focused our attention on *Dkk3*, encoding a peculiar member of the dickkopf family of Wnt signaling modulators (12), since this gene was previously shown to be selectively expressed in the ZG of the human adrenal cortex (13) and to inhibit aldosterone biosynthesis in cultured human adrenocortical cells (15). Significantly increased expression of *Dkk3* mRNA in female *Kcnk3* null mice adrenals compared with WT adrenals was confirmed by RT-qPCR (Supplementary Material, Fig. S3). The Wnt/β-catenin pathway has been implicated by several studies in the control of aldosterone secretion and glomerulosa cell differentiation in the mouse adrenal gland (reviewed in 16). However, the activation status of canonical

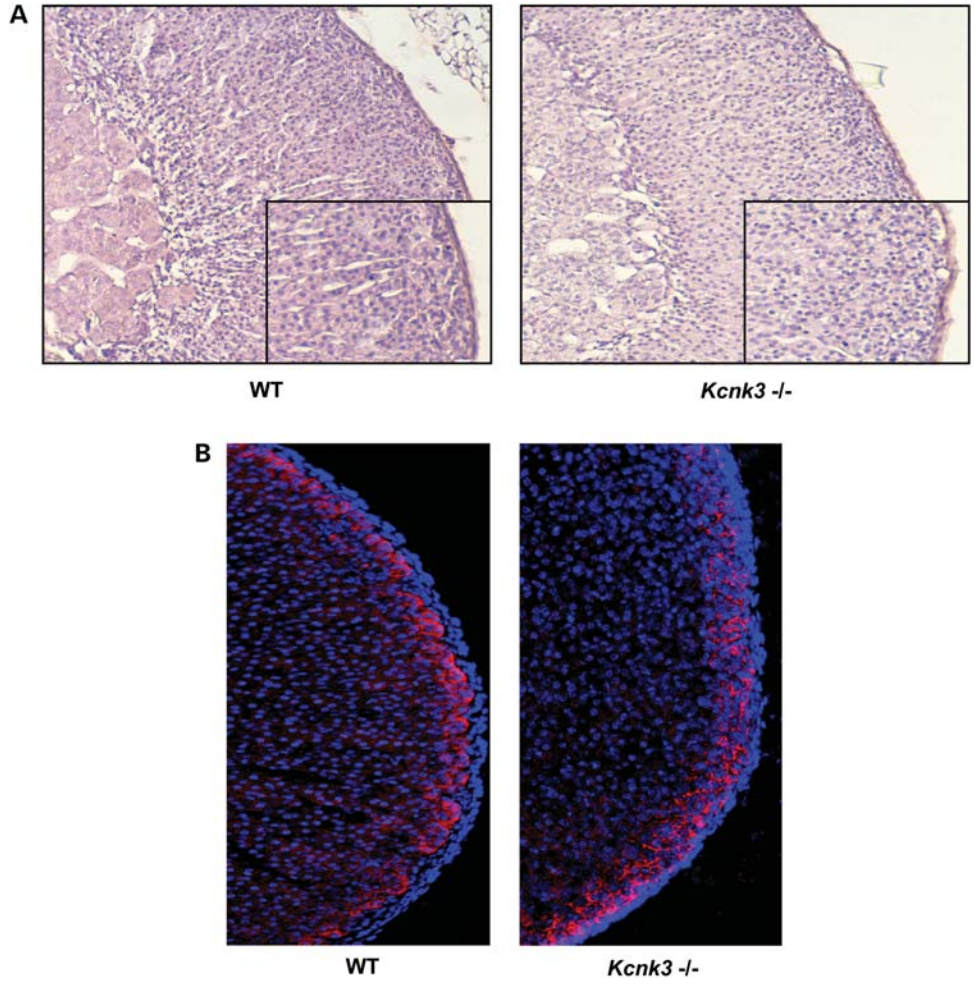

Figure 1. Normal morphological and molecular differentiation of the adrenal cortex of female *Kcnk3* null mice. (**A**) and (**B**) hematoxylin–eosin staining of the adrenal cortex of a 2-month-old female (A) WT and (B) *Kcnk3* null mice. 10× magnification. Insets: higher (×20) magnification of each panel. (**C**) and (**D**) Immunofluorescence staining for the ZG marker Dab2 (in red) in a 2-month-old female (C) WT and (D) *Kcnk3* null mice. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (in blue). ×10 magnification.

Wnt signaling, as monitored by nuclear staining of β-catenin, is normal in the adrenal cortex of female *Kcnk3* null mice (Supplementary Material, Fig. S4), being restricted to the outer part of the gland, as previously described (17).

Increase of intracellular calcium concentration induces Dkk3 expression in adrenocortical cells

Membrane depolarization in adrenal ZG cells induces opening of voltage-dependent calcium channels, which increase intracellular calcium concentration stimulating *Cyp11b2* expression and aldosterone production (reviewed in 5). Adrenocortical cells from *Kcnk3* null mice are depolarized compared with WT cells both under basal and stimulated (K^+ and AngII) conditions (6) and knockdown of its human homolog *KCNK3* in H295R cells was shown to increase intracellular calcium concentration (18). Since calcium induces *Dkk3* mRNA expression in human endothelial cells (19), we reasoned that calcium stimulation may be responsible for *Dkk3* transcript upregulation in the adrenal of *Kcnk3* null mice. Consistent with this hypothesis, treatment of mouse adrenocortical ATC-1 and Y-1 cells with the calcium ionophore induced a significant increase of *Dkk3* mRNA expression (Fig. 3).

Inactivation of the Dkk3 gene in the Kcnk3 null background extends the hyperaldosteronemic phenotype to the male sex without affecting functional zonation of the adrenal cortex

To investigate the impact that lack of the *Dkk3* gene has on the hyperaldosteronemic phenotype and altered zonation of *Kcnk3* null mice, we measured plasma aldosterone levels in WT, *Kcnk3* null, *Dkk3* null (20) and compound *Dkk3*/*Kcnk3* null mice both under basal conditions and after treatment with a potassium-rich diet. As previously reported (6), female, but not male, *Kcnk3* null mice had severely increased plasmatic aldosterone levels which could not be further increased significantly by a potassium-rich diet. *Dkk3* null mice of both sexes had normal plasma aldosterone levels which were increased by a potassium-rich diet. Compound *Kcnk3*/*Dkk3*

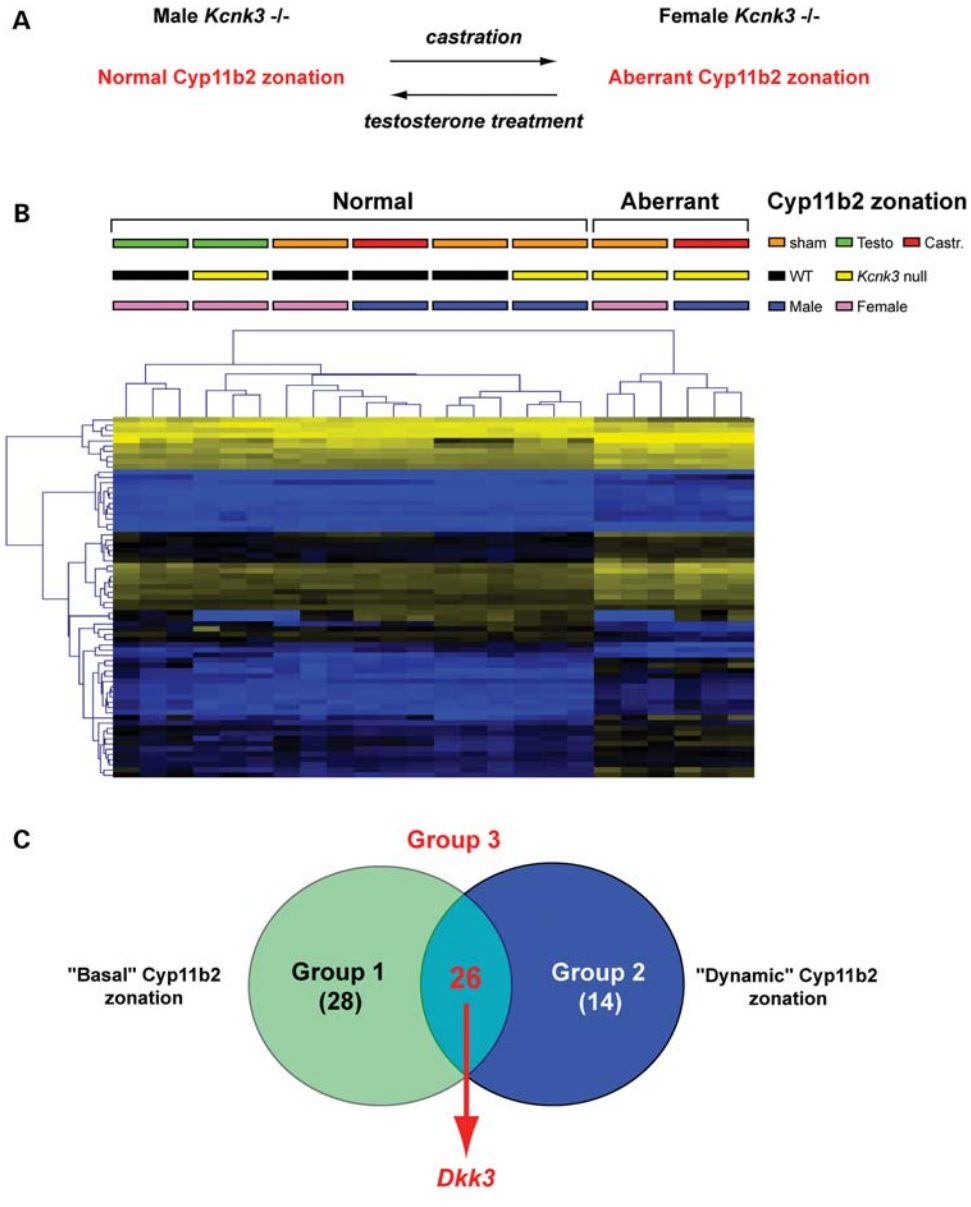

Figure 2. Altered gene expression associated with aberrant *Cyp11b2* zonation in adrenal glands from *Kcnk3* null mice. (**A**) Schematic representation of the adrenal phenotype associated with basal or hormonal treatments of male and female KcnK3 null mice. (**B**) Hierarchical clustering of samples using the 68 genes discriminating normal from aberrant *Cyp11b2* zonation. RNA samples from adrenal glands of male and female WT or *Kcnk3* null mice under basal conditions or after hormonal treatment (castration for males and testosterone injection for females) were harvested ($n = 3$, total of 24 samples) and expression profiles were determined with Agilent arrays. Gene expression patterns were determined by the two-way hierarchical clustering method for the eight experimental groups represented according to the gender (male/female), *Kcnk3* status (WT/KO) and hormonal treatment (sham/testo/cast) against the 68 selected genes reported in Supplementary Material, Table S1. Each square represents the expression level of a given gene and sample, colors vary from blue to yellow for the lowest to the highest gene expression levels, respectively. The distance corresponds to a Manhattan distance. (**C**) Venn diagram showing the repartition of the 68 selected genes into subgroups according to their regulation by sex hormones. Four distinct contrasts (C1–C4) were used in order to group the 68 genes reported in Supplementary Material, Table S1: C1: sham KO females versus sham KO males; C2: sham KO females versus sham WT females; C3: castrated KO males versus sham KO males and C4: sham KO females versus testosterone-treated KO females. Group 1 (genes differentially expressed only in C1 and C2) corresponds to genes associated with 'basal Cyp11b2 zonation'; Group 2 (genes differentially expressed only in C3 and C4) corresponds to genes associated with 'dynamic Cyp11b2 zonation'. Group 3 includes 26 genes significantly modulated under all four conditions. Adjusted *P*-value cutoff for selection in each contrast is 0.05.

null female animals had a similar phenotype to *Kcnk3* null mice. Remarkably, male compound *Kcnk3*/*Dkk3* null mice had increased aldosterone levels, which were not further significantly incremented by the potassium-rich diet (Fig. 4A). Conversely, corticosterone levels did not differ among the different groups of animals (Supplementary Material, Fig. S5). Furthermore, the ARR was also elevated in male compound *Kcnk3*/*Dkk3* null mice (Fig. 4B). Those animals had increased adrenal *Cyp11b2* mRNA expression albeit to a lesser degree than female *Kcnk3* and compound *Kcnk3/Dkk3* null mice (Fig. 4C). *Cyp11b2* expression was not simply a consequence of increased depolarization, since membrane potential of

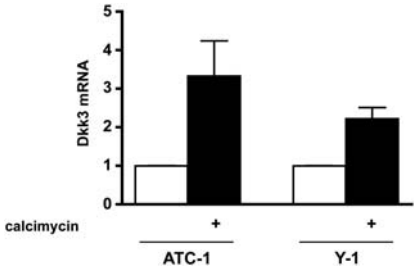

Figure 3. Increase of intracellular calcium concentration stimulates Dkk3 mRNA expression in mouse adrenocortical cell lines. ATC-1 and Y-1 cells were stimulated with calcimycin (0.1 μM) for 24 h before measurement of *Dkk3* mRNA levels by RT-qPCR, using the $2^{-?Ct}$ method and *Tbp* as a reference mRNA ($n = 4$). Calcimycin stimulation (black histograms) significantly increased *Dkk3* mRNA levels compared with basal (white histograms) in both the cell lines ($P = 0.0286$ for ATC-1 and $P = 0.0159$ for Y-1, Mann–Whitney test).

adrenocortical cells in compound *Kcnk3*/*Dkk3* null mice was similar to *Kcnk3* null mice (Supplementary Material, Fig. S6). Also, male compound *Kcnk3*/*Dkk3* null mice did not exhibit an altered functional zonation of their adrenal glands, since Cyp11b2 was expressed in the ZG, similarly to WT animals (Fig. 4D).

DISCUSSION

In this work, we took advantage of the unique sex hormone-dependent hyperaldosteronemic phenotype displayed by female *Kcnk3* null mice to identify *Dkk3* as a modifier gene whose functional inactivation extends the phenotype to the male sex. Marked sex differences in adrenal cortex structure, steroidogenic capacity, response to stress, enzymatic activities and propensity to disease have long been known to exist in several animal species including humans (21–25). A peculiar feature of the hyperaldosteronemic phenotype of *Kcnk3* null mice is its restriction to females and its depedence on sexual hormone administration. In those animals, hyperaldosteronism is dependent upon aberrant expression of Cyp11b2, the rate-limiting enzyme in aldosterone biosyntheis, in the adrenal *zona fasciculata* and suppression of its physiological expression in the ZG (6). Full reversion of the phenotype can be obtained by testosterone administration to female *Kcnk3* null mice. Conversely, male *Kcnk3* null mice become hyperaldosteronemic and display aberrant functional zonation, in terms of Cyp11b2 expression, after castration (6). Using gene expression profiling to identify genes whose expression is modulated coordinately with the hyperaldosteronemic phenotype in the adrenals of *Kcnk3* null mice, we identified *Dkk3* as a modifier gene whose inactivation in the *Kcnk3* null background extends the phenotype to the male sex. Remarkably, *Dkk3* inactivation did not induce aberrant functional zonation in compound *Kcnk3*/*Dkk3* null male mice but quite unexpectedly increased *Cyp11b2* expression in its physiological site of expression, the ZG, which lacks *Cyp11b2* expression in female *Kcnk3* null mice (6).

Dkk3 encodes a peculiar member of the *dickkopf* protein family whose founding component, *Dickkopf-1 (Dkk1)*, was identified for its activity as an embryonic head inducer and Wnt antagonist in *Xenopus* (26). However, *Dkk3* appears to represent a divergent member of the Dkk family according to several structural and evolutionary criteria (12) and conflicting results have been published about the ability of *Dkk3* to antagonize or activate the Wnt pathway in different biological systems (reviewed in 27). A close relationship appears to exist between canonical Wnt signaling and differentiation of aldosterone-producing cells in the adrenal cortex under both physiological and pathological conditions (reviewed in 16). Nevertheless, our results (Supplementary Material, Fig. S3) suggest that aberrant expression of Cyp11b2 and increased aldosterone production in the adrenal cortex of female *Kcnk3* null mice, where *Dkk3* expression is highly upregulated, can take place in the absence of alterations of canonical Wnt signaling, as monitored by β-catenin staining, and that *Dkk3* probably has a Wnt-independent role in modulating the hyperaldosteronemic phenotype of those mice. Our data suggest a model whereby *Dkk3* acts as a repressor of *Cyp11b2* expression in the adrenal ZG. In *Kcnk3* null mice adrenocortical cells are depolarized (6), with subsequent calcium entry (18) triggering increased *Dkk3* expression (Fig. 3). *Dkk3* acts to repress *Cyp11b2* expression in the ZG, but in females *Cyp11b2* is ectopically expressed in the *zona fasciculata,* probably due to the absence of the compensatory action of the TASK3 potassium channel (6), which forms heterodimers with TASK1 (28) and is upregulated in male compared with female WT and *Kcnk3* null mice (6). This sexually dimorphic pattern of TASK3 expression is maintained in compound *Kcnk3*/*Dkk3* null mice (Supplementary Material, Fig. S7). Other factors [e.g. downregulation of the expression of *Agtr1b* (see Supplementary Material, Table S1) and low levels of circulating renin (6)] may contribute to the extinction of *Cyp11b2* expression in the ZG of female *Kcnk3* null mice. In the absence of *Dkk3*, Cyp11b2 expression is released from inhibition in the ZG of male *Kcnk3* null mice, leading to hyperaldosteronism, while compensatory factors still inhibit extension of the Cyp11b2 expression domain to the *zona fasciculata* (Fig. 5).

Potassium channels have a pivotal role in setting the background conductance in adrenocortical ZG cells (reviewed in 5) and studies from genetically modified mice have suggested an important function for different types of those channels in the pathogenesis of hyperaldosteronism (6–9,29,30). Moreover, the clinical relevance of somatic *KCNJ5* mutations in the pathogenesis of a large proportion of aldosterone-producing adenomas has recently been elucidated (10,11). Here we have identified the first example of a gene, *Dkk3*, which can act as a modifier of the hyperaldosteronemic phenotype in a mouse model for altered potassium channel function. Further studies will be required to investigate the potential involvement of human *DKK3* in the pathogenesis of PA.

MATERIALS AND METHODS

Mice

Kcnk3 null (6) mice were crossed with *Dkk3* null (20) mice. All strains were in the C57BL/6 background. Mice were kept on a standard diet with free access to chow and water. For experiments involving administration of a potassium-rich diet, potassium chloride was supplemented (3%) in drinking

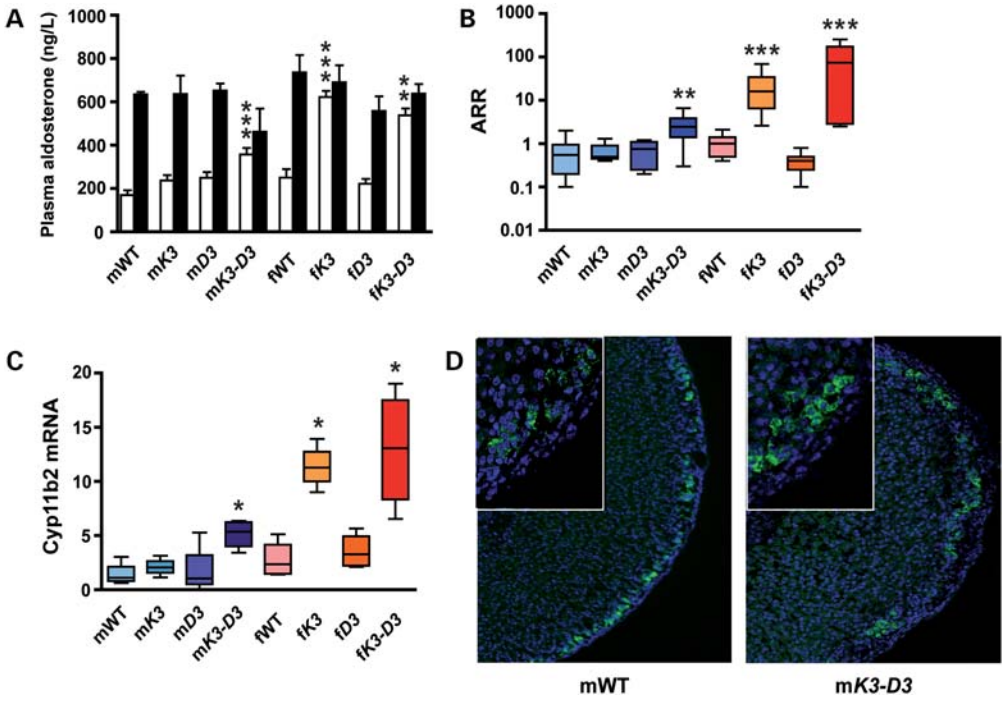

Figure 4. Compound *Kcnk3*/*Dkk3* null male mice are hyperaldosteronemic. (**A**) Plasma aldosterone levels of WT, *Kcnk3* null (*K3*), *Dkk3* null (*D3*) and compound *Kcnk3*/*Dkk3* null (*K3-D3*) mice of both sexes (m and f) under normal (white histograms) or potassium-rich (black histograms) diet ($n = 3–19$). Aldosterone levels were significantly higher in male compound *Kcnk3*/*Dkk3* null mice ($P = 0.0004$) and in female *Kcnk3* null ($P = 0.001$) and compound *Kcnk3/Dkk3* null ($P = 0.0023$) mice compared with WT mice of the same sex, respectively. (**B**) ARR in the same mice groups under normal diet ($n = 5–15$). ARR was significantly higher in male compound *Kcnk3*/*Dkk3* null mice ($P = 0.0017$) and in female *Kcnk3* null ($P < 0.0001$) and compound *Kcnk3/Dkk3* null ($P = 0.0005$) mice compared with WT mice of the same sex, respectively. (**C**) *Cyp11b2* mRNA levels measured by RT-qPCR in the adrenal gland of the same mice groups ($n = 3$). *Cyp11b2* was significantly upregulated in adrenals from male compound *Kcnk3*/*Dkk3* null mice (mean 4.042, $P = 0.023$) and in female *Kcnk3* null (mean 12.445, $P = 0.029$) and compound *Kcnk3/Dkk3* null (mean 4.882, $P = 0.032$) mice compared with WT mice of the same sex, respectively. (**D**) Immunofluorescence staining for the *Cyp11b2* protein (green) in 2-month-old WT (left) and male compound *Kcnk3*/*Dkk3* null (right) mice. Nuclei were counterstained with DAPI (blue). ×20 magnification. Insets: ×40 magnification. Cyp11b2 expression is restricted to the outer part of the adrenal cortex in both animals.

water. For hormonal treatments, 5-week-old male mice were anesthetized with isoflurane, castrated via scrotal incision and later sacrificed at the age of 10 weeks. Female mice (4 weeks old) were injected twice a week with 1 mg testosterone (Androtardyl, Schering) dissolved in 50 μl sesame oil for a period of 3 weeks. After euthanasia, the adrenal glands were collected and snap-frozen in liquid nitrogen. All experimental protocols were approved by the local council for animal care and were conducted according to French and European laws for animal experimentation.

Gene expression profiling

RNA isolation

Total RNA were extracted from adrenal glands using the guanidinium thiocyanate method (31). Integrity of RNA was assessed by using an Agilent BioAnalyser 2100 (Agilent Technologies) (RIN above 8).

Samples labeling and microarrays hybridization

RNA samples were labeled with Cy3 dye using the low RNA input quickamp kit (Agilent) as recommended by the manufacturer. Eight hundred and twenty-one nanograms of labeled cRNA probe were hybridized on 8 × 60K high-density SurePrint G3 gene expression mouse Agilent microarrays. Three biological replicates were performed for each experimental condition. Experimental data have been deposited in the NCBI gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under series record GSE37825.

Statistical analysis and biological theme analysis

Normalization was performed using the Limma package available from Bioconductor (http://www.bioconductor.org). Inter slide normalization was performed using the quantile methods. The mean of ratios from all comparisons were calculated and the B-test analysis was performed. Differentially expressed genes were selected using Benjamini–Hochberg correction of the *P*-value for multiple tests, based on the *P*-value <0.05. Hierarchical clusterings were done with the MultiExperiment Viewer (MeV) program version 4.3, using a Manhattan distance metric and average linkage. Data from expression microarrays were analyzed for enrichment in biological themes (gene ontology molecular function and canonical pathways) and build biological networks built using Ingenuity Pathway Analysis software (http://www.ingenuity.com/) and Mediante (http://www.microarray.fr:8080/merge/index).

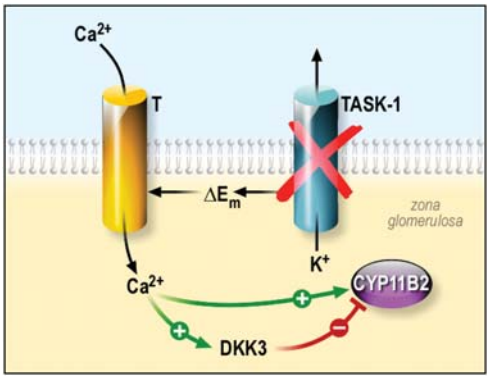

Figure 5. A model for *Dkk3* role in the regulation of *Cyp11b2* expression in adrenal ZG. Membrane depolarization (ΔE_m) consequent to inactivation of TASK1 potassium channels increases intracellular calcium concentration by inducing opening of T-type voltage-dependent calcium channels. Increased intracellular calcium stimulates DKK3 expression, which, in turn, represses CYP11B2. In the absence of DKK3, CYP11B2 is constitutively increased and not sensitive to a potassium-rich diet.

Hormone assays

For aldosterone and corticosterone assays, blood was collected into heparin-treated capillary tubes from mice tails after small lateral incision. Samples were centrifuged and plasma was frozen and kept at −20°C. Aldosterone concentrations were determined in unextracted plasma using a commercial EIA method (EIAsy Aldosterone kit, Diagnostics Biochem Canada), displaying no cross-reactivity with corticosterone. Corticosterone levels were measured with a commercial EIA method (Enzo Life Sciences). Plasma renin activity was determined as previously described (6).

Immunohistochemistry

It was performed as previously described (6) to detect Cyp11b2 on adrenal slices using an antibody generously provided by Dr. Gomez-Sanchez or an affinity-purified rabbit antibody recognizing the Cyp11b2 MAP-peptide KVRQN ARGSLTMDVQQ and produced as described (32). Dab2 (13) was detected using a commercial rabbit polyclonal antibody (Santa Cruz). β-Catenin was detected using a commercial mouse monoclonal antibody (Becton Dickinson).

Cell culture

ATC-1 cells were maintained in DMEM-F12 (Invitrogen) supplemented with 2.5% fetal bovine serum (Invitrogen), 2.5% horse serum (Invitrogen), 1% ITS+ supplement (Becton Dickinson) and penicillin-streptomycin (Invitrogen) at 37°C in a 5% CO_2–95% air atmosphere. Y-1 cells were maintained in DMEM/Ham's F-10 1:1 medium (Invitrogen), supplemented with 15% horse serum (Invitrogen), 2.5% fetal bovine serum (Invitrogen) and penicillin-streptomycin (Invitrogen) at 37°C in a 5% CO_2–95% air atmosphere. To increase intracellular calcium concentration, cells were plated at the density of 3×10^5 in 6-well plates, stimulated with 0.1 μM calcimycin (Sigma-Aldrich) for 24 h and then harvested for total RNA extraction using the RNeasy Mini kit (Qiagen).

RT-qPCR

It was performed as previously described (6) using primers TCTCTACTCCATGGGCCGAA (forward)—AGCGCTGA GGCATATAGCGT (reverse) for *Cyp11b2*, ACGGTCACTT GGACTCCGG (forward)—TCATTGAGCGTAGCTTCCT CC (reverse) for *Dkk3* and AGGCCAGACCCCACAACTC (forward)—GGGTGGTGCCTGGCAA (reverse) for *Tbp* as a reference gene.

Statistics

The Mann–Whitney test was used to assess the significance of differences between the groups. A *P*-value of <0.05 was accepted to indicate statistical significance. The REST software (33) using 2000 iterations was used to measure the significance of differences between groups in *Cyp11b2* RT-qPCR experiments.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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