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Rami El Zein

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INSERM U970, Centre de recherche cardiovasculaire de Paris

Role of mineralocorticoid and retinoic acid signaling in the physiology of the adrenal cortex and the development of primary aldosteronism

Par Rami El Zein

Thèse de doctorat de physiopathologie

Dirigée par le Dr. Maria-Christina Zennaro

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A mes parents,

A mes sœurs et mon frère,

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1 List of abbreviations

11- β HSD2: 11-beta-hydroxysteroid dehydrogenase	<i>ATP1A1</i> : ATPase Na ⁺ /K ⁺ Transporting Subunit Alpha 1
5-HT: 5-hydroxytryptamine	<i>ATP2B3</i> : ATPase Plasma Membrane Ca ²⁺ Transporting 3
5-HTR4: 5-hydroxytryptamine receptor type 4	ATRA: All-trans retinoic acid
9-cis RA: 9-cis Retinoic acid	AVS: Adrenal venous sampling
ACE: Angiotensin I converting Enzyme	BAH: Bilateral adrenal hyperplasia
ACTH: Adrenocorticotrophic hormone	BCL6: BCL6 Transcription Repressor
AD4BP: Adrenal 4 binding protein	BMI: Body mass index
AF: Activating function	BP: Blood pressure
AKR1B7: Aldo-Keto Reductase Family 1 Member B7	BSA: Bovine serum albumin
AML: Acute myeloid leukemia	<i>CACNAID</i> : Calcium Voltage-Gated Channel Subunit Alpha1 D
Ang II: Angiotensin II	<i>CACNAIH</i> : Calcium Voltage-Gated Channel Subunit Alpha1 H
APA: Aldosterone producing adenoma	CaMK: Calmodulin-dependent protein kinases
APC: Adenomatous polyposis coli	cAMP: Cyclic adenosine 3,5-monophosphate
APCC: Aldosterone-producing cell clusters	CAR: Constitutive androstane receptor
APL: Acute promyelocytic leukemia	β -Catenin: β -Cadherin-Associated Protein
AR: Androgen receptor	Cav 1.3: Voltage-dependent L-type calcium channel subunit alpha-1D
ARB: Angiotensin Receptor Blockers	Cav 3.2: Voltage-dependent T-type calcium channel subunit alpha-1H
Arg: Arginine	CBP: CREB-binding protein
<i>ARMC5</i> : Armadillo Repeat containing 5	CD: Collecting duct
ARR: Aldosterone to renin ratio	ChIP: Chromatin immunoprecipitation
AS: Aldosterone synthase	ChIP-Seq: Chromatin immunoprecipitation followed by high throughput sequencing
ASDN: Aldosterone sensitive distal nephron	
Asp: Aspartic acid	
AT1R: Angiotensin II receptor type 1	
AT2R: Angiotensin II receptor type 2	
ATP: Adenosine triphosphate	

CK1 β : Casein kinase 1 β
CLCN2: Chloride Voltage-Gated Channel 2
 CLC2: Chloride channel protein 2
 CM Junction: Cortico-medullary junction
 CNT: Connecting tubule
 CPA: Cortisol-producing adenoma
 CRABP: Cellular retinoic acid-binding protein
 CREB: Cyclic AMP-responsive element-binding protein
CTNNB1: Catenin (Cadherin-Associated Protein), Beta 1
CYP11A1: Cytochrome P450 11A1
CYP11B1: Cytochrome P450 11B1
CYP11B2: Cytochrome P450 11B2
CYP17A1: Cytochrome P450 17A1
CYP21A2: Cytochrome P450 21A2
 Dab-2: Disabled Homolog 2
 DAPI: 4',6-diamidino-2-phenylindole
 DAX1: DSS-AHC Critical Region on the X Chromosome Protein 1
 DBD: DNA-binding-domain
 DCT: Distal convoluted tubule
 DHEA: Dehydroepiandrosterone
 DHEAS: Dehydroepiandrosterone sulfate
 DNA: Deoxyribonucleic acid
 DZ: Definitive zone
 EB1: Microtubule plus-end binding protein
 ECM: Extracellular matrix
 EDNRC: Endothelin receptor type B-like protein
 ENaC: Amiloride-sensitive sodium channel subunit alpha
 ER: Endoplasmic reticulum
 ER: Estrogen receptor
 ERC: Epithelial sodium channel regulatory complex
 ERK: Extracellular signal-regulated kinases
 ESC: Embryonic stem cells
 FH: Familial hyperaldosteronism
FSCN1: Fascin 1
 FXR: Farnesoid X receptor
 FZ: Fetal Zone
 Fz: Frizzled
 GABA: Gamma-Aminobutyric acid
 GILZ: Glucocorticoid induced leucine zipper
 GIRK: G protein-activated inward rectifier potassium channel 4
GLCC1: Glucocorticoid induced transcript 1
 Gly: Glycine
GNAI2: Guanine nucleotide-binding protein α -12
 GNRHR: Gonadotropin-releasing hormone receptor
 GPCR: G-protein coupled receptor
 GR: Glucocorticoid receptor
 GRA: Glucocorticoid-remediable aldosteronism
 GRE: Glucocorticoid response element
 GRM3: Metabotropic glutamate receptor 3
 GSK-3 β : Glycogen synthase kinase-3 β
 GTP: Guanosine triphosphate

HAC: Human adrenocortical cell line
HDAC: Histone Deacetylase
HES: Hematoxylin-eosin-safran
His: Histidine
HK: Human kidney cell line
HL60: Human leukemia cell line
Hr: Hour
HRE: Hormone response element
HSD3B1: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
HSD3B2: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
HSP: Heat shock protein
HT: Arterial Hypertension
ICAM: Intercellular Adhesion Molecule
IF: Immunofluorescence
IGF: Insulin-like growth factor
IHA: Idiopathic hyperaldosteronism
IHC: Immunohistochemistry
Ile: Isoleucine
IP3: Inositol 1,4,5-triphosphate
IRES: Internal ribosome entry site
JDP2: Jun dimerization protein 2
JNK: C-Jun N-terminal kinase
Kbs: Kilo base pair
***KCNJ5*:** Potassium Voltage-Gated Channel Subfamily J Member 5
Kir: Inward rectifier K(+) channel
KO: Knock-out
LBD: Ligand-binding-domain
LEF: Lymphoid enhancer factor
Leu: Leucine
LHCGR: Leuteinizing hormone-chorionic gonadotropin receptor
LSR: lipolysis stimulated lipoprotein receptor
LXR: Liver X receptor
Lys: Lysine
M: Molar
mAPA: micro-APA
MAPK: Mitogen-Activated Protein Kinase
MBS: Mineralocorticoid binding sites
MC2R: Melanocortin receptor 2
Met: Methionine
miRNA: Micro-RNA
MR: Mineralocorticoid receptor
MRA: MR antagonist
MRAP: Melanocortin-2 receptor accessory protein
MRE: Mineralocorticoid response element
mRNA: Messenger ribonucleic acid
mTOR: Mammalian target of rapamycin
N: number
NDRG2: N-Myc Downstream-Regulated Gene 2 Protein
NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS: Next generation sequencing
NGS: Normal goat serum
Nos1ap: Nitric oxide synthase 1 adaptor protein
NPM1: Nucleophosmin 1

NR0B1: Nuclear Receptor Subfamily 0 Group B Member 1

NR1B1: Nuclear Receptor Subfamily 1 Group B Member 1

NR3C2: Nuclear Receptor Subfamily 3 Group C Member 2

NR4A1: Nuclear receptor subfamily 4 group A 1

NR4A2: Nuclear receptor subfamily 4 group A 2

NR5A1: Nuclear Receptor Subfamily 5 Group A Member 1

NUMA1: Nuclear Mitotic Apparatus Protein 1

P300: Binding protein 300

PA: Primary Aldosteronism

pAATL: Possible APCC-to-APA translational lesions

PASNA: Primary Aldosteronism, Seizures and Neurological Abnormalities

PBS: Phosphate buffered saline

PCP: Planar cell polarity

PCR: Polymerase chain reaction

p-CREB: phospho-CREB

PI3K: Phosphoinositide 3-kinase

PIAS1: Protein Inhibitor of Activated STAT 1

PKA: Protein kinase A

PMCA3: Plasma membrane calcium-transporting ATPase 3

PML: Promyelocytic leukemia protein

PMS2: Postmeiotic segregation increased 2

PPAR: Peroxisome proliferator activated receptor

PPP4R4: Protein Phosphatase 4 Regulatory Subunit 4

PR: Progesterone receptor

PRKACA: Protein Kinase CAMP-Activated Catalytic Subunit Alpha

PRKARIA: Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha

PRKAR1B: cAMP-dependent protein kinase type I β -regulatory subunit

PTGER1: Prostaglandin E receptor 1

qPCR: Quantitative polymerase chain reaction

qRT-PCR: Quantitative real time polymerase chain reaction

RA: Retinoic acid

RAAS: Renin-Angiotensin-Aldosterone System

RAR: Retinoic acid receptor

RAR α : Retinoic acid receptor alpha

RARE: Retinoic acid response element

RBaK: Retinoblastoma-associated Kruppel-associated box gene

RBP: Retinol binding protein

RHOC: Ras homolog gene family, member C

RPA3: Replication protein A3

RSPO: R-spondin

RXR: Retinoic X receptor

SCNN1A: Sodium channel epithelial 1 alpha subunit

Ser: Serine

sER: Smooth Endoplasmic reticulum

SF1: Steroidogenic factor 1

SGK1: Serum and glucocorticoid regulated kinase 1

SHH: Sonic Hedgehog

SLC35F1: Solute Carrier Family 35 Member F1

SRC: steroid receptor co-activator

StAR: Steroidogenic acute regulatory protein

STAT: Signal Transducer and Activator of Transcription

STAT5B: Signal Transducer and Activator of Transcription 5B

SUMO: Small Ubiquitin-like Modifier

Supv3 11: Suv3 like RNA helicase

T4: L-thyroxine

TACR1: Tachykinin receptor 1

TASK: TWIK-related acid-sensitive potassium channel

TBS: Tris buffer solution

TCF: T-cell factor

TDGF-1: epidermal growth factor-like cripto protein CR-1

TGF-B: Transforming growth factor beta

Thr: Threonine

TR: Thyroid receptor

TSH: Thyroid stimulating hormone

Tyr: Tyrosine

TZ: Transitional zone

Val: Valine

VDR: Vitamin D receptor

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

VSNL1: Visinin like 1

WES: Whole exome sequencing

Wnt: Wingless

WT: Wild type

WT1: Wilm's Tumour-1

XZ: X-zone

ZBTB16: Zinc Finger and BTB Domain Containing 16

ZF: Zona fasciculata

ZG: Zona glomerulosa

ZNF12: Zinc finger protein 12

ZR: Zona reticularis

2 Résumé

Titre : Rôle de la signalisation minéralocorticoïde et rétinolique dans la physiologie du cortex surrénalien et le développement de l'hyperaldostéronisme primaire.

L'hyperaldostéronisme primaire est la cause majeure d'hypertension artérielle secondaire. Des mutations somatiques récurrentes dans les gènes *KCNJ5*, *CACNA1D*, *ATP1A1* et *ATP2B3* ont été identifiées dans les adénomes produisant de l'aldostérone (APA). Bien que le rôle de ces mutations dans la régulation de la biosynthèse de l'aldostérone soit bien établi, les mécanismes impliqués dans la prolifération et la formation des APA restent à démontrer. L'objectif principal de mon projet de thèse était d'identifier le rôle de la signalisation des récepteurs à l'acide rétinolique alpha ($RAR\alpha$) et minéralocorticoïde (MR) dans le développement des APA.

Nous avons identifié la signalisation $RAR\alpha$ comme étant un élément central dans la formation des nodules dans les surrénales de patients ayant un APA. L'inactivation de $RAR\alpha$ chez la souris induit une désorganisation structurelle et fonctionnelle majeure dans le cortex surrénalien dans les deux sexes, avec des modifications de l'architecture vasculaire et de la matrice extracellulaire, et une réduction de l'expression des gènes de la stéroïdogénèse. Ces anomalies sont dues à une augmentation de la prolifération, une réduction de l'expression de *Vegfa* et de la signalisation Wnt non canonique, sans modification de la voie Wnt canonique et de la signalisation PKA. La désorganisation de la corticosurrénale persiste avec l'âge, alors que les anomalies moléculaires touchant la voie Wnt et la voie du *Vegfa* et la réduction de l'expression des gènes de la stéroïdogénèse régressent. Notre étude suggère que $RAR\alpha$ contribue au développement normal du cortex surrénalien, en modulant les signalisations Wnt et *Vegfa*.



Enfin, pendant ma thèse j'ai également contribué à l'identification du rôle du canal chlorure *ClC-2* dans l'hyperaldostéronisme primaire.

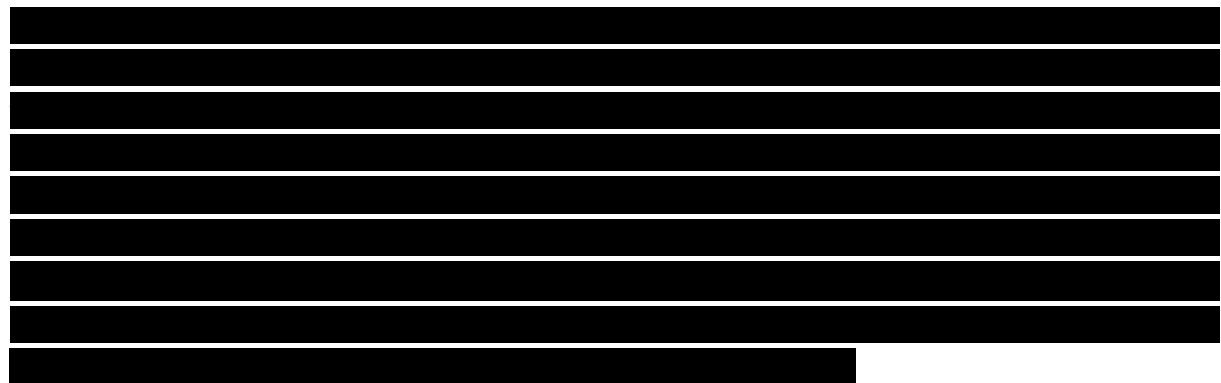
Les résultats obtenus au cours de mon projet de thèse ont permis une meilleure compréhension des mécanismes impliqués dans les APA et ouvrent de perspectives pour l'identification de nouvelles cibles thérapeutiques dans l'hyperaldostéronisme primaire.

3 Abstract

Title: Role of mineralocorticoid and retinoic acid signaling in the physiology of adrenal cortex and the development of primary aldosteronism

Primary aldosteronism is the major cause of secondary arterial hypertension. Recurrent somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* have been identified in aldosterone producing adenoma (APA). Although the role of these mutations in regulating aldosterone biosynthesis has been clearly established, the mechanisms involved in proliferation and APA formation still remain to be elucidated. The main aim of my PhD project was to identify the role of the retinoic acid receptor alpha ($RAR\alpha$) and the mineralocorticoid receptor (MR) in the development of APA.

We identified $RAR\alpha$ signaling as a central molecular network involved in nodule formation in adrenals from patients with APA. Inactivation of $Rar\alpha$ in mice led to major structural and functional disorganization of the adrenal cortex in both sexes, with modifications of the vessel architecture and extracellular matrix and reduced expression of steroidogenic genes. These abnormalities were due to increased proliferation, decreased *Vegfa* expression and modifications in extracellular matrix components. *Rara* inactivation reduces non-canonical Wnt signaling, without affecting the canonical Wnt pathway nor PKA signaling. Adrenal cortex disorganization persisted with aging, while molecular abnormalities affecting Wnt signaling and *Vegfa* and steroidogenic gene expression regressed. Our study suggests that $Rar\alpha$ contributes to normal adrenal cortex development, by modulating Wnt and *Vegfa* signaling.



Finally, during my PhD I have also contributed to the identification of the role of the chloride channel *ClC-2* in primary aldosteronism.

Results obtained during my PhD have allowed a better understanding of the mechanisms underlying APA development, and open new perspectives for the identification of therapeutic targets in primary aldosteronism.

4 Introduction

4.1 Arterial hypertension

Arterial hypertension (HT) is a major health problem that affects 25% of the adult population (1), resulting in 10.4 million deaths in 2013 (2). Hypertension is defined as an elevated BP of equal to or above 140 mmHg systolic blood pressure and/or 90 mmHg diastolic pressure. Up to 53% of patients suffering from high blood pressure (BP) remain unaware of the illness and are untreated (3). In some cases, patients do not present any symptoms before severe damage has been made to target organs such as the heart, the kidneys and the brain, which manifest with heart attacks, kidney failure or strokes.

No treatment was available for HT before the late 50s or early 60s, despite the discovery of renin by Goldblatt in 1940 (4). Harry Goldblatt had established the first animal model of hypertension on dogs where he induced hypertension by constriction of both renal arteries (5). This model helped in uncovering the renin-angiotensin-aldosterone system and BP control, and led thereafter to the development of enzyme inhibitors for the treatment of chronic hypertension.

Following this stage, the pharmaceutical industry witnessed a rise in drugs that are focused on treating HT especially since the first clinical trial on thiazide diuretics in 1957. Since then, a diverse and vast disposition of drugs for the treatment of hypertension was available, besides diuretics, renin angiotensin aldosterone system antagonists, more precisely, Angiotensin Receptor Blockers (ARBs) and Angiotensin I Converting Enzyme (ACE) inhibitors and direct renin inhibitors, but also, vasodilators, sympatholytic drugs such as β - or α -adrenergic blocking agents and calcium channel blockers. Different guidelines on patient treatment exist to assure the best outcome and patient care, however, optimal BP in as much as two thirds of hypertensive patients is not achieved despite using different drug combination (6).

In many cases, the cardiovascular complications of HT are not reduced due to inadequacies in prevention, diagnosis and control of the disorder. Identification of secondary causes is therefore key for implementing efficient treatments in patients with HT. Secondary causes of hypertension include a number of adrenal disorders, in which increased and autonomous hormone production increases blood pressure. Those are Primary Aldosteronism, Cushing's

syndrome and Pheochromocytoma. In most cases, these diseases are curable and surgical or medical intervention normalizes blood pressure. However, in many cases these endocrine disorders remain undiagnosed.

4.2 Regulation of blood pressure and the renin-angiotensin-aldosterone system

One of the major regulators of blood pressure is the renin-angiotensin-aldosterone system (RAAS). Renin is synthesized and stored in juxtaglomerular cells of the kidney, highly specialized cells of the afferent arteriole derived from smooth muscle cells. Renin is released in the following conditions:

- when there is a reduction in the renal perfusion pressure, which is detected by the baroreceptors on the afferent arteriole
- in case of reduction in sodium reabsorption at the macula-densa, which is due to a reduction in the glomerular filtration rate
- in case of stimulation of the sympathetic nervous system, which is mediated by the β -adrenergic receptors (7).

The RAAS can act via many effectors, one of which is Ang II, which is a potent vasoconstrictor of arterioles. On a systemic level, arteriolar vasoconstriction induces an increase in total peripheral resistance and therefore increases BP without affecting blood volume. Ang II exerts its effects by binding to cell surface receptors, which are expressed on different target organs, mainly, heart, kidney, blood vessels, adrenal glands, liver and the cardiovascular control centers in the brain. Altogether, these effects lead to an elevation in BP. Ang II induces its effects by binding to two G-protein coupled receptors, the Ang II type 1 (AT1) receptor and the type 2 (AT2) receptor. Interestingly, pharmacological studies have shown that Ang II exerts most of its actions via the AT1 receptor, and that AT1 and AT2 receptors signaling have opposing effects, whereas the AT1 receptor mediates vasoconstrictor responses, AT2 generally exerts blood pressure lowering responses by mediating vasodilator effects (8, 9).

In addition to vasoconstriction, AngII acts on the adrenal cortex to stimulate aldosterone production. Aldosterone is the one of the major hormones contributing to BP regulation and is

one of the most potent sodium-retaining factors in the organism. Under physiological conditions, aldosterone is produced in response to a decrease in blood pressure, a reduction in sodium or an increase in potassium levels. Aldosterone acts on the so-called aldosterone-sensitive distal nephron, to promote sodium reabsorption and potassium secretion (10). These actions are mediated by the mineralocorticoid receptor (MR), a member of the nuclear receptor superfamily of transcription factors. Sodium reabsorption is coupled to water reabsorption, thus increasing blood volume and blood pressure (Fig. 1).

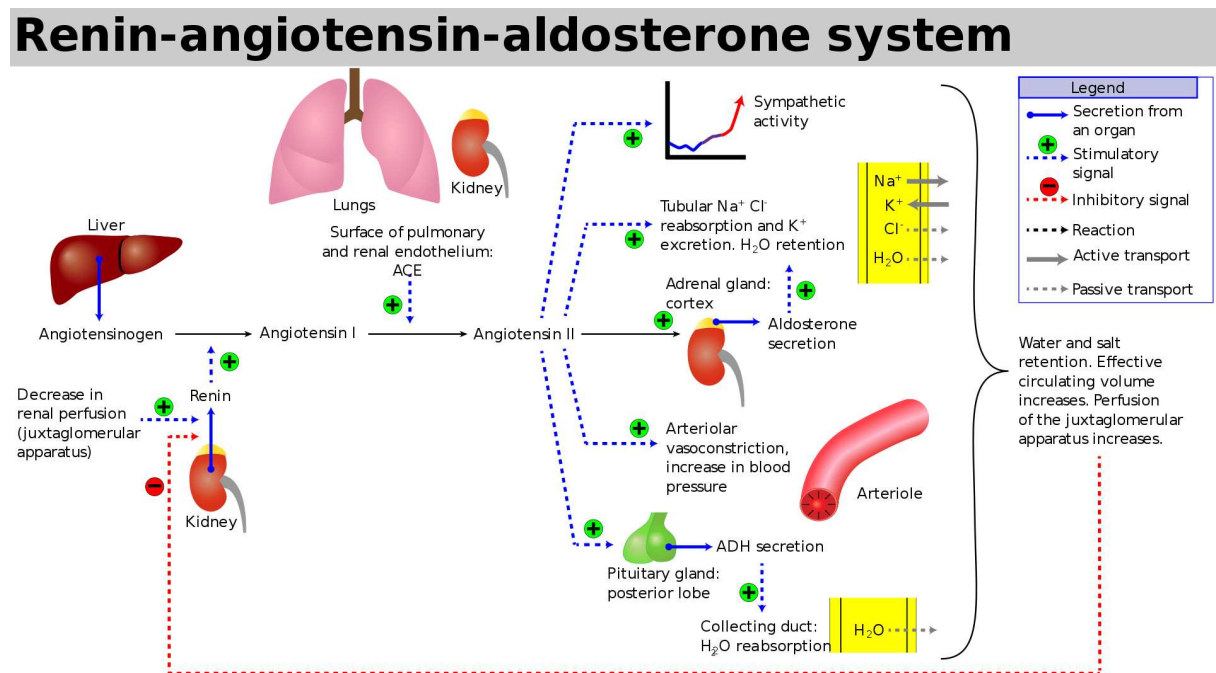


Figure 1. Renin angiotensin aldosterone system (RAAS). Initiation and effects in the organism (from Wikimedia Commons, the free media repository; https://commons.wikimedia.org/w/index.php?title=File:Renin-angiotensin-aldosterone_system.svg&oldid=288052845).

4.3 Primary aldosteronism

PA, also known as Conn's syndrome, was described by Jerome Conn in 1955 in a thirty-four-year-old patient who entered the hospital at the university of Michigan complaining of seven years of episodic muscle weakness that often resulted in virtual paralysis of her lower legs, in addition to muscle spasms and cramps in her hands (11). In 1964, Conn suggested that up to 20% of hypertensive patients may have PA without hypokalemia. The widespread use of the Aldosterone to Renin Ratio (ARR) in the 90s showed that PA is more common than what was thought. Primary aldosteronism (PA) is the most common form of secondary HT, with

prevalence estimates of up to 10% of cases in referred hypertensive patients, 4%-5% of patients in primary care (12-14), and as high as 20% of patients with resistant HT (15, 16).

PA is due in the majority of cases either to an Aldosterone Producing Adenoma (APA) or to Bilateral Adrenal Hyperplasias (BAH, also called idiopathic hyperaldosteronism – IHA). Both conditions account to 95% of cases of PA (17). The main clinical symptoms of PA are hypertension which is due to high aldosterone levels, associated to suppressed renin and hence a high aldosterone to renin ratio and in some cases hypokalemia and metabolic alkalosis. The ARR has become the main screening tests for PA, alongside adrenal venous sampling (AVS) for subtype diagnosis (18).

PA is associated with an elevated risk of cardiovascular complications; these complications occur independently from the increase in blood pressure and include heart failure, myocardial infarction, coronary artery disease, atrial fibrillation and renal damage (19). The correct diagnosis of PA is very important, as patients can be easily treated by aldosterone antagonists in the case of BAH or laparoscopic adrenalectomy in the case of APA. Early diagnosis of PA can improve the prognosis and prevent organ damage, and several studies insisted on the importance of screening most hypertensive patients for PA to confirm or exclude the diagnosis (20, 21). Furthermore, PA during pregnancy is very dangerous for both the mother and the fetus, a case of PA was overlooked resulting in delivery of an infant at a young gestational age. (22)

Although major advances have been made in the management of PA following the establishment of guidelines and algorithms for best patient care, the prognosis of PA is very variable and depends on different criteria such as age, sex, body mass index (BMI), and duration of hypertension. (21, 23). Indeed, younger and female patients show a better clinical outcome after adrenalectomy in comparison to older or male patients (21, 24, 25).

4.4 The adrenal gland

4.4.1 Adrenal gland zonation and function

The adrenal glands are a pair of triangular structures located on top of the kidney. From the 10th week of gestation the adrenals are divided anatomically and functionally in two parts: the

outer part or adrenal cortex and the inner part or adrenal medulla, which have different developmental origins. The cortex produces the three main steroid hormones, aldosterone, cortisol and dehydroepiandrosterone (DHEA) in the zona glomerulosa, the zona fasciculata and the zona reticularis respectively. The uniqueness of each of these zones lies in the capacity to produce specific hormones through their specific stimulatory and regulatory signals, as well as the zone-specific expression of steroidogenic enzymes. This is known as the functional zonation of the adrenal cortex. The medulla, on the other hand, secretes catecholamines (adrenaline and noradrenaline), which control short-term response to stress and blood pressure.

The adrenal cortex, which forms ~90% of the human adult adrenal gland, is composed of three concentric zones: The Zona Glomerulosa (ZG), the Zona Fasciculata (ZF) and Zona Reticularis (ZR). All three zones are enclosed within a capsule made up of fibroblasts and myofibroblasts. The outer layer, the ZG, is a narrow zone constituting ~15% of the whole cortex, contains small cells generally arranged in large clusters. ZG cells present intense nuclei with basophilic cytoplasm and these cells have low lipid content. The ZG produces the mineralocorticoid hormone aldosterone, which is mainly responsible for regulating blood volume and water/salt balance. The second layer, the ZF, is formed of lipid-rich polygonal cells that are arranged in radial columns. The ZF produces glucocorticoids, cortisol in humans and corticosterone in mice and rats; glucocorticoids are mainly implicated in regulating metabolic functions, the cardiovascular system and the immune response. The inner most cortical zone is the ZR, consists of polyhedral cells arranged in a net-like structure. The ZR produces glucocorticoids and C19 adrenal androgens including dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) as well as androstenedione (Fig. 2).

The medulla composes the inner mass of the adrenal gland; it is composed of cells that originate from the neural crest. The medulla produces catecholamines such as adrenaline, noradrenaline and dopamine. The medulla is separated from the cortex with little to no connective tissue, meaning that the two zones are in direct contact.

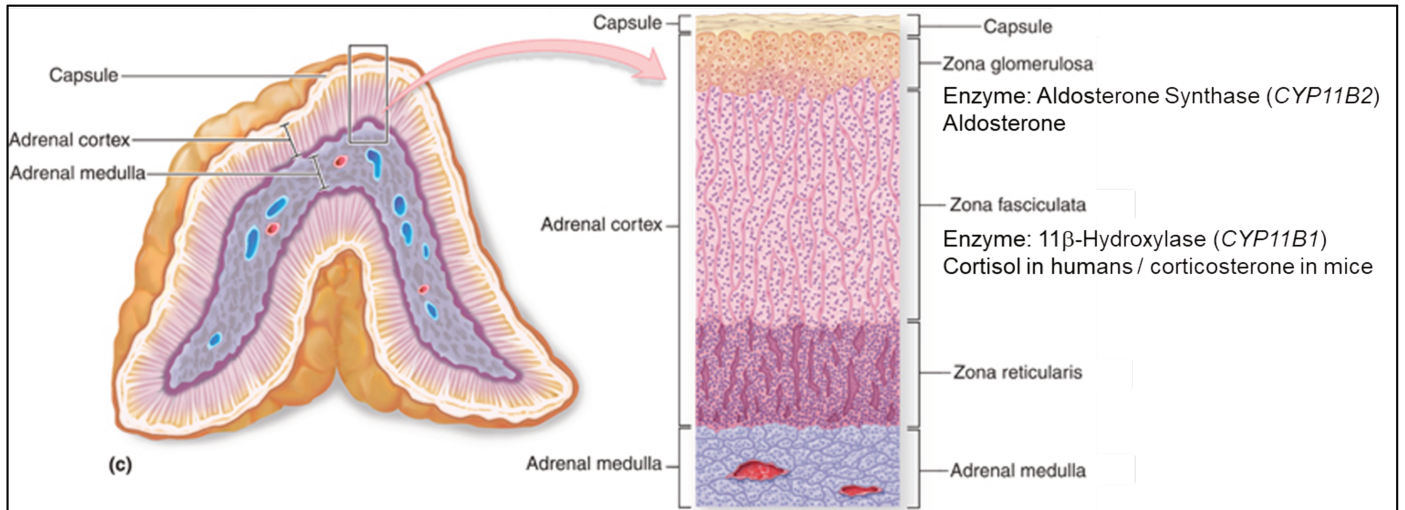


Figure 2. Structure and cellular distribution of the adrenal gland. (Modified from: Junqueira's basic histology: Text and Atlas, <http://www.accessmedicine.com>)

4.4.2 Vascularization of the adrenal cortex

The adrenal cortex is a highly vascularized tissue, where almost every single cell is in contact with a blood vessel to assure hormone distribution to the blood following its production in the adrenocortical cells. Sparrow and Coupland showed in 1987 that only 7.4% of the adrenal blood passes directly to the medulla, whereas the larger part passes through the cortex (26). The adrenal arteries split up on the capsule to form a dense arterial plexus, trabecular in shape in the ZG and rather in the shape of fenestrated capillaries in the ZF. In the ZR there exists an interconnecting network of blood vessels forming another arterial plexus, while small venules pass between the medullary cells and end up in the adrenal vein. This delicate and well ramified vasculature in the adrenal gland ensures that every cell is in contact with a blood vessel (27), creating an efficient environment for adrenal hormone delivery to the blood stream and oxygenation to the adrenal cells (Fig. 3).

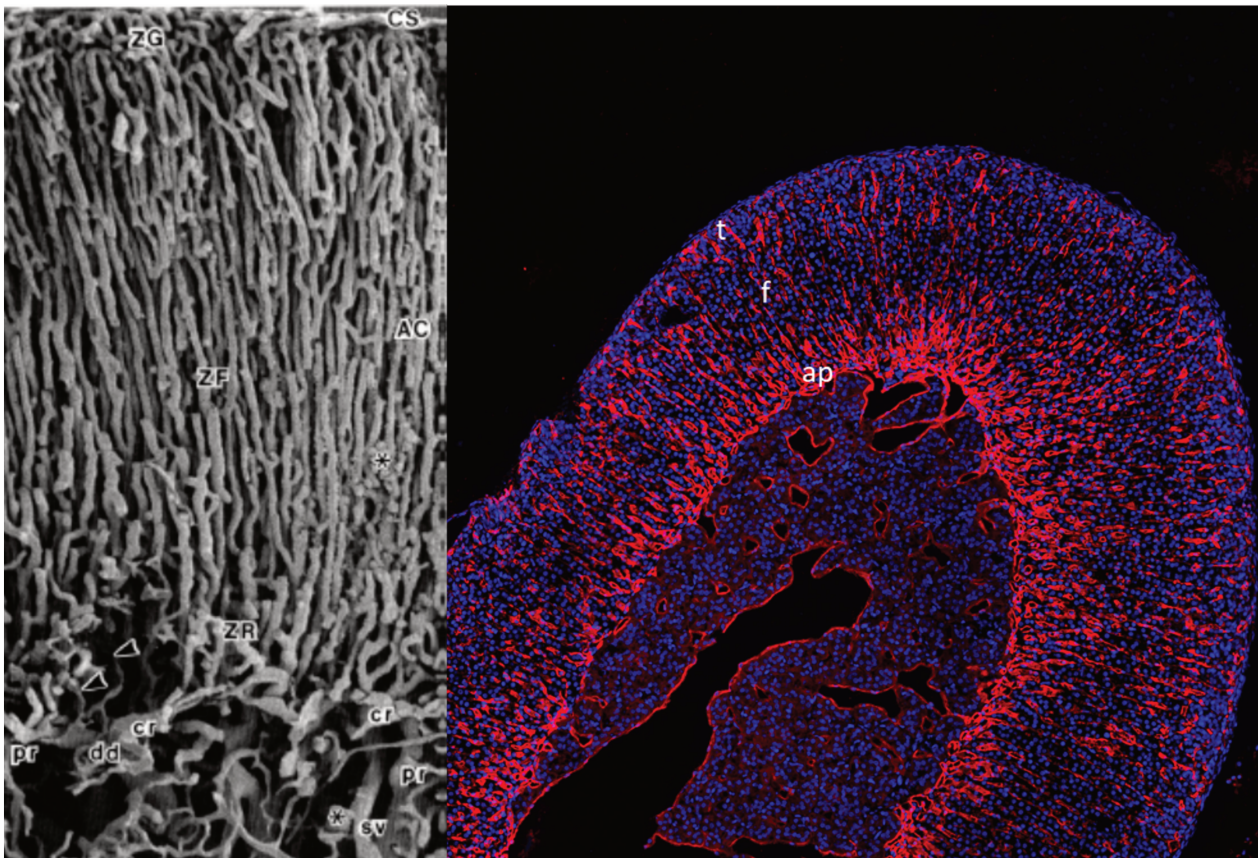


Figure 3. Vasculature of the adrenal gland. Left panel: Methyl methacrylate (resin or plexiglass) microcorrosion cast of a rat adrenal gland, CS-cortical surface, AC-cortical vascular bed, cr-corticomedullary venous radicals, sv-subcortical veins, pr-premedullary venous radicles, dd-medullary capillaries (28). Right panel: Podocalyxin staining from an adult mouse adrenal, t-trabecular, f-fenestrated and ap-atrial plexus.

4.4.3 Innervation of the adrenal gland:

Before the 1970s, little was known about the adrenal gland innervation, however, thanks to the introduction of nerve fiber tracing techniques as well as other methods, the adrenal innervation began to emerge. Evidence points to the existence of both sympathetic and parasympathetic innervations in the adrenal gland. Cholinergic terminals are found throughout the cortex and medulla and is regarded as the main type of innervation in the adrenals. In the cortex, adrenergic terminals are also found to be associated with cortical cells and blood vessels (29, 30). These nerve endings could modulate steroidogenesis indirectly by affecting mast cell degranulation or vascular tone. Interestingly, there is an upregulation in APA of the expression of genes that code for neurotransmitter G-protein coupled receptors (GPCR), such as the metabotropic glutamate receptor 3 (*GRM3*) and tachykinin receptor 1 (*TACR1*) the receptor for substance P, thus suggesting a potential role for these receptors in APA and/or steroidogenesis (31).

4.4.4 Adrenal cortex extracellular matrix composition

Different studies have indicated that components of the adrenal gland extracellular matrix (ECM), could influence the development of the adrenal gland. In particular, it has been shown that collagen IV favors secretion of ACTH and DHEAS and that laminin promotes cell proliferation, while fibronectin promotes cell death (32, 33). Experiments in primary cultures of fetal adrenal cells grown on Petri dishes coated with laminin, collagen IV or fibronectin show that cell morphology and the steroidogenic profile of the cells are modified by the different culture conditions, thus suggesting that ECM is an important regulator of adrenal gland physiology, cell turnover and homeostasis.

4.4.5 Adrenal cortex development and remodeling

Starting from the 10th week of embryonic human life, the fetal adrenal cortex is divided into 2 zones, the definitive zone and the larger inner fetal zone. The definitive zone seems to remain with minimal activity until the end of the second trimester (28 weeks of gestation). In the early postnatal life, the definitive zone differentiates into the ZG and the ZF and this occurs in parallel to an involution and disappearing of the fetal adrenal. The ZR emerges at age 6-8 years inducing the adrenarche. The process causing this development is not fully understood, due to the lack of animal models, as mice for example do not express the Cyp17 enzyme (34). The adrenal gland is a very dynamic organ; cells undergo constant turnover to replace dying cells. Both layers (ZG and ZF) are continuously renewed throughout life and undergo dynamic hormonal feedback regulation. Different theories exist regarding the turnover of cells, such as the model of centripetal zonation that was first proposed by Salmon and Zwemer in 1941. Different studies supported this theory, showing that cells have a tendency to proliferate in the outer cortex near the capsule into ZG, and then centripetally move into the ZF, and finally undergo apoptosis at the corticomedullary junction (35). Another zonal model of lineage development was suggested by Deane and Greep in 1946, where each zone is thought to develop independently. Using lineage tracing to map cell fate of Shh and Gli1 expressing progenitor cells, King et al showed that there were radial stripes that seem to migrate through the ZG into the ZF, supporting the centripetal model. Many studies suggest that the conversion of one fully differentiated cell type into another is possible, following overexpression of specific transcription factors (36). These studies underscore the role of transcriptional modulators in cell fate determination. In 2012, Sisakhtnezhad and Matin

demonstrated that a direct conversion between cell types could occur spontaneously *in vivo* during early development, chronic injury and regeneration (37). However, the mechanisms behind these conversions remain poorly understood. SF1 (*NR5A1*, AD4BP) is well known to be an essential regulator of adrenal development (38). In adult mice, Sf1 is essential for regulating steroidogenic gene expression and adrenocortical cell homeostasis. A study has shown that unilateral adrenalectomy on WT mice induced a significant compensatory adrenal growth in the remaining adrenal. However, this was not the case in Sf1 heterozygous mice, which were not able to undergo compensatory adrenal growth following the loss of one adrenal gland (39). Sf1 has also been shown to play a role in lineage conversion by converting embryonic and mesenchymal stem cells into steroid producing cells (40, 41). Using lineage tracing studies, Freedman et al showed that ZG cells undergo lineage conversion into ZF cells in postnatal mice. They also highlighted the importance of Sf1 in this process, where the loss of Sf1 in ZG cells still led to a fully functional ZF, suggesting that ZF could differentiate in a ZG non-dependent fashion. This could be due to the loss of the ability of ZG cells lacking Sf1 to differentiate, and highlights the plasticity and adaptability of the adrenal gland to compensate and give a fully functional ZF despite lack of ZG lineage conversion (42). In humans, during embryonic life the adrenal cortex consists of 3 layers, the outer definitive zone (DZ), the transitional zone (TZ) and the fetal zone (FZ). The latter undergoes involution after birth through apoptosis (43). The adult adrenal cortex develops from the DZ and the TZ, which convert into the ZG and the ZF, respectively. In mice, apart from the ZG and ZF, there is a third layer that develops after birth and is thought to be analogous to the FZ, namely, the X-zone (XZ). The XZ degenerates at puberty in male mice, while in females, it undergoes gradual slow degeneration as the mouse grows older or vacuolar degeneration at the first pregnancy (44). However, the XZ persists in both sexes if they undergo castration, suggesting that sex hormones are important in the process of the degeneration of the XZ (45). Once developed, the mature adrenal cortex is constantly supplied with steroidogenic cells to compensate for the apoptosis at the cortico-medullary junction. This process involves recruitment of cells from the capsular stem cells and subcapsular ZG progenitor cells through RSPO/Wnt/ β -catenin and SHH signaling pathways. These mechanisms allow maintaining adrenal size, function and homeostasis. Supporting this theory, studies have shown that most cell mitoses occur on the outer part of the adrenal cortex, and most cell deaths occurs in the inner part, and that most cells accumulated in the ZR were, in fact, cells that have migrated from the outer parts of the cortex (46, 47). Other studies have shown that most cell division

occurs in the ZG, by showing that ERK-1 and ERK-2, which are involved in the regulation of cell division and proliferation, were exclusively expressed in the ZG (48).

The adrenal cortex is highly sexually dimorphic. Many studies reported sexual dimorphism in mice adrenals and female adrenal glands are larger, with higher plasma levels of corticosterone, aldosterone and ACTH than male mice (49). Moreover, sex hormones have been shown to affect the adrenal cortex physiology. Female adrenal cortex cells undergo 3-fold faster turnover compared to male mice (50), and several mouse models of adrenal disease show different phenotypes between both sexes.

4.5 Steroidogenesis

Adrenal steroids are synthesized from cholesterol by a series of enzymatic steps involving cytochrome P450 (CYP) family enzymes and hydroxysteroid dehydrogenases. These reactions take place either in the mitochondrial membrane or in the endoplasmic reticulum (ER) (Fig.4).

4.5.1 Aldosterone biosynthesis

Aldosterone is produced in the ZG of the adrenal cortex, the major enzyme in aldosterone biosynthesis is Aldosterone Synthase (AS), encoded by *CYP11B2*. Cholesterol is transported to the inner mitochondrial membrane by the transporter enzyme steroidogenic acute regulatory protein (StAR). The next step is the removal of cholesterol's side chain by CYP11A1 generating pregnenolone. Pregnenolone is then translocated to the ER where 3 β -hydroxysteroid dehydrogenase enzymes will convert it to progesterone. Afterwards, the 21-hydroxylase (*CYP21A2*) converts progesterone to 11-deoxycorticosterone. This is transported back to the inner membrane of the mitochondria where AS will catalyze the three final steps in aldosterone biosynthesis. 11-deoxycorticosterone is hydroxylated at the C11 position giving corticosterone, hydroxylation at the C18 position to generate 18 (OH) corticosterone, which is finally oxidized at the C18 position to produce aldosterone.

Aldosterone biosynthesis is mainly regulated by the renin-angiotensin system through stimulation of ZG cells by Angiotensin II (Ang II), or by extracellular potassium levels, and to a lesser extent by the adrenocorticotrophic hormone (ACTH). Angiotensinogen is secreted by

the liver into the blood stream where it will be cleaved by renin, secreted by the kidneys, into Angiotensin I. Renin secretion is stimulated by sympathetic innervation due to decreased renal perfusion pressure, decreased sodium delivery to the distal tubule or catecholamines. Angiotensin-converting enzyme (ACE), located at the surface of renal and pulmonary endothelium, will hydrolyze Angiotensin I into Ang II. Ang II on its own can stimulate vasoconstriction, increase contraction of the heart, but also stimulate aldosterone synthesis via its receptor AT1, a 7 transmembrane GPCR, which is expressed at the surface of ZG cells. Binding of Ang II to AT1 activates the Gαq-phospholipase C-mediated signaling pathway, which increases inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol concentrations. This cascade results in the increase of intracellular calcium levels via calcium release from intracellular stores. Furthermore, Ang II inhibits the background TWIK-related acid-sensitive potassium channels (TASK), Na⁺, K⁺ -ATPase as well as the potassium channel GIRK4 (encoded by *KCNJ5*), inducing cell membrane depolarization, which leads to opening of voltage-gated Ca²⁺ channels and increased intracellular calcium concentrations (51). ZG cells are very sensitive to changes in extracellular K⁺ levels due to their strong potassium conductance, which is controlled by potassium channels such as TASK1 and TASK3, which are highly expressed in the ZG cells. Increases in extracellular K⁺ close these channels, which in turn induces cell membrane depolarization (52).

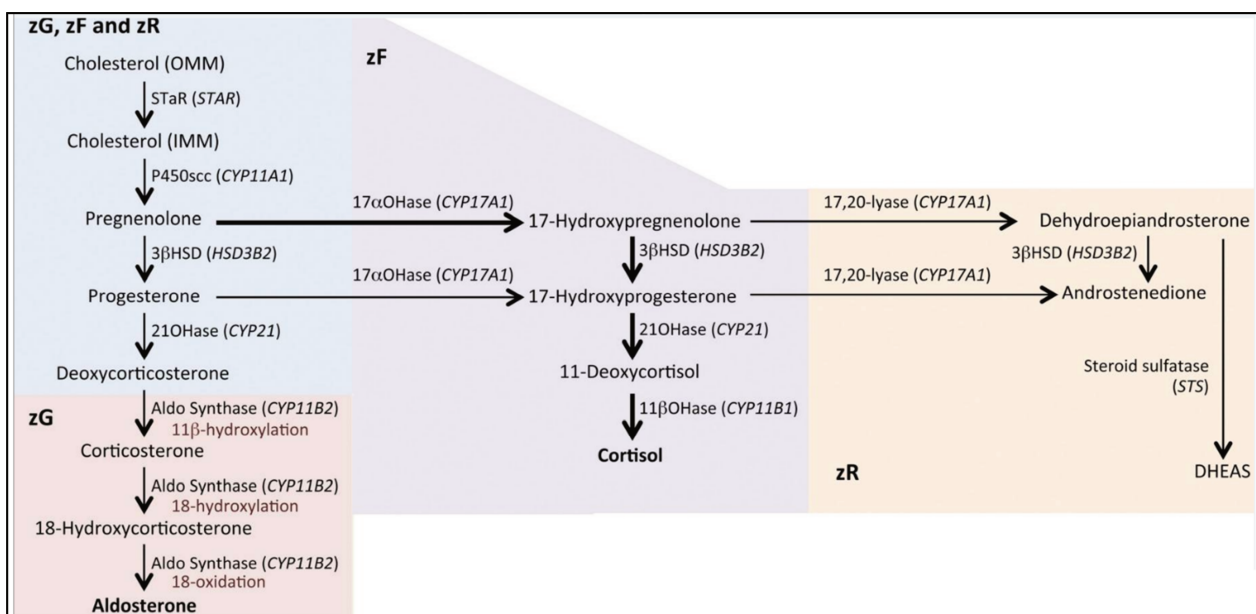


Figure 4. Steroidogenic pathway for the biosynthesis of aldosterone, cortisol and DHEAS in humans (53).

In resting conditions, the cell membrane of a ZG cell is in hyperpolarized state, close to the equilibrium potential of potassium channels, which are highly expressed in these cells (Fig. 8A). Cell membrane depolarization leads to opening of voltage-gated calcium channels and an increase in intracellular calcium levels, which activate calcium signaling. This chain of events leads to the release of deesterified cholesterol from its cytoplasmic stores and its translocation to the inner mitochondrial membrane. Increased calcium signaling also upregulates transcription factors regulating the expression of cytochrome P450 enzymes. Elevated calcium levels activate calmodulin and CaMK (calmodulin-dependent protein kinases); which phosphorylate transcription factors such as nuclear receptor subfamily 4 group A 1 and 2 (*NR4A1* and *NR4A2* coding for NUR77/NGF1B and NURR1 respectively) and the cyclic AMP-responsive element-binding protein (CREB). These transcription factors are major regulators inducing the expression of *CYP11B2* (54) (Fig. 5).

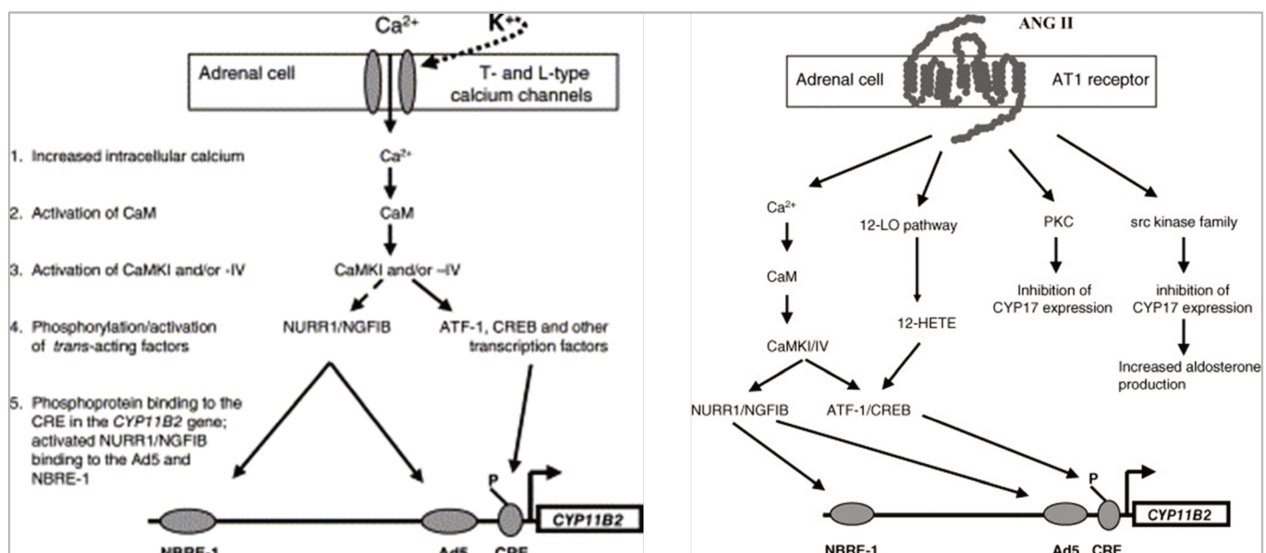


Figure 5. Regulation of aldosterone synthase expression. Left and right panels: the actions of K^+ and Ang II on *CYP11B2* expression, respectively (54).

ACTH can also induce aldosterone production in an acute and transitory manner via cAMP signaling (55). However, chronic administration of ACTH, over the course of 3 days or more, suppresses aldosterone production with a decreased expression of Ang II receptor in the ZG and a decrease of the renin-angiotensin-system activity. This chronic exposure to ACTH induces ZG cells to differentiate into ZF-like cells, which alters their cellular functioning and steroidogenesis (56-60).

Reports have shown that a paracrine regulation of aldosterone by other factors exists. Among these regulators, Serotonin (5-HT) which is produced in mastocytes, contributes to steroid production (61). Serotonin acts through the 5-HT type 4 receptor (HTR4) which is expressed in adrenocortical cells. Binding to this receptor activates the cAMP pathway and induces calcium influx into the cells, therefore regulating aldosterone and cortisol production. Agonists of this receptor such as cisapride and zacopride have also been shown to induce aldosterone production in healthy volunteers (62-65).

4.5.2 Cortisol production

In humans, cortisol is produced with a circadian rhythmicity at ~10 mg/day (66) and is the major glucocorticoid hormone produced by the ZF. It has major roles in the regulation of metabolism, the cardiovascular system, the immune system, inflammatory responses.

Cortisol is also a derivative of cholesterol and is synthesized in the ZF of the adrenal cortex. Cortisol synthesis is similar to aldosterone synthesis, with one major difference due to the presence of 17 α -hydroxylase (encoded by *CYP17A1*) in the ER of ZF and ZR cells, which converts progesterone into 17-hydroxyprogesterone. Hydroxylation by the 21-hydroxylase will generate 11-deoxycortisol, which is then hydroxylated in the mitochondria by the 11 β -hydroxylase (*CYP11B1*) at the C11 position to form cortisol. Interestingly, the CYP17 enzyme is not expressed in the cortex of mice and rats and this results in corticosterone being the major glucocorticoid produced by the ZF of these animals. The main regulator of cortisol biosynthesis is the hypothalamic pituitary axis. ACTH binds to its receptor, the melanocortin receptor 2 (MC2R). Unlike AT1R, MC2R is a G α s type GPCR and binding of ACTH causes the α subunit to associate with adenylate cyclase. ATP is converted to cAMP which activates downstream signaling pathways, most importantly the cAMP-dependent protein kinase (PKA). PKA consists of two subunits, one regulatory subunit and one catalytic subunit. In unstimulated conditions, PKA is in a tetrameric form, consisting of 2 regulatory subunits and 2 catalytic ones. Following cAMP binding to the regulatory subunits, conformational changes occur and the catalytic subunits are no longer bound and inhibited by the regulatory subunits. The catalytic subunits of PKA phosphorylate and activate cholesteryl ester hydrolases and StAR, increasing the amount of cholesterol delivered to the inner mitochondrial membrane. cAMP can also upregulate the transcription of *StAR*, *CYP11A1* and *CYP17A1*. Increased

amounts of glucocorticoids can induce a negative feedback on the production of ACTH and therefore cortisol production itself (67) (Fig. 6).

MRAP (melanocortin-2 receptor accessory protein) is a single transmembrane protein. MRAP is essential for the activity of MC2R, as it binds to MC2R in the ER and exports it to the cell membrane (68). Both MRAP and MC2R are highly expressed in the adrenal cortex, where MRAP/MC2R complexes are necessary to mediate the actions of ACTH (69). Mutations in MC2R are associated to cases of familial glucocorticoid deficiency (70).

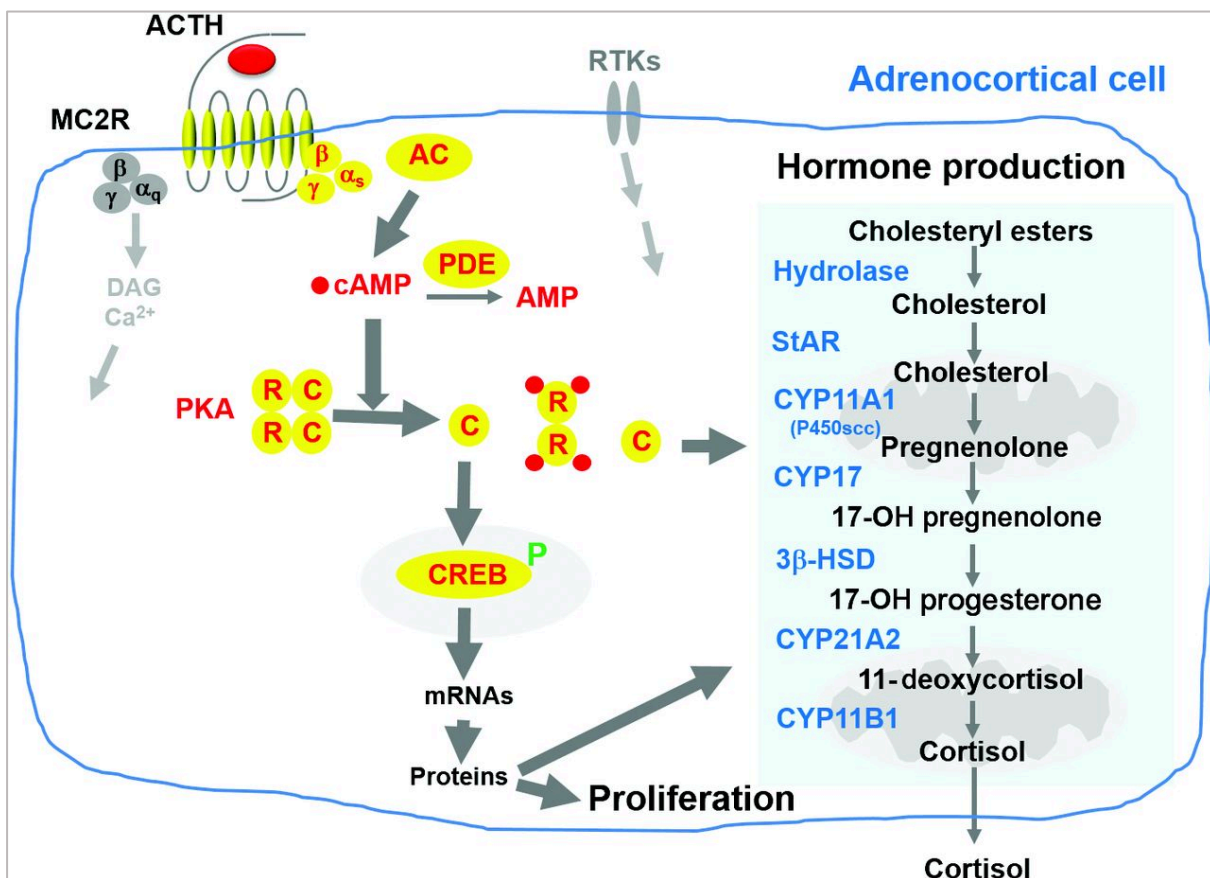


Figure 6. ACTH regulation of *CYP11B1* expression and cortisol production (71).

4.6 Signaling pathways and transcription factors in adrenal gland development and steroidogenesis

4.6.1 Wnt signaling pathway

Wnt signaling plays an important role in proliferation, mitosis, cell polarity, apoptosis, inflammation and differentiation. In the adrenal cortex, Wnt signaling plays an important role since early development and in the functional activity of the adrenal cortex. There exists two

separate Wnt signaling pathways, a canonical β -catenin dependent pathway and another non-canonical β -catenin-independent pathway. Depending on the Wnt-receptor complex, one of the two pathways is activated. Many Wnt ligands and receptors have been identified, which have been attributed to specific canonical or non-canonical Wnt signaling. However, studies have reported controversial data concerning this matter; whether or not the activated pathway depends on the tissue or cellular type is yet to be confirmed. The canonical Wnt/ β -Catenin signaling pathway is a central contributor to the development and functioning of the adrenal cortex and to steroid biosynthesis (72). In the absence of a Wnt ligand, β -catenin activity is suppressed by action of the destruction complex which phosphorylates the amino terminal region of β -catenin. The destruction complex consists of CK1 β (casein kinase 1 β), GSK-3 β (glycogen synthase kinase-3 β), APC (adenomatous polyposis coli), the scaffolding protein Axin and the adenomatous polyposis coli binding protein EB1. β -catenin is subsequently ubiquitinated and degraded by the proteasome. Binding of a Wnt ligand to its cell-surface receptor frizzled (Fz) results in the activation of the signaling pathway by inhibition of the phosphorylation of β -catenin. β -catenin dissociates from the destruction complex and translocates to the nucleus, where it acts as a transcriptional co-activator and enhances the expression of Wnt target genes, notably the transcription factors TCF (T-cell factor) and LEF (lymphoid enhancer factor) (72) (Fig. 7A).

The non-canonical β -catenin-independent Wnt signaling pathway has been shown to be responsible for embryonic development, tissue development and homeostasis. It is mediated either by JNK signaling (planar cell polarity pathway), or by intracellular calcium signaling (73). Interestingly, the non-canonical signaling pathway, when activated, has been shown to antagonize the canonical pathway (74) (Fig. 7B).

Besides its function as a transcriptional co-activator with the transcription factors TCF and LEF, β -catenin regulates the interaction of cadherin complexes, implicated in cell-cell junctions and in cellular cytoskeletons. Canonical Wnt signaling has been suggested to work exclusively in the ZG, as β -catenin is solely expressed in the ZG of the adrenal cortex (72).

Wnt4 has been shown to have an important role in the organogenesis of several tissues (75-78). Wnt4-deficient mice show an underdeveloped zona glomerulosa; this is associated to reduced expression of *CYP11B2* and abnormalities in its localization. These mice also exhibit an abnormal ectopic expression of adrenal-like cells that are in fact of adrenal origin in the gonads, which is attributed to abnormal adrenocortical progenitor cell migration during

development, suggesting a role of Wnt4 in cell sorting during development. Indeed, adrenal cortex cells and gonads derive from the same progenitor cells during development (79). Although Wnt4 and β -catenin are co-expressed in the adrenal ZG, suggesting that they belong to the same signaling pathway, a study showed that Wnt4 antagonizes β -catenin activity by disrupting its recruitment at or near SF-1 binding sites. This indeed indicates that Wnt4 could in fact activate the non-canonical Wnt signaling (75).

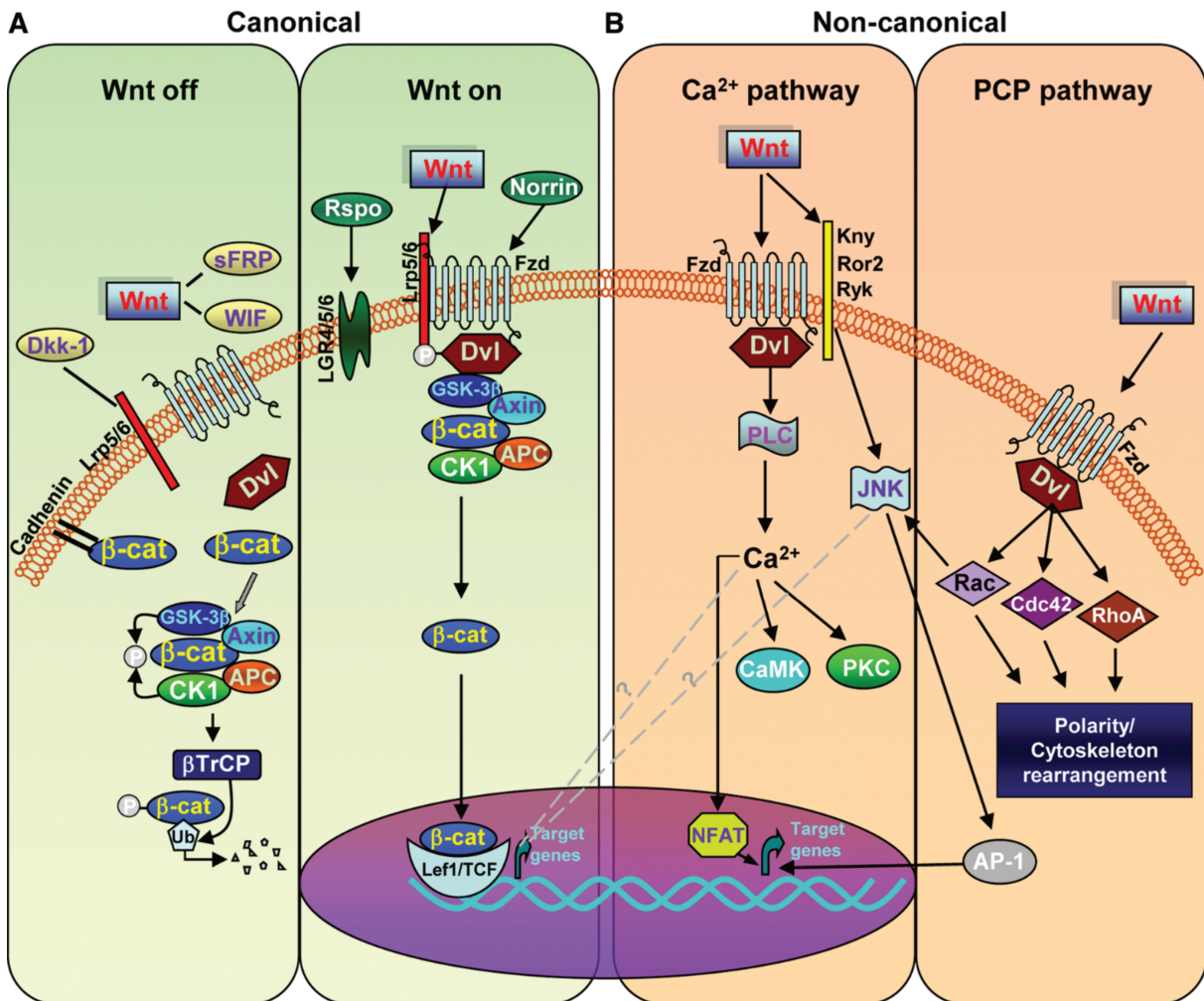


Figure 7. Wnt signaling pathway. A. The canonical Wnt/ β -catenin signaling cascade. Wnt off: in the absence of active Wnt signaling, β -catenin is phosphorylated at its inactivation sites by the destruction complex and then degraded by the proteasome. Wnt on: Following binding of Wnt ligand to its receptor Frizzled and other coactivators (LRP family members), the destruction complex is inhibited from phosphorylating β -catenin, which translocates to the nucleus where it regulates target gene expression with the transcription factors TCF and LEF. B. Non-canonical Wnt signaling pathway: non-canonical Wnt signaling passes through one of two pathways, the Wnt/ Ca^{2+} pathway or the planar cell polarity (PCP) pathway. The Wnt/ Ca^{2+} pathway involves activation of G-proteins, this activates intracellular calcium signaling as well as JNK/AP-1 signaling. The Wnt/PCP pathway involves polarity and cytoskeletal rearrangement; it can also activate AP-1 signaling. Canonical and non-canonical signaling pathways have also been shown to negatively interact: when non-canonical signaling is activated it could inhibit the transcriptional effects of the canonical pathway (80).

4.6.2 PKA signaling

Protein Kinase A (PKA) signaling has been shown to be important for the differentiation and homeostasis of the ZF and its function. In particular, PKA signaling has to be activated for the conversion of ZG cells into ZF cells and to maintain normal zonation of the cortex (81). PKA is a symmetrical tetramer complex that consists of two catalytic subunits (PRKACA) that are inactive due to binding to two other regulatory subunits (PRKARA). Different isoforms exist for each regulatory or catalytic subunits. Binding of ACTH to its receptor MC2R and co-receptor MRAP, activates cAMP, which binds to two sites on each of the regulatory subunits inducing their dissociation from the complex, freeing the catalytic subunits, the effectors of PKA signaling (82). PKA regulates transcription by phosphorylating transcription factors such as the cAMP-response element-binding protein (CREB), which interacts with CREB-binding protein (CBP) and p300 to regulate target genes expression (83).

In the adrenal cortex, PKA is active particularly in the ZF. Interestingly, the over-activation of PKA signaling by knocking out the regulatory subunit of PKA (*Prkar1a*) in mice in an adrenal specific manner induce disorganized zonation and Cushing's syndrome (84). PKA has been also shown to accelerate the regeneration of the adult adrenal cortex and to favor ZF differentiation (85). It has been suggested that PKA acts as a tumor suppressor in the adrenal cortex through repressing the activity of Wnt signaling (81). Modifications in PKA signaling are frequently reported in adrenal gland disorders, including cortisol producing adenoma and Carney complex, where different activating mutations of subunits of PKA induce cortisol excess (86).

Wnt and PKA play essential and complementary roles in the functional zonation of the adrenal cortex, with Wnt signaling favoring a ZG phenotype, while PKA activation promotes a ZF phenotype (81).

4.6.3 Vascular signaling

The adrenal gland one of the most highly vascularized organs in the body (87), where each cell is in direct contact with a vessel (27). One of the major contributors to vessel development and maintenance is vascular endothelial growth factor (VEGF) signaling. It has been shown that VEGF was more abundant in APA compared to normal adrenals, and inhibiting the effects of VEGF and VEGFR resulted in a decrease in cell growth (88). Interestingly, in the adrenal gland, it has been shown that VEGFA not only participates in the development of the vasculature, but has also a role in steroidogenesis. In H295R

adrenocortical cells, stimulation with VEGF resulted in a significant increase in aldosterone biosynthesis, this effect being additive to that of Ang II when the cells were incubated with both stimulants. Moreover, inhibition of VEGF by the overexpression of soluble fms-like tyrosine kinase-1 resulted in a decrease of aldosterone synthesis in rats (89).

ACTH, which is required for the maintenance of a normal adrenal cortex vasculature, stimulates the expression of VEGF mRNA through a post-transcriptional mechanism (90, 91). It has been proposed that reduction of VEGF is a secondary effect of ACTH deprivation. Besides VEGF signaling, the epidermal growth factor-like crypto protein CR-1 (TDGF-1) has been shown to be upregulated in APA, and had effects on H295R cells such as reduction of apoptosis and increased aldosterone production (92).

4.6.4 Steroidogenic Factor-1

Steroidogenic Factor-1 (SF-1) is one of the major drivers of adrenal cortex development and very important for steroidogenesis and function of the hypothalamic-pituitary-adrenal and gonadal axis (93, 94). It is an orphan nuclear hormone receptor also known as Ad4BP or NR5A1 and is highly expressed in the adrenal cortex and the gonads, where it regulates the transcription of genes involved in both steroidogenesis and reproduction. It is also expressed in other tissues such as the brain, the skin and the spleen (95). Mouse embryonic stem cells and bone marrow cells overexpressing SF-1 differentiate into steroidogenic cells that respond to ACTH and produce deoxycorticosterone and 17OH progesterone (40, 96). SF-1 overexpression has also been shown to increase *CYP11B1* levels and decrease *CYP11B2* and *CYP21A2* in basal conditions and following Ang II treatment in H295R cells (97). Suppression of SF-1 expression led to the repression of *CYP11B1* and *CYP11B2* as well as other steroidogenic enzymes, indicating that SF-1 is also important in regulating *CYP11B2* expression (98).

Sf-1 KO mice lack adrenal glands and gonads and show increased levels of ACTH. These mice survive intrauterine life, possibly due to the passage of maternal steroids by the placenta, but die shortly after birth (~8 days) when their steroid levels are diminished due to the lack of adrenal glands (99).

SF-1 activity could also be altered post-translationally by its state of sumoylation; ube9 and PIAS1, two enzymes involved in sumoylation (the conjugation of small ubiquitin-like

modifier 1 – SUMO1) of SF-1 have been shown to be co-expressed in the ZG. Sumoylation of SF-1 has been shown to regulate its activity and been proposed to increase steroidogenesis (100).

4.6.5 Other signaling pathways

Many signaling pathways have been linked to the development, function and organization of adrenal cortex. SHH is mostly expressed during the early development of the young adrenal cortex and is barely detectable in the subcapsular cortex in the outer ZG of the adult adrenal gland. However, it is highly expressed in APA and its adjacent cortex, suggesting that these cells have acquired characteristics of stem and progenitor cells, which could underlie the excessive proliferation of the ZG and hence nodule formation (101).

The GATA transcription factors are a family of 6 zinc finger transcription factors that regulate gene expression, differentiation, cellular proliferation and apoptosis in various tissues. The KO of any of these factors (except *Gata5*) is lethal during embryonic life, thus highlighting their role in development (102). In the adrenal gland, *Gata4* and *Gata6* are expressed (103). *Gata4* has been shown to interact with SF-1 and has been shown to induce the expression of the *CYP17* enzyme and *StAR* (104). *Gata6* is expressed during development and also regulates steroidogenesis by enhancing the expression of *CYP17A1*, *CYP11A1* and *StAR*, also by interacting with SF-1 (104, 105). Interestingly, *Gata4* is upregulated in adrenal cortex tumors, while *Gata6* is downregulated (106). *Gata4*, which is a transcription factor and a marker of gonadal differentiation, should not be expressed in the adult adrenal gland; if present, it could indicate dedifferentiation in the sense of the common adreno-gonadal precursor cell type (107). During mouse embryonic development, the adrenal primordium expresses *Wnt4*, SF-1 and *WT1* (Wilm's Tumor-1), which participate in the differentiation of adrenocortical and gonadal cells (108). The *WT1* gene is a transcription factor and mutations in this gene are associated with defects in kidney and gonad development (109). However, *Wt1* KO mice do not survive past the 14th day post conception due to heart failure, and partially rescued mice by a human *WT1* transgene survive with urogenital malformations and an impaired adrenal development (110).

PI3K (phosphoinositide 3-kinase)/AKT/mTOR (mammalian target of rapamycin) signaling is a major regulator of metabolism and cell proliferation and is implicated in the regulation of the activity of growth factor receptors such as receptors of vascular endothelial growth factor (VEGF) and insulin-like growth factors (IGF) (111, 112). The expression of AKT seems to be

mostly abundant in the ZR in normal adrenals glands and scarce in the ZG and ZF (113), indicating that PI3K/AKT/mTOR signaling could contribute to androgen production through IGF in the ZR. The expression of AKT has been found to be significantly elevated in adrenocortical carcinomas (113). Interestingly, it has been reported that PI3K/AKT pathway stimulates aldosterone biosynthesis (114). Moreover, mTOR signaling is upregulated in APA and IHA, and on a proteomic level, it was among the mostly deregulated signaling pathways in APA (115). Furthermore, inhibition of mTOR by rapamycin led to a decrease of cell proliferation and aldosterone production in H295 cells (116).

4.7 APA, somatic and germline mutations, familial forms of PA

The discovery and application of high throughput sequencing techniques has helped in uncovering underlying genetic mechanisms in many diseases. This emergence was notably important in identifying somatic mutations implicated in the development of APA. Ion channels and ATPase pumps are major contributors to the normal function of ZG cells, given their role in maintaining ZG cell membrane potential and ionic homeostasis. During the last ten years, different studies have identified somatic mutations in APA, affecting mainly 4 genes (*KCNJ5*, *CACNAID*, *ATP1A1* and *ATP2B3*), coding for potassium and calcium channels and ATPases (Fig. 8). Those mutations are found in nearly 90% of APA when applying CYP11B2 immunohistochemistry guided next-generation sequencing (NGS) for genetic screening.

4.7.1 Somatic mutations in PA

Using whole exome sequencing (WES), Choi and colleagues identified two somatic mutations in *KCNJ5*, coding for the potassium channel GIRK4, in 8 out of 22 APA (117). This channel is the G protein-activated inward rectifier potassium channel 4 (also known as Kir3.4), and is mainly expressed in the ZG. GIRK4 belongs to the family of inwardly rectifying potassium channels, which induce large inward currents at the cell membrane. GIRK4 comprises two membrane-spanning domains (M1 and M2), cytoplasmic N- and C- termini that participate to the pore structure and one pore forming region (H5). Mutations occurring near to or within the channel pore affect its selectivity filter, notably between amino acids Thr149 and Gly153. This induces a loss of channel selectivity, which renders the channel permeable to sodium,

leading to sodium entry into the cell. This again leads to cell membrane depolarization and thus opening of voltage gated calcium channels, with increased intracellular calcium concentrations and activation of calcium signaling in the cell (117). Oki and colleagues transfected adrenocortical HAC15 tumor cells with *KCNJ5* mutants, this increased *CYP11B2* expression and aldosterone production in a calcium dependent manner (118). In some cases, *KCNJ5* mutations reduce the abundance of GIRK4 at the cell membrane (119). However, *KCNJ5* mutations do not affect cell proliferation, and surprisingly their overexpression in HAC15 leads to reduced cell viability or sodium dependent cell death (117, 120). Therefore, *KCNJ5* mutations do not explain cell proliferation, but only the mutation's contribution to aldosterone biosynthesis. Interestingly, it has recently been shown that knockout of *Kcnj5* in mice results in lower plasma aldosterone levels in female, but not in male mice. This sexual dimorphism could be due to the activity of the peroxisome proliferator activated receptor (PPAR) α which could modify signaling pathways and alter aldosterone production in female adrenal glands. Interestingly, in female mice, treatment with Ang II resulted in higher aldosterone levels in KO mice compared to the WT. Similar to humans, *Kcnj5* is exclusively expressed in the ZG of mouse adrenals cortex. Further testing on H295R cells showed that activation of PPAR α by Fenofibrate increased the expression of *CYP11B2* and stimulated aldosterone production (121).

One of the calcium channels involved in ZG cell physiology is Cav 1.3 (Calcium channel, voltage-dependent, L type, alpha-1d subunit), which is encoded by *CACNAID*. Cav 1.3 is a L-type calcium channel composed of four repeat domains (I – IV), each of these domains consists of six transmembrane segments (S1-S6) with a membrane associated loop between S5 and S6 (122). Whereas the S4 segments are involved in voltage sensing, the S5 and S6 domains as well as the loop in between line the pore of the channel (123). More than 20 different somatic *CACNAID* mutations have been identified in APA. These are gain-of-function mutations, which lead to channel activation at less depolarized cell membrane potential and delay channel inactivation, leading to increased calcium signaling in the cell and induction of aldosterone biosynthesis (124-126).

The $\alpha 1H$ subunit of the voltage gated calcium channel 3.2 (Cav 3.2), encoded by *CACNAIH*, is also prone to genetic alterations, as it has been recently shown to be involved in familial forms of PA, which will be discussed further below (127, 128). *CACNAIH* is located on chromosome 16 and codes for the pore-forming $\alpha 1$ subunit of the T-type voltage-dependent calcium channel Cav3.2, which consists of a single polypeptide chain of four homologous

domains (I-IV); each domain consists of 6 transmembrane spans (S1-S6), where S4 serves as a voltage sensor, and cytoplasmic N- and C- termini. Mutations in *CACNA1H* induce a change in the electrophysiological properties of the channel, in particular a shift in the activation threshold of the channel to more negative currents, and a delay in their inactivation. As a consequence, the channel is activated at less depolarized states of the ZG cellular membrane, leading to activation of calcium signaling and overproduction of aldosterone.

The Na⁺, K⁺-ATPase alpha-1 Subunit and plasma membrane transporting ATPase 3 (PMCA3) are both members of the P-type family of ATPases, and are encoded respectively by *ATP1A1* and *ATP2B3*. Both pumps consist of 10 transmembrane domains (M1 – M10) and intracellular C- and N- termini. Na⁺, K⁺-ATPase is an important regulator of plasma membrane potential in ZG cells by its activity on ionic exchange and homeostasis. PMCA3 regulates intracellular calcium concentrations by extruding calcium from the cell.

In physiological conditions, when ATP1A1 is activated by ATP, it exchanges Na⁺ and K⁺ ions by extruding 3 Na⁺ ions against 2 K⁺ ions. Mutations in *ATP1A1* lead to a loss of function of the pump and induce a proton leak. This induces cell membrane depolarization and increased Ca²⁺ influx (124, 129). However, transfection of adrenocortical H295R cells with two of the described *ATP1A1* mutations surprisingly did not show changes in basal calcium concentrations. Moreover, it barely had an effect on potassium stimulated calcium influx into the cells, even though it led to cell membrane depolarization and aldosterone biosynthesis. In contrast, the mutated pump led to a proton leak of H⁺ ions into the cells, which caused intracellular acidification, suggesting that intracellular acidification could be responsible for the increased aldosterone biosynthesis (130).

Like ATP1A1, PMCA3 (*ATP2B3*) is expressed at the cell membrane and is also involved in maintaining intracellular ionic homeostasis, by extruding intracellular calcium in exchange with hydrogen ions. Mutations in *ATP2B3* are found in region coding for the M4 transmembrane domain; they consist mainly in small deletions between Leu422 and Leu433. One particular mutation studied by Tauber and colleagues, Leu425_Val426del, resulted in a pathological Na⁺ and possibly Ca²⁺ leak into the cells and a reduced calcium export, resulting in loss of the physiological function of the pump. This ultimately leads to increased calcium concentrations in the cell and to autonomous aldosterone biosynthesis. Interestingly, transfection of H295R cells with this mutation caused the cells to express not only more *CYP11B2* but also more *CYP11B1* as well (129, 131), a finding consistent with the literature,

where one study showed that ATPase mutations can indeed induce high expression of *CYP11B2* as well as *CYP11B1* (132).

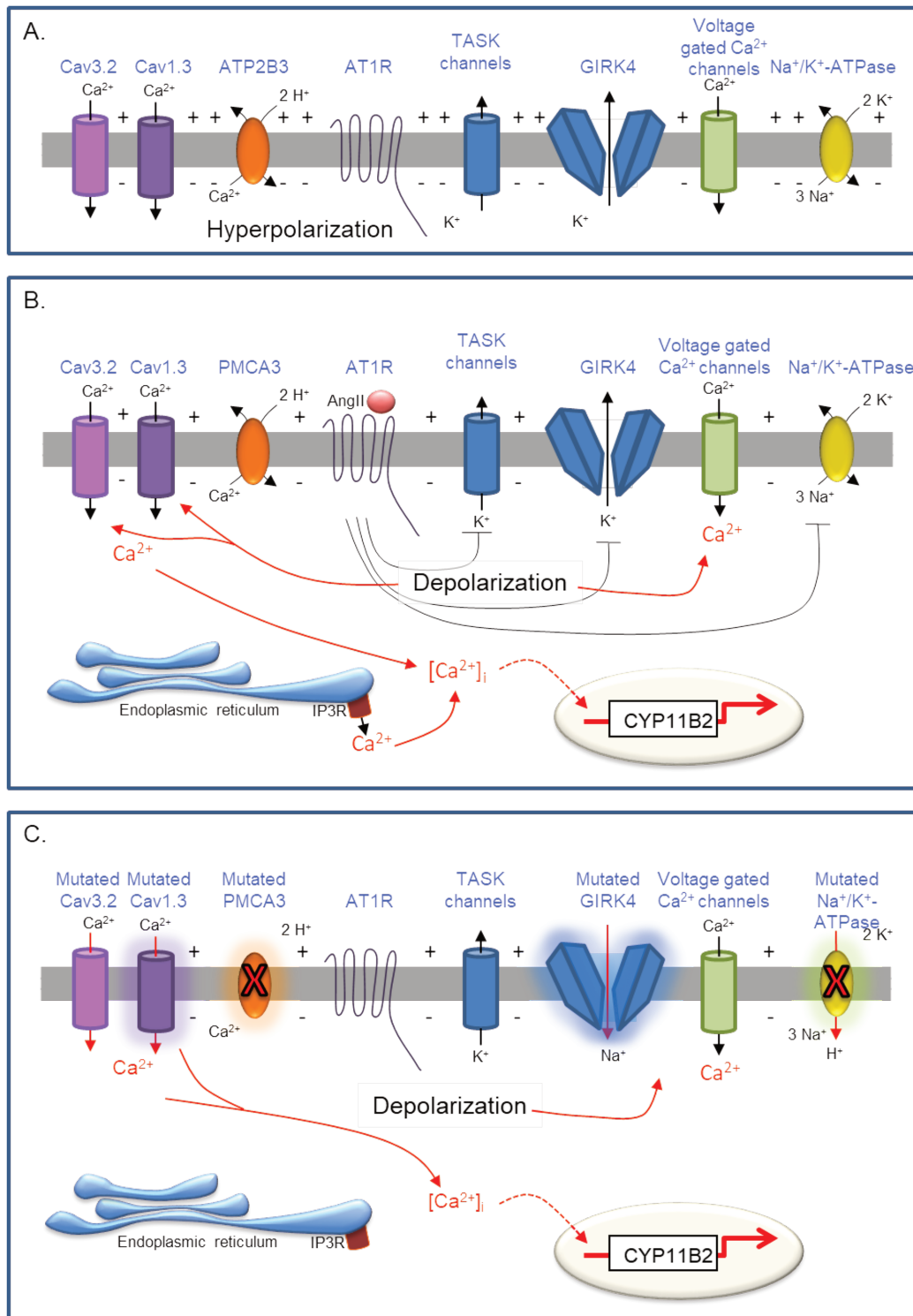


Figure 8. Aldosterone synthase expression in physiological and pathological conditions. A. Zona glomerulosa cell potential in resting conditions. B. Ang II binding to AT1R or increases in extracellular K⁺ levels lead to cell membrane depolarization and activation of intracellular calcium signaling and CYP11B2 expression. Ang II also induces, through inositol triphosphate (IP3), the release of Ca²⁺ from the sarco/endoplasmic reticulum. C. Mutations in ion channels (KCNJ5, CACNA1D and CACNA1H) and in ATPases (ATP1A1 and ATP2B3) lead to a constitutively depolarized cell membrane or directly to increased Ca²⁺ signaling, leading to increased expression of CYP11B2 and autonomous aldosterone production (133).

Although Wnt signaling has been shown to be constitutively active in up to 70% of APA (134), somatic mutations in *CTNNB1* coding for β -catenin in APA occur at a frequency of ~5% (135, 136). Somatic *CTNNB1* mutations have been shown to be associated with very high levels of expression of the leuteinizing hormone-chorionic gonadotropin receptor (LHCGR) and the gonadotropin-releasing hormone receptor (GNRHR) in APA. They were found in pregnant and menopausal women with severe hypertension, suggesting that these two conditions may trigger the development or reveal an underlying PA (137). Primary cell cultures from APA transfected with the mutant *CTNNB1* expressed high levels of LHCGR, as opposed to their untransfected counterparts that did not express the receptor. All mutations occur in the phosphorylation consensus motif of β -catenin and thus impair β -catenin from being degraded leading to an upregulated Wnt activity (137).

Mutations have also been found in the *PRKACA* gene, encoding the alpha-isoform of the catalytic (C α) subunit of Protein Kinase A (PKA), in two APA out of 122 APAs (1.6%) (138). One of the mutations, p.Leu206Arg, corresponds to the most frequent *PRKACA* mutations described in cortisol-producing adenomas (CPA). The patient carrying the mutation indeed showed both PA and Cushing's syndrome (139-141). The second mutation, p.His88Asp, was found in a patient with APA but without cortisol hypersecretion. The role of these mutations in aldosterone biosynthesis remains to be established. Further mechanistic testing showed that p.His88Asp was not associated to any gain-of-function despite the strong expression of CYP11B2.

PRKACA mutations and *CTNNB1* mutations are found in CPA as well. There is evidence of an overlap of genetic determinants between aldosterone and cortisol excess, such as the cortisol co-secretion observed in a subset of APA, particularly APA harboring *KCNJ5* mutations. How the same mutations can lead to the production of two different hormones and the mechanisms behind it remain to be elucidated (142).

4.7.2 Inherited forms of PA

Similar to somatic mutations in PA, our knowledge on inherited forms of PA has increased exponentially in the last few years, from two forms (glucocorticoid remediable and non-glucocorticoid remediable), to 4 different subtypes of familial hyperaldosteronism (FH). Although most of the cases are sporadic, PA can be transmitted as a Mendelian trait in familial forms of the disease. These forms account for 4-5 % of cases and are transmitted in

an autosomal dominant manner (143-145). To date, four different forms of FH have been defined, based on their underlying genetic alterations.

4.7.2.1 Familial hyperaldosteronism type I, the chimeric gene

FH-I, also known as glucocorticoid-remediable aldosteronism (GRA), is an autosomal dominant disease with a prevalence of $\approx 1\%$ in adult PA patients and up to 3% in pediatric cohorts (143, 145-147). This disease is characterized by early and severe hypertension, associated to suppressed plasma renin, hypokalemia, metabolic alkalosis, and high serum levels of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol; suppression of aldosterone by high levels of dexamethasone is efficient in reducing plasma aldosterone levels (148). The genetic cause underlying FH-I is a chimeric *CYP11B1/CYP11B2* gene. Indeed, *CYP11B2* and *CYP11B1* lie in close proximity on chromosome 8. Under physiologic conditions, *CYP11B2* encoding aldosterone synthase is expressed exclusively in the ZG under the control of Ang II and extracellular K^+ , while *CYP11B1* encoding 11- β hydroxylase is expressed in the ZF under the control of ACTH. In GRA, in addition to the two normal genes, a chimeric gene is formed by unequal crossing over during meiosis, which is composed of the regulatory regions of *CYP11B1* and the coding regions of *CYP11B2* and is expressed in the ZF under the control of ACTH. Consequently, *CYP11B2* is ectopically expressed in the ZF under the control of ACTH, and follows the circadian rhythm of cortisol (149, 150). Patients with FH-I are at increased risk of cardiovascular and cerebrovascular events as a consequence of high aldosterone levels in young patients (151, 152). Replacement doses of exogenous glucocorticoids can reduce ACTH levels by negative feedback in the hypothalamus, thereby, diminishing the expression of the chimeric gene and suppressing the ectopic aldosterone production.

4.7.2.2 Familial hyperaldosteronism type II and *CLCN2* mutations

FH-II is a non-glucocorticoid remediable form of FH transmitted in an autosomal dominant manner, diagnosed in $\approx 4\%$ of adult patients with PA. Generally, patients present a family history of PA, with either APA or BAH sometimes in the same family (143-145, 153). FH-II is difficult to distinguish from sporadic PA, the diagnosis is made in the presence of two or more affected family members, after exclusion of other genetic forms involving *KCNJ5* or *CACNA1H* mutations. A genetic locus on chromosome 7p22 has been found to be associated

with FH-II in 2000. The authors suggested many candidate genes in this locus that could be implicated, such as retinoblastoma-associated Kruppel-associated box gene (*RBaK*), postmeiotic segregation increased 2 (*PMS2*), guanine nucleotide-binding protein α -12 (*GNAI2*), replication protein A3 (*RPA3*), zinc finger protein 12 (*ZNF12*), glucocorticoid induced transcript 1 (*GLCC1*), fascin 1 (*FSCN1*), and the cAMP-dependent protein kinase type I β -regulatory subunit (*PRKAR1B*). However, no causal mutations were identified in any of these genes (154). The ambiguity of the underlying causes of this disease were still unknown until very recently, where two studies identified, using WES, a new gene that was associated with FH-II. Different gain-of-function mutations in the *CLCN2* gene, encoding the chloride channel CLC-2, were identified in patients with FH-II and in young onset PA. These findings also showed for the first time the implication of a chloride channel in the pathogenesis of PA and specifically the production of aldosterone.

Our laboratory identified a *de novo* mutation in the *CLCN2* gene, p.Gly24Asp, in a 9 year-old female patient who suffered from severe hypertension due to PA (155). This is a gain-of-function mutation affecting a highly conserved region in CLC-2, which leads to a constitutive activation of the channel (156). We found that this channel had a high expression in the human and mouse adrenal zona glomerulosa. Patch clamp analyses on cells from mouse ZG *ex vivo* slices showed *bona fide* chloride currents, which were completely abolished in *Clcn2* knockout mice, indicating that CLC-2 is the major chloride conducting channel in the ZG (155). We also showed that transfection of the mutant CLC-2 channel in the adrenocortical cell line H295R-S2, leads to increased chloride currents and depolarization of the ZG cell membrane, leading to opening of voltage-gated calcium channels and induction of autonomous aldosterone production.

In the other study, Scholl and colleagues performed WES on the original FH-II family described by Stowasser et al in 1992 (157). WES revealed a germline mutation in the *CLCN2* gene, p.Arg172Gln, in affected family members. This same mutation was identified in three additional cases with early onset PA, from which one was a familial case and another *de novo*. Apart from this mutation, the authors also identified 4 other mutational variants occurring in the *CLCN2* gene in 4 unrelated patients with early onset PA, p.Tyr26Asn, p.Ser865Arg, p.Lys362del and p.Met22Lys. (158) All in all, Scholl et al demonstrate that 17 patients within 8 families had *CLCN2* mutations.

These studies report for the first time an implication of an anion channel in PA, where mutations induce increased chloride conductance at resting potentials, leading to ZG cell

membrane depolarization, and expressing aldosterone synthase. Based on these results, screening for *CLCN2* mutations should be included in the panel of susceptibility genes which are screened in patients with early onset PA.

4.7.2.3 Familial hyperaldosteronism type III and *KCNJ5* mutations

FH-III is also a non-glucocorticoid remediable form of FH with early onset severe HT, hyperaldosteronism, hypokalemia and high levels of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol. (159) The adrenals show massive bilateral hyperplasia of the adrenal cortex, and patients generally need a bilateral adrenalectomy to normalize their blood pressure. As in somatic *KCNJ5* mutations, the germline mutations affecting this channel are also gain-of-function mutations, affecting the selectivity filter of the channel, which leads to Na⁺ leaking, cell membrane depolarization and autonomous aldosterone production. (117, 118) However, other germline *KCNJ5* mutations have been described, some of which are not associated with a severe phenotype of PA. (160) These patients were thought to have FH-II due to the lack of bilateral adrenal phenotype, however, following the genetic diagnosis they were re-classified as mild forms FH-III.

4.7.2.4 Familial hyperaldosteronism type IV and *CACNA1H* mutations

As FH-II and III, FH-IV is also a non-glucocorticoid remediable form of FH that is inherited in an autosomal dominant manner but with a reduced penetrance. Scholl and colleagues performed WES on a cohort of 40 patients diagnosed with PA at an early age, all having 10 years of age or less. They identified recurrent mutations in the *CACNA1H* gene in 5 blood unrelated patients in this cohort. Shortly after, our group reported mutations in the same gene in two unrelated patients that were originally diagnosed with FH-II, as well as two other patients, one being *de novo* and the other was a female patient with APA and a germline mutation of *CACNA1H*. (127, 128) Interestingly, germline *CACNA1H* mutations have been linked to several mental and neurological disorders, notably, autism, epilepsy and amyotrophic lateral sclerosis. (161-163) The mutations that were described in Scholl's and Daniil's papers result in a decrease in the deactivation of the channel, consequently, the channel remains open for longer periods with increases in Ca²⁺ influx, and higher levels of *CYP11B2*. Intriguingly, Reimer and colleagues showed that treatment with the non-selective calcium channel blocker Mibefradil of HAC15 adrenocortical carcinoma cells overexpressing

a Cav 3.2 channel carrying the p.Met1549Val mutation, reduced the expression of *CYP11B2* and aldosterone biosynthesis, indicating that calcium channel blockers may be used in patients with FH-IV (164).

4.7.2.5 Germline mutations in sporadic forms of PA

De novo germline *CACNAID* mutations have been described in two very young children with Primary Aldosteronism, Seizures and Neurological Abnormalities (PASNA). One of the subjects, had a *CACNAID* mutation (p.Ile770Met) suffered from cerebral palsy and complex seizures right after birth, with HT, with hyperaldosteronism and hyperkalemia. The other patient was a 3-year-old female who had severe HT (199/78 mmHg), high aldosterone levels as a newborn, high aldosterone to renin ratios, hypokalemia with biventricular hypertrophy and neurological abnormalities. This patient also harbored a germline mutation in the *CACNAID* gene (p.Gly403Asp) (165, 166).

Germline mutations have also been identified in *ARMC5* (Armadillo Repeat containing 5) in sporadic cases of PA (138, 167). This gene that codes for an apoptosis regulator which belongs to the armadillo/ β -catenin-like superfamily. *ARMC5* mutations have been mainly described in adrenocortical disease which is associated with cortisol excess and sporadic and inherited forms of primary bilateral macronodular hyperplasia. Generally, mutations in *ARMC5* promote cell survival and steroid hormone production *in vitro* (168, 169). However, more studies have to be attributed to decipher the real role of *ARMC5* in PA, as a study suggested that the mutations that have been identified in this gene in PA are likely non-pathogenic (170).

Despite the major advances in our knowledge concerning FH, a few points need to be addressed, the difference of the presentation of the diseases, and the difference in the penetrance as well as the clinical heterogeneity. Yet, the advances that have been so far made are quite promising, especially if it could contribute to the development of new diagnostic and therapeutic approaches.

4.7.3 Genomic and proteomic changes in APA

A recent study performing quantitative proteome and phosphoproteome analysis on APA and adjacent non-tumoral adrenal cortex from 6 PA patients, identified different proteomic

changes in APA. In particular, it discovered upregulation of enzymes involved in increased steroidogenesis, such as CYP11B2, HSD3B2, CYP21A2, and genes that are involved in cholesterol uptake such as LSR (lipolysis stimulated lipoprotein receptor). Some of these enzymes were regulated by phosphorylation, notably CYP21A2 and HSD3B2. Components of the extracellular matrix (ECM) were also altered: the authors reported a downregulation of ECM components and enzymes involved in ECM turnover, as well as collagen and collagen fibril assembly. Interestingly, there was also a deregulation of kinases affecting cytoskeletal remodeling. Moreover, RHOC (Ras homolog gene family, member C), a GTPase that controls actin organization, was upregulated in APA, and significantly induced *CYP11B2* but not *CYP11B1* expression in adrenocortical carcinoma H295R cells transfected with a RHOC-encoding vector. Whether RHOC exerts its effects by affecting the cytoskeleton of the cells or by affecting signaling pathways remains to be uncovered (115). Furthermore, APA express high levels of proteins involved in N-glycosylation, which affects the activities of AT1R and MC2R and other membrane receptors, as well as proteins that are involved in cholesterol and steroid metabolism and thus affect steroidogenesis in the adrenal cortex (115, 171). The authors also show that APAs had a low level of GABAergic signaling, which could modulate aldosterone biosynthesis, as activation of GABAergic signaling has been shown to decrease steroidogenesis in rat adrenal glands (172).

GPCRs, notably MC2R, are known effectors in the overproduction of cortisol in cortisol producing adenomas (CPA). GPCRs have been shown to be aberrantly expressed in APA and play a role in the regulation of aldosterone production. Ye et al. showed that several GPCR, such as *HTR4*, *GnRHR*, *LHCGR*, *MC2R*, metabotropic glutamate receptor 3 (*GRM3*), endothelin receptor type B-like protein (*EDNRC*), had significantly higher expression in APA when compared to normal adrenal glands. The ectopic expression of these receptors is hypothesized to upregulate aldosterone production in APA where the renin-angiotensin system is suppressed (173). Interestingly, the *CYP11B2* gene is reported to be hypomethylated in APA, along with a hypomethylation of GPCR and GPCR-related genes, such as *HTR4*, *MC2R*, tachykinin receptor 1 (*TACR1*) the receptor for substance P, *GRM3* and the prostaglandin E receptor 1 (*PTGER1*). Concomitantly, mRNA levels of *HTR4* and *PTGER1* were upregulated, showing a significant correlation to their hypomethylation status. Remarkably, it is well known that *HTR4* increases aldosterone production by activating the cAMP pathway and calcium signaling (64), whereas prostaglandin E2 increases aldosterone production by inducing the conversion of cholesterol to pregnenolone through its receptor

PTGER1 (174). These alterations in GPCRs and their related proteins could lead to increased expression of *CYP11B2* and stimulation of aldosterone biosynthesis in APA (31). The changes in methylation and gene expression seemed to be independent from the mutational status (*KCNJ5* or *ATP1A1*) of APA; moreover, transfection of HAC15 cells with mutated *KCNJ5* channels (T158A) did not affect methylation of *CYP11B2* or *CYP11B1* genes (175). Furthermore, the methylation levels of *CYP11B2* were not associated to transcription levels of *CYP11B2* in APA, thus, the role of the methylation status of *CYP11B2* in APA remains to be elucidated.

Different studies showed an upregulation of several genes regulating the expression of *CYP11B2*, such as *NURR1* and *NGFIB* (176), as well as essential transcription factors for adrenal cortex development and steroidogenesis, such as the steroidogenic factor SF-1, *DAX1* and *VSNL1* (visinin like 1), an SF-1 target gene that has been shown to increase aldosterone biosynthesis in H295R cells, (177, 178). It has further been reported that *CYP17A1* expression is higher in APA with ZF-like cells harboring *KCNJ5* mutations in comparison to *ATP1A1* and *CACNA1D* mutated adenomas (179, 180).

4.7.4 The pathogenesis of APA

The relationship between the occurrence of somatic mutations and aldosterone biosynthesis is very well understood; however, the impact of these mutations on cell proliferation, nodule and APA formation is still far from being understood. Intriguingly, APA show a very pronounced molecular heterogeneity in terms of both its mutational status and aldosterone synthase expression within the APA itself, and in the adjacent tissue. Studies have shown the occurrence of different somatic mutations within different aldosterone producing nodules in the adrenal gland (181, 182). These findings, suggest that somatic mutations could occur as several independent events in a previously remodeled adrenal cortex. Other studies have reported similar findings, where one study described one patient who was diagnosed with PA and Cushing's syndrome with two adrenocortical adenomas, one harboring a *KCNJ5* mutation and the other a *PRKACA* mutation (183). In another study, the same group reported an APA that presented heterogeneity in aldosterone synthase expression, with the mutation being only present in the aldosterone synthase-expressing region (184). Our group has also described a case of a young patient with severe lateralized PA, bilateral macronodular adrenal hyperplasia and Gardner syndrome. This patient carried a germline *APC* mutation and a somatic *KCNJ5* mutation leading to the development of APA (185). This case further supports a two-hit

model for APA development with the *APC* mutation favoring nodule formation and the *KCNJ5* mutation causing aldosterone overproduction (Fig. 9).

Another hypothesis on the pathogenesis of APA was suggested by Nishimoto and colleagues in a study that described aldosterone-producing cell clusters (APCC) as being the origin of APA formation (186). APCC are structures that consist of cell clusters in contact with the inner part of the capsule and the outer part of the ZF. Cells in the outer part resemble ZG cells, while those in the inner part resemble ZF cells. These structures are mainly identified by a very strong staining for aldosterone synthase which they produce autonomously, however, they lack 11 β -hydroxylase expression (186, 187) (Fig. 9).

APCC are distinctive from APAs in their size, cellular arrangements, and their enzyme expression profiles. APCC are hypothesized to increase in number in normal adrenal glands with age; in the human adrenal gland, at a young age and up to 11 years, aldosterone synthase expression is rather continuous in the ZG, while with age the ZG loses the continuity of the aldosterone synthase expression in favor of an irregular discontinuous expression with increased numbers of APCC. This discontinuation is suggested to be due to a negative feedback by the autonomous production of aldosterone by APCC (188).

Although there are no sex differences in the prevalence of APA, APCC seem more frequent in female adrenal cortex (189). APCC are found in normal adrenal cortex and in adrenal cortex with APAs. Sequencing of DNA extracted from APCC from normal adrenal glands showed that up to 35% of these APCCs harbor mutations of APA-driver genes such as *ATP1A1*, *ATP2B3* and *CACNA1D*, but not *KCNJ5*, which is the most frequently mutated gene in APA. It is suggested that APCC with *KCNJ5* mutations are rare events, or that APCC harboring *KCNJ5* mutations rapidly progress to APA and thus are hard to be witnessed. Moreover, transcriptome analysis on APCC showed, in addition to high expression of *CYP11B2* in comparison to ZG and ZF, that APCC express high levels of *MC2R*, *SLC35F1* and *PPP4R4*, implicated in steroid production, glucose transport and regulation of intracellular phosphorylation and dephosphorylation, respectively (189). The transition from APCC to APA is thought to occur through the development of structures called possible APCC-to-APA translational lesions (pAATL) (190). These structures consist of an outer APCC-like portion sprouting into an inner micro-APA (mAPA)-like portion. In a case of a pAATL, a *KCNJ5* mutation occurred only in the mAPA-like portion and not the APCC-like portion (190); this could suggest that the APA and the pAATL did not share the same origin. In another case,

both portions of the pAATL harbored an *ATP1A1* mutation, indicating that both portions had the same origin. Clearly, APA development from APCC through pAATL and mAPA is interesting and has to be further investigated to gain a better understanding of the genomic and proteomic events involved in this transition (190).

Remarkably, it has been recently shown that accumulation of APCC could be the cause of idiopathic hyperaldosteronism (IHA). In a multicenter study on 15 IHA adrenal glands that were examined by CYP11B2 immunohistochemistry and NGS, Omata and colleagues showed that IHA adrenal glands presented a significantly higher number of APCC than normotensive controls. All IHA adrenal glands harbored at least one APCC positive for CYP11B2 or mAPA. Interestingly, in this cohort the authors identified somatic *CACNA1D* mutations in 58% of cases and a mutation in *KCNJ5* in one case. These data suggest that IHA may not only be due to hyperplasia but also to the accumulation and enlargement of APCC (191).

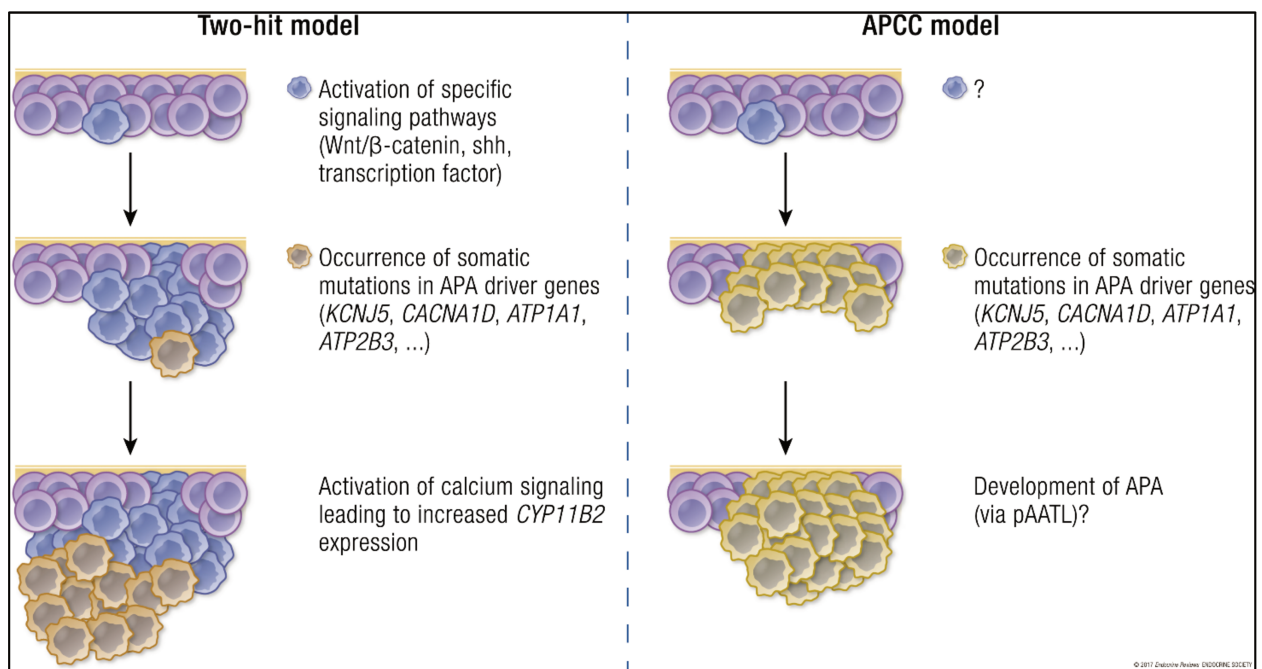


Figure 9. The current model for APA development. Left panel (Two-hit model): Two hit model of APA development, where activation of specific pathways such as Wnt pathway leads to increased cell proliferation, this creates a suitable environment for the occurrence of somatic mutations. Right panel (APCC model): In ZG cells, the occurrence of mutations in certain APA-driver genes would lead to the formation of APCC which produce aldosterone autonomously, APCC would eventually evolve into APA by passing through an intermediate phase of pAATL (126).

4.7.5 Genotype-phenotype correlations in patients with APA

It is important to unravel clinical and biochemical markers of PA, as this could be beneficial for the management of the disease. *KCNJ5* mutations, for example, seem to occur more frequently in women and younger patients and tend to be also associated with higher plasma levels of aldosterone, larger tumors and higher left ventricular mass index. *KCNJ5* mutations tend to be more prevalent than other mutations in Caucasians and much more in east Asian patients and this type of mutation has also been linked to a better outcome in younger patients with APA (125, 192-197). *CACNA1D* mutations on the other hand, have been associated with smaller APA (125). Williams and colleagues have performed a promising study that could be translated to clinical care, where they identified a 7-steroid fingerprint capable of predicting the underlying mutational status of APA with high accuracy through steroid profiling in adrenal and peripheral venous plasma samples by liquid chromatography-tandem mass spectrometry (132). Consistently with what was previously reported, *KCNJ5* mutations were associated with significantly higher levels of hybrid steroids like 18-hydroxycortisol and 18-oxocortisol compared to other genotypes.

5 Thesis project

5.1 Background

Arterial hypertension is a major cardiovascular risk factor that affects 25% of the global population. Primary aldosteronism (PA) is the most common form of secondary arterial hypertension, accounting for 5-10% of the hypertensive patients and up to 20% of patients with resistant hypertension (198). PA results from autonomous production of aldosterone by the adrenal cortex. This is associated to low renin levels and in some cases hypokalemia. The identification and diagnosis of secondary forms of arterial hypertension is of importance as this allows the adequate treatment and targeting of the underlying disease, and prevention of cardiovascular complications and organ damage. Despite our vast knowledge on PA, the disease remains poorly diagnosed due to the complexity of the process and the correct diagnosis is often concluded late, in some cases several years after the patients had been suffering from hypertension (20). Additionally, PA is associated with high cardiovascular complications such as myocardial infarction, heart failure and atrial fibrillation; more importantly, these events occur independently from high blood pressure, they are rather associated to deleterious effects of excess aldosterone on heart and vessels (19).

5.2 Rational of the study

In the last few years, major advances have been made in the comprehension of the genetics of APA. By whole exome sequencing, recurrent somatic mutations have been identified in genes that code for ion channels (*KCNJ5*, *CACNA1D*) and ATPase pumps (*ATP1A1*, *ATP2B3*). These genes regulate the intracellular ionic homeostasis and membrane potential. Somatic mutations lead to the activation of calcium signaling, which is the main trigger of aldosterone synthase expression. This ultimately leads to the autonomous production of aldosterone. Despite the fact that the role of these mutations in aldosterone biosynthesis is well established, their role in cell proliferation and nodule formation is far from being understood.

Our group has shown that the cortex of adrenals harboring APA undergoes significant remodeling, characterized by increased nodulation, diminished vascularization and ZG

hyperplasia (187). Our group has also shown that multinodular adrenals harbor different somatic mutations, suggesting a polyclonal cellular origin of these nodules (182). These findings suggest that somatic mutations could be a secondary event in an already disorganized adrenal cortex through mechanisms that are unknown to date.

5.2.1 Aims Part I

We aimed at identifying signaling pathways involved in nodule formation and adrenal cortex remodeling. To this end, we performed a large scale analysis integrating transcriptomic and histological analysis with clinical and biological data from patients with APA. This led to the identification of retinoic acid receptor α (RAR α) signaling as a molecular network involved in nodule formation. My objective was to identify the role of RAR α in the adrenal cortex development and function. To understand the role of RAR α , we studied the adrenal gland phenotype of Rar α KO mice, on a morphological, functional and molecular level.

5.2.2 Aims Part II

It has been clearly established that aldosterone and MR are both implicated in inducing structural deleterious modifications in different tissues (199). Our group has shown that MR is expressed in the adrenal cortex, specifically in the ZG and in APA (187). Given the role of MR in tissue remodeling and its expression in the ZG and APA, we hypothesized that aldosterone could be involved in the pathophysiology of APA through MR by an autocrine-paracrine mechanism. To investigate this hypothesis, we sought to identify the role of MR, in regulation of aldosterone production and cell proliferation in the adrenal cortex. To this aim, we have generated ZG-specific Mr knock-out mice and investigated their adrenal gland phenotype and function.

PART 1: ROLE OF RETINOIC ACID RECEPTOR ALPHA
SIGNALING IN THE ADRENAL CORTEX

6 THESIS WORK

6.1 The nuclear receptor superfamily of transcription factors

The nuclear receptor superfamily is the largest family of transcription factors in eukaryotes and regulates the expression of specific target genes. They regulate different physiological processes, such as development, inflammation, apoptosis, metabolism, cell differentiation. Nuclear receptors are zinc finger transcription factors which bind to specific response elements on DNA and act in a cell type-specific manner on regulatory regions of target genes. Although most nuclear receptors are activated by ligand binding, for some of them, the so called orphan receptors, the ligand is unknown. A few of them do not require ligand binding for their transcriptional activity (200). Nuclear receptors bind DNA either as homo- or heterodimers. Different dimerization patterns, as well as different ligands, modulate the transcriptional activity of nuclear receptors, by modulating transcriptional coregulatory recruitment and DNA binding site selection (201).

Nuclear receptors typically consist of a DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), a hinge region in between the DBD and LBD for some of them, and an N-terminal domain of variable length. The DBD consists of two well conserved zinc finger motifs rich in cysteine (202). These motifs interact with the double helix of DNA and have very important roles in determining the specificity of the nuclear receptor to a given DNA sequence (203). Among the known examples of zinc finger short protein motifs are the P-box which is present in nuclear receptors of the steroid receptor subfamily, such the mineralocorticoid receptor (MR), the glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR); this sequence allows these receptors to recognize their response element (AGAACA) (204). Another group of nuclear receptors also have a P-box which differs from the first group by two amino-acids, this motif facilitates the recognition of the AGGTCA sequence, which is an elementary sequence for receptors such as retinoic acid receptor alpha (RAR), retinoic X receptor (RXR), vitamin D receptor (VDR), thyroid hormone receptor (TR) and estrogen receptor (ER).

The LBD consists of eleven to twelve helices and two beta-sheets, which form a triple sandwich structure composed of three sheets of antiparallel helices which form a hydrophobic cavity for ligand binding within the core of the LBD (205). The binding of the ligand induces conformational changes that depend on the nature of the ligand. Agonists induce

conformational changes that promote receptor activation by generating interfaces for coactivator recruitment. On the other hand, antagonists induce a disruption in the structure of the LBD and disrupt the recruitment of transcriptional co-regulators (206).

The middle hinge region is thought to be a flexible region in between the DBD and LBD. The N-terminal hinge domain is involved in nuclear receptor dimerization, co-regulator recruitment, and binding site recognition (207). The C-terminal part of the hinge region is suggested to participate in the regulation of ligand interactions (208, 209).

The nuclear receptor superfamily consists mainly of four subclasses of receptors, which are separated based on their dimerization preferences and their DNA-binding properties. The first subfamily consists of receptors that form dimers with RXR, this group recognizes direct repeats as a response element. The second subfamily includes steroid receptors, these function as ligand induced dimers, and they recognize and bind to specific hormone response elements (HRE) which are mostly inverted palindromic repeats. The third subfamily of nuclear receptors bind as homodimers primarily to direct repeats. The fourth subfamily members bind as monomers to extended core sites.

6.2 Retinoic acid receptors

Retinoic signaling is mediated by two subfamilies of retinoic receptors, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), which act as RAR/RXR heterodimers or RXR/RXR homodimers (210). Each family of receptors consists of 3 subtypes : α , β and γ . Retinoid receptors share the common structure of most nuclear receptors, apart from the activation functions (AF domains); they have a well conserved DBD as well as a conserved LBD, which contains not only the ligand-binding pocket, but also the main dimerization domain and the ligand-dependent transactivation domain (211, 212). Retinoid receptors are primarily found in the nucleus bound to retinoic acid response elements (RARE). Whereas in the absence of a ligand, the DNA-bound receptors repress transcription due to recruitment of corepressors, following ligand binding the receptors dissociate from corepressors and recruit transcriptional coactivators and chromatin remodeling complexes; this complex will reorganize and decompact the chromatin and guide RNA polymerase II and other transcription factors to the promoter region of target genes (213-215) (Fig. 10). The main ligands of retinoid receptors are all-trans retinoic acid (ATRA or tretinoin), the abundant

natural retinoid, or its 9-cis homologue. RAR are activated by ATRA and 9-cis RA, while RXR are only activated by 9-cis RA. At high concentrations, ATRA (10^{-5} M) is converted to 9-cis RA (216). Retinoic acid receptors play roles in a variety of processes, notably, development, differentiation, proliferation and apoptosis; they also have the capacity to integrate and to cross-talk with other signaling pathways.

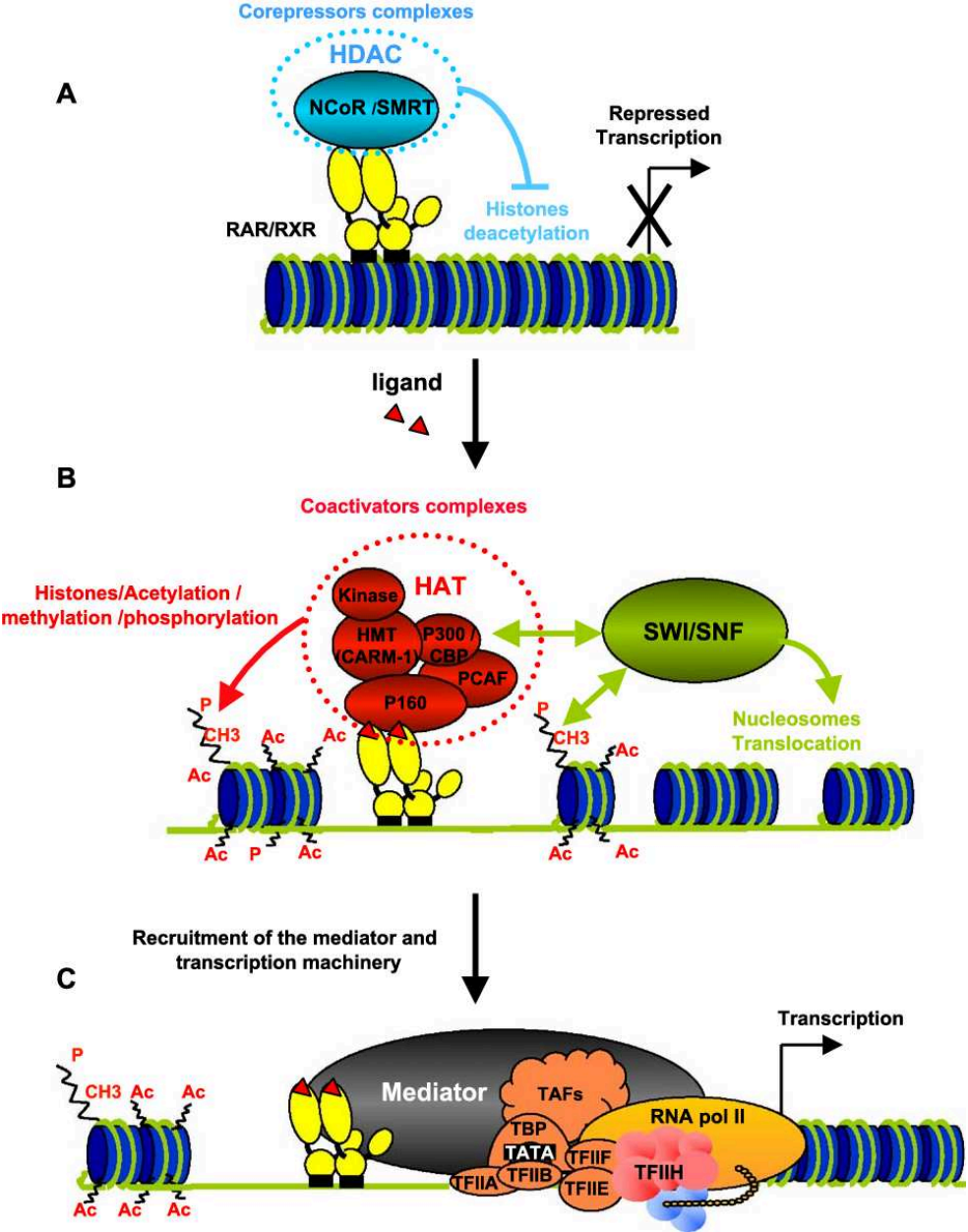


Figure 10. Three step genomic activity of RAR. A. In the absence of a ligand, retinoic receptors are bound to response elements on target genes and are associated to a corepressor complex (HDAC complex) which inhibits transcription. B. Retinoic acid binding leads to HDAC complex dissociation, this allows the recruitment of coactivator complexes and opens and decompresses the chromatin. C. The final step is the dissociation of the coactivator complex, and the recruitment of RNA polymerase II and other transcription factors, leading to the initiation of transcription (217).

A wide variety of experimental and clinical approaches have shown that Vitamin A (retinol) and its derivatives play a crucial role in embryonic development and organogenesis, metabolic homeostasis, proliferation, differentiation and apoptosis (218). Vitamin A deficiency leads to congenital ocular malformations, as well as alterations in the development of cardiac, respiratory and urogenital systems (219). Retinol is mainly stored in the liver, from there it is transported by the plasma retinol binding protein (RBP) and enters the cells via RBP membrane receptors. In the process of retinoic acid production, retinol undergoes a series of enzymatic steps. Retinol is first oxidized to retinal through a reversible step by retinaldehyde, then in an irreversible manner by retinaldehyde dehydrogenase to RA. RA is suggested to have a short half-life of 0.5 hr (220), suggesting that RA is produced at the site or near to the site of its action. RA is transported to the nucleus by the cellular RA-binding protein (CRABP) where it binds to its receptor (221). Retinoid signaling sometimes in a non-genomic manner, interacting with other signaling pathways such as AP1, NF κ B, TGF- β , PI3K and intracellular calcium signaling, cAMP, VEGF and Wnt; these interactions in addition to RAR ubiquitous expression support the involvement of RAR in different processes in the organism as early as the embryonic stages (222-229). RXR interacts and forms dimers not only with RAR, but also with a big variety of nuclear receptors such as peroxisome proliferator activated receptor (PPAR), vitamin D receptor (VDR), constitutive androstane receptor (CAR), farnesoid x receptor (FXR) and liver x receptor (LXR) (230-236).

Different knockout models of mice have been generated to understand the roles of retinoid receptors in the organism. RAR β KO mice are growth deficient and exhibit abnormalities in their eyes and cervical vertebrae and motor and locomotion coordination (237). RAR γ KO mice have growth deficiency, skeletal and epithelial defects and male sterility (238). RXR α KO is lethal for the mice in the fetal period due to underdeveloped myocardium (239). It was shown that RXR α mediates the teratogenic effects of retinoids, since mouse embryos lacking RXR α were immune to retinoic acid induced limb defects (240). RXR β KO led to the death of almost half of the embryos in utero, with males being sterile due to testicular defects (241). However, mice with impaired RXR β function, due to a defect in the transcription activation function 2 (AF-2), do not display the spermatid defects observed in RXR β KO mice. These mice do, however, have abnormal lipid metabolism in Sertoli cells, suggesting the existence of a functional interaction between RXR β and other nuclear receptors which control lipid metabolism (242). RXR γ mice on the other hand, are fertile, but show high serum levels of

thyroid stimulating hormone (TSH) and L-thyroxine (T4) as well as high metabolic rates compared to control mice (243).

RAR α , encoded by the *NR1B1* gene, was the first member of the family to be cloned in 1987. It was found to be induced by retinoic acid and to resemble to other nuclear receptors for steroid and thyroid hormones and vitamin D3 (244). RAR α has the uniqueness among other RARs that it is ubiquitously expressed in early and adult life, especially the more abundant isoform RAR α 1, whose promoter region resembles the promoter region of a housekeeping gene, in contrast to the more restricted transcript levels of the RA-inducible isoform RAR α 2 (245-248). RAR α shows a high degree of conservation between humans and mice (249).

RAR α - and RAR α 1- null mice were generated in the laboratory of P. Chambon. RAR α 1 KO mice appeared to be normal, possibly due to compensation by other isoforms of the receptor. However, homozygous RAR α complete KO mice, were sterile due to testicular abnormalities, including degeneration of the germinal epithelium of the testes. Moreover, the seminiferous tubules were atrophic and did not carry spermatozooids, suggesting that RAR α KO disrupted spermatogenesis in these mice. Although KO mice represented 25% of the embryos and fetuses during gestation or after cesarean delivery, more than 90% of homozygous mice died before the age of 2 months apparently due to unexplained cannibalism by their mothers. These mice also present webbed-digits which was restricted to soft bones, they were emaciated and lethargic and showed slower growth rates than their WT and heterozygote littermates. RAR α KO mice also present impaired alveolar formation, homeotic transformations, malformation of the vertebrae and the squamosal bone and laryngeal cartilage and pterygoquadrate cartilage (250).

RAR α , like other retinoid receptors, has been suggested to have different functions. Besides its heterodimerization with RXR after ATRA binding, it has also been shown to heterodimerize with ER α in an ATRA-independent manner. This could explain the role of RAR α in breast cancer and the inhibitory activity of RAR α on the growth of breast cancer cells; it has also been shown that RAR α is a target gene of ER α (251, 252). RAR α has also been shown to interact with mRNA in the cytosol of neuronal cells, where it is able to control translation, and thus is implicated in spinal formation following activation with ATRA independently from transcriptional regulation (253).

RAR α was also found to be expressed on cell membrane lipid rafts where it forms complexes with G α q proteins; in RA-resistant breast cancer cells, the formation of RAR α /G α q complexes is suppressed. The activity of this complex is involved in rapid non genomic effects such as the induction of the activity of p38MAPK/MSK1 pathway. Interestingly, the activation of this pathway induces the phosphorylation of RAR and the expression of their target genes. These effects occur rapidly, indicating a non-genomic effect. The reason why RAR α is the only subtype of RAR that is expressed on the cellular membrane was attributed to its N-terminal domain, which is not conserved across RAR and appears to be necessary for targeting RAR α to lipid rafts (254).

RAR have been shown to be involved in tumorigenesis and cancer. In skin cancer, transcript levels of RAR α and RAR γ are decreased in mice and their mRNA levels are significantly reduced in human squamous cell carcinomas (255, 256). The *RAR α* gene has been shown to be susceptible to form fusions with various genes, such as in acute promyelocytic leukemia (APL), which accounts for up to 10 to 15 % of acute myeloid leukemia (AML) cases. APL is sensitive to ATRA, which induces the differentiation of immature leukemic promyelocytes into mature granulocytes. In most cases of APL, a fusion gene is created between RAR α and the promyelocytic leukemia protein (PML) (257). Other genes that have been reported to form fusion proteins with *RAR α* are *ZBTB16*, *NPM1*, *NUMA1*, *STAT5B*, *BCL6*, *PRKARIA* (258). The resulting fusion proteins behave as transcriptional repressors of retinoic acid signaling, thus inhibiting the differentiation of promyelocytes. However, treatment with ATRA triggers the differentiation into granulocytes in most patients (259, 260).

Interestingly, it has been shown that ATRA induces angiogenesis and tube formation via RAR through enhancement of VEGF signaling, indicating that RAR can interact with VEGF signaling and induce vascularization (228).

RAR α as well as other receptors of retinoic signaling, have also been shown to interact with Wnt signaling. In one study on embryonic stem cells (ESC), RA treatment induced the activity of the non-canonical Wnt signaling pathway, resulting in the suppression of the canonical pathway (261) (Fig. 11). RAR α has also been shown to interact with Wnt signaling in esophageal carcinoma, where RAR α is highly expressed. Knockout of RAR α in an esophageal carcinoma cell line activated GSK3 β , part of the destruction complex involved in Wnt signaling, leading to an increase in the degradation of β -catenin (262). Other studies showed that GSK3 β , in turn, phosphorylates RAR α in a way that resembles a negative

feedback, where the treatment combination of ATRA and GSK3 β inhibitors ameliorate the prognosis of the treatment in acute myeloid leukemia (263, 264).

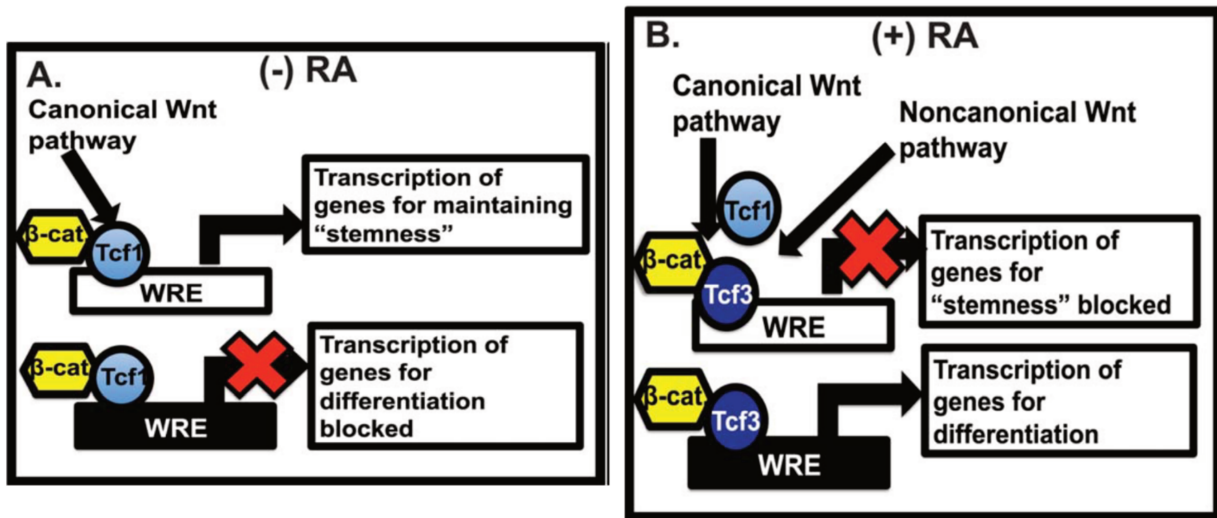


Figure 11. Effect of activation of retinoic acid signaling on the Wnt signaling pathway. A. In the absence of RA signaling, canonical Wnt signaling is activated. B. When RA signaling is active, it promotes the activity of non-canonical Wnt signaling; in turn, non-canonical Wnt signaling inhibits the activity of the canonical Wnt pathway by reducing the transcription of canonical pathway target genes and transcription factors (261).

Intriguingly, a proteomic characterization of adrenal glands in mice over different time points during embryonic life and at the first postnatal day showed that retinoic acid signaling components, notably involved in retinol binding (Rbp1, Crabp1 and Crabp2) are decreased during development of the adrenal gland. This suggests that these components are important for the early development of the adrenal gland but possibly less involved during postnatal life. This study also showed that proteins involved in fatty acid binding and cholesterol transporter activity (including Star) were increased over the course of adrenal gland development (265).

6.3 Results

Role of retinoic acid receptor α (RAR α) in adrenal cortex development and function

Article: Retinoic acid receptor α as a novel contributor to adrenal cortex structure and function through interactions with Wnt and Vegfa signalling

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Retinoic acid receptor α as a novel contributor to adrenal cortex structure and function through interactions with Wnt and Vegfa signalling

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Primary aldosteronism (PA) is the most frequent form of secondary arterial hypertension. Mutations in different genes increase aldosterone production in PA, but additional mechanisms may contribute to increased cell proliferation and aldosterone producing adenoma (APA) development. We performed transcriptome analysis in APA and identified retinoic acid receptor alpha (RAR α) signaling as a central molecular network involved in nodule formation. To understand how RAR α modulates adrenal structure and function, we explored the adrenal phenotype of male and female Rar α knockout mice. Inactivation of Rar α in mice led to significant structural disorganization of the adrenal cortex in both sexes, with increased adrenal cortex size in female mice and increased cell proliferation in males. Abnormalities of vessel architecture and extracellular matrix were due to decreased Vegfa expression and modifications in extracellular matrix components. On the molecular level, *Rar α* inactivation leads to inhibition of non-canonical Wnt signaling, without affecting the canonical Wnt pathway nor PKA signaling. Our study suggests that Rar α contributes to the maintenance of normal adrenal cortex structure and cell proliferation, by modulating Wnt signaling. Dysregulation of this interaction may contribute to abnormal cell proliferation, creating a propitious environment for the emergence of specific driver mutations in PA.

Primary aldosteronism (PA) is the most common and curable form of secondary arterial hypertension, with prevalence estimations of up to 10% of cases in referred hypertensive patients, 4% of patients in primary care^{1,2} and 20% of patients with resistant hypertension^{3,4}. Rapid diagnosis and treatment are important to prevent severe cardiovascular consequences of long term aldosterone exposure, which are independent of blood pressure levels and are due

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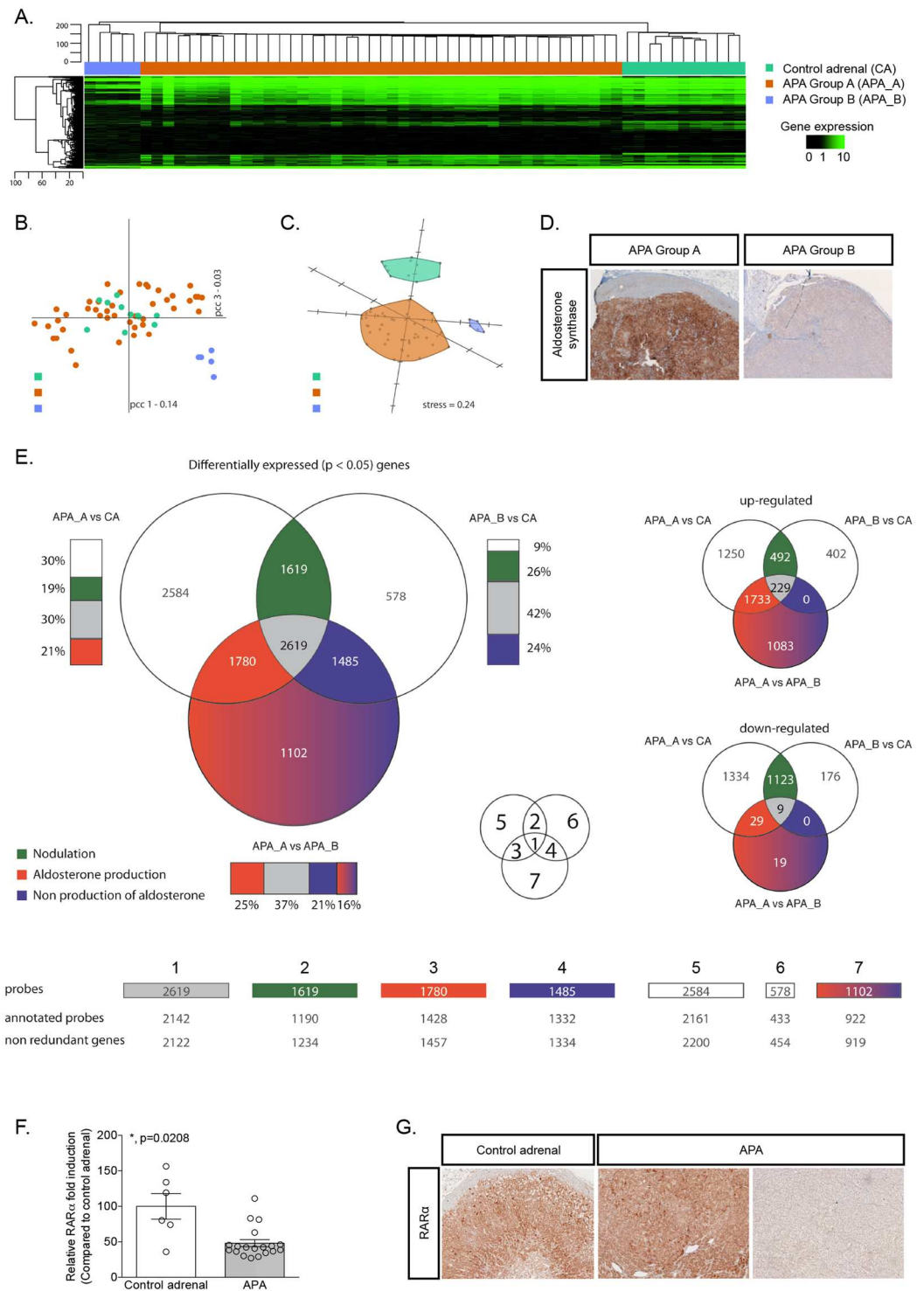


Figure 1. Transcriptome analysis reveals two distinct subgroups of APA. **(A)** Hierarchical clustering using Euclidean distance and complete linkage of 48 APA and 11 control adrenals. Heat-map of the whole gene expression is displayed using black for low expression and green for high expression. **(B)** Principal Component Analysis in the correlation space over the entire transcriptome. The first two principal components and their associated inertia are displayed. **(C)** Singular-value-decomposition-initialized multidimensional scaling under the correlation space over all genes^{86,87}. Kruskal error is indicated as “stress”. **(D)** Aldosterone synthase immunohistochemistry performed on adrenals from APA_A and APA_B. **(E)** Three groups (APA_A, APA_B and CA) are pair-wise compared to obtain a list of statistically significantly expressed candidate genes for each of the three comparisons. The common and comparison-specific genes are classified in seven different groups some of which are associated to a biological process. Venn-diagrams are displayed for statistically significantly differentially expressed candidate genes. The smaller Venn diagrams on the right-hand side of the figure represent the overlap for up-regulated and down-regulated genes. Bars are displayed next to the represented

sets to indicate the relative proportion of the overlapping regions to the corresponding list of candidate genes. Figures displayed are the number of genes that populate each of the selected sets. (F) Expression of *RAR* α was investigated by RT-qPCR on mRNA extracted from 6 control adrenals and 19 APA. Values are presented as the mean \pm SEM; p values were calculated using a two-sided Mann-Whitney test. * $p < 0.05$. (G) *RAR* α immunohistochemistry performed on control adrenal and in APA.

to deleterious cardiovascular effects of the hormone^{5,6}. Indeed, patients with PA have an increased risk of developing stroke, coronary artery disease, atrial fibrillation, left ventricular hypertrophy, and heart failure^{2,5,7–9}. In the majority of cases, PA is due to an aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH)¹⁰. Patients with PA present hypertension, a high aldosterone to renin ratio and variable hypokalemia¹¹. Over recent years, while exome sequencing (WES) unraveled different somatic mutations in genes encoding ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*)^{12–15} in about 50% of APA¹⁶. Recently, use of aldosterone synthase immunohistochemistry-guided next-generation sequencing identified somatic mutations in up to 88% of APA¹⁷. In physiological conditions, these channels and pumps play an important role in regulating intracellular ion homeostasis as well as maintaining cell membrane potential. When mutated, they induce cell membrane depolarization leading to opening of voltage-gated calcium channels, or directly affect intracellular calcium concentrations. This ultimately leads to activation of calcium signaling, the main trigger of aldosterone production. Although the link between mutations and aldosterone production has been clearly established, their role in promoting abnormal cell proliferation and/or APA development is not well understood. Activating mutations in the *CTNNB1* gene (encoding β -catenin) were also identified in 2–5% of APA^{18,19}, and the Wnt/ β -catenin signaling pathway has been shown to be constitutively active in ~70% of APA^{20,21}. This signaling pathway plays an important role in the development of the adrenal cortex and in aldosterone biosynthesis²². Recent studies have suggested a two-hit mechanism of APA development, whereby a first hit induces adrenal cortex remodeling and/or increases nodule formation and a second hit, involving mutations in APA driver genes, specifies the hormonal secretory pattern^{23,24}.

In mice, the adrenal cortex is composed of two distinct functional zones, the zona glomerulosa (ZG) and the zona fasciculata (ZF), with different functions. The ZG is located under the capsule and produces mineralocorticoids that play a major role in the regulation of blood pressure by regulating sodium and potassium homeostasis. The ZF produces glucocorticoid hormones that are involved in stress response and energy homeostasis. Adrenal cortex undergoes continual renewal, with stem/progenitor cells that first differentiate into ZG cells and then migrate centripetally acquiring ZF cells characteristics²⁵. Different studies report sexual dimorphism in mouse adrenal cortex, with adrenals being larger in females than in males and plasma ACTH, corticosterone and aldosterone levels being higher²⁶. At the transcriptome level, a core sexually dimorphic expression program has been identified²⁶. Moreover, sexual dimorphism in the adrenal cortex pathophysiology has been reported in several genetically modified mouse models^{21,27–29}. Interestingly, adrenal cortex renewal has been recently shown to be 3-fold faster in females than in males, highlighting the role of sex hormones in this process³⁰.

Here we have performed a large-scale study integrating transcriptome, histological and immunohistological analyses with clinical and biological information of patients with APA to better understand the mechanisms involved in increased cell proliferation in BAH and APA development and to identify specific signaling pathways responsible for abnormal cell proliferation and nodule formation. We identified retinoic acid receptor (*RAR*) α signaling as a central molecular network involved in nodule formation. Analysis of the adrenal phenotype of mice lacking *Rar* α revealed structural and functional disorganization of the adrenal cortex at 12 weeks of age, which was associated with modifications of the extracellular matrix and vessel architecture in both male and female mice. This was accompanied by increased adrenal cortex weight in female, and increased cell proliferation in male mice. In males, morphological abnormalities were associated with alterations in non-canonical Wnt signaling as well as reduced expression of steroidogenic genes, without modifications in canonical Wnt signaling nor PKA signaling. Abnormalities of vessel architecture and extracellular matrix were due to decreased *Vegfa* expression and modifications in extracellular matrix components. With aging, adrenal cortex disorganization was still present, but without evidence of modification of Wnt signaling and *Vegfa* or steroidogenic gene expression. In female *Rar* α ^{-/-} mice, morphological abnormalities of the adrenal cortex were also present at both ages. However, even at 12 weeks, no alterations in Wnt signaling and *Vegfa* expression were observed, suggesting that molecular abnormalities may have occurred at an earlier time point in adrenal cortex development. Our study suggests that *Rar* α contributes to the maintenance of normal adrenal cortex structure and cell proliferation, by modulating Wnt signaling. Dysregulation of this interaction may contribute to abnormal cell proliferation, creating a propitious environment for the emergence of specific driver mutations in PA.

Results

Decrease of retinoic acid receptor α expression is associated with nodulation in APA. We compared the transcriptome profile of 48 APA and 11 control adrenals. Unsupervised clustering revealed the presence of at least three different groups of samples (Fig. 1A). The first one being composed exclusively of control adrenals, whereas the 48 APA were subdivided into two groups of 43 and 5 samples defined as APA group A (APA_A) and APA group B (APA_B), respectively. The APA_B is spatially separated from the rest of the samples (Fig. 1B), and from this perspective control adrenals seem to be very similar to samples in APA_A. However, using the first three dimensions of a Principal Component Analysis (PCA), APA_A are clearly separated from control adrenals and from APA_B (Fig. 1C). By comparing clinical and biochemical characteristics of patients from APA_A and APA_B, we found no difference between groups except for the tumor size [15 mm (9–18) for APA_A versus 40 mm (32–52.5) for APA_B (Table 1)]. Interestingly, another major difference characterizing these two groups of tumors was the level of expression of *CYP11B2* (aldosterone synthase) detected by immunohistochemistry,

	Group A	Group B	P value
Gender (male/female), %	51/49	40/60	1
Age at PA dg (ys)	39 (33, 44.5)	43 (36.5, 51)	0.29
Lowest plasma K (mmol/l)	3.2 (2.9, 3.5)	3.1 (3.0, 3.55)	0.78
Systolic BP (mmHg)	150 (135, 167)	154 (143, 175)	0.49
Diastolic BP (mmHg)	90 (82, 99)	95 (85, 102)	0.56
Treatment score (n)	2 (1, 3.5)	1 (0, 2)	0.07
Urinary aldosterone (nmol/24 h)	95 (76, 126)	115 (87.5, 153.5)	0.57
Plasma aldosterone (pmol/l)	845 (508, 1127)	1168 (633.5, 1797)	0.32
Plasma renin (mU/l)	2.2 (1.4, 4.3)	1.7 (1.2, 3.4)	0.42
ARR (pmol/mU)	271.8 (179.8, 602.5)	831.0 (385.5, 1152)	0.16
Adenoma size (mm)	14.5 (9.0, 18.0)	40 (32.0, 52.5)	<0.0001
Systolic BP at FU (mmHg)	131 (120, 149)	116 (112, 142)	0.31
Diastolic BP at FU (mmHg)	84 (80, 96)	73 (72, 94)	0.34
Treatment score at FU (n)	0 (0, 2)	0 (0, 2)	0.63
Plasma K at FU (mmol/l)	4.1 (3.8, 4.3)	4.0 (3.6, 4.2)	0.45
Plasma aldosterone at FU (pmol/l)	130 (85, 251)	74 (36, 200)	0.15
Plasma renin at FU (mU/l)	11.0 (6.8, 17.2)	9.0 (7.9, 101.3)	0.80
ARR at FU (pmol/mU)	14.1 (7.0, 17.8)	9.0 (7.9, 101.3)	>0.99

Table 1. Clinical and biochemical correlates of patients from group A and B. PA: primary aldosteronism; HTN: hypertension; K: potassium; BP: blood pressure; ARR: aldosterone to renin ratio; FU: follow-up. Categorical data reported as number (percentage) and compared with Fischer's exact test; quantitative data reported as median (interquartile range) and compared with the Mann-Whitney test (p significant if <0.05).

which was higher in APA_A compared to APA_B (Fig. 1D). The three groups were pairwise compared to obtain a list of statistically significantly differentially expressed genes for each of the three comparisons (Supplementary Tables 1–3). The common and comparison-specific genes were then classified into 7 different groups, some of which were associated to a specific biological process (Fig. 1E and Supplementary Fig. 1; Supplementary Tables 4–10). In particular, we made the hypothesis that genes commonly differentially expressed in APA_A (high aldosterone production) and APA_B (low aldosterone production) compared to control adrenals would be involved in nodulation (Fig. 1E, subgroup 2) rather than steroid biosynthesis. Within this group, the expression of retinoic acid receptor α ($RAR\alpha$) was significantly down regulated in APA ($\log Q[APA_A \text{ vs } CA] -3.8852$; $\log Q[APA_B \text{ vs } CA] -3.0056$). RT-qPCR performed on 6 control adrenals and 19 APA confirmed the down-regulation of $RAR\alpha$ mRNA expression in APA (Fig. 1F). Immunohistochemistry showed that $RAR\alpha$ was expressed throughout the adrenal cortex, and to a lesser extent in the medulla; its expression was heterogeneous in APA (Fig. 1G). Our transcriptome data also revealed that all the enzymes necessary for the biosynthesis of RA are expressed in control adrenal cortex as well as in APA (Table 2), supporting the possibility of RA biosynthesis and action in the adrenal gland. Modulation of $RAR\alpha$ target genes was investigated by retrieving, from our transcriptome data, the expression of 511 genes described as $RAR\alpha$ target genes and expressed in humans³¹ (Supplementary Table 11). Among them, 147 were found to be differentially expressed between APA_A and control adrenals (67 were up-regulated and 80 down-regulated) and 123 between APA_B and control adrenals (10 up-regulated and 113 down-regulated).

Significant disorganization of the adrenal cortex in 12 weeks old male and female $Rar\alpha^{-/-}$ mice.

To evaluate the role of $Rar\alpha$ in adrenal cortex structure and function, we compared the adrenal phenotype of 12 weeks old male and female $Rar\alpha^{+/+}$ and $Rar\alpha^{-/-}$ mice. As previously described³², the $Rar\alpha^{-/-}$ mice exhibited growth retardation characterized by lower total body weight (Table 3). Relative adrenal weight of $Rar\alpha^{+/+}$ and $Rar\alpha^{-/-}$ was similar in male mice, while it was significantly increased in female $Rar\alpha^{-/-}$ mice (Table 3) compared to wild type littermates. HES staining revealed marked disorganization of the adrenal cortex, characterized by a loss of the radial organization of the zona fasciculata (ZF) in both male and female mice (Fig. 2A and Supplementary Fig. 2), with a conserved zona glomerulosa (ZG). Confirming the HES observation, the expression of Disabled-2 ($Dab-2$), a marker of ZG³³, was not affected in $Rar\alpha^{-/-}$ mice (Fig. 2A,B), indicating that only the ZF was affected in both males and females. We also investigated the expression of 20- α HSD, a marker of the fetal X-zone³⁴, which regresses at puberty in males and after the first gestation in females. No modification of 20- α HSD expression was observed in $Rar\alpha^{-/-}$ mice compared to wild type littermates, with the X-zone being visible in females but not in males in both genotypes (Supplementary Fig. 3), suggesting that the development and the regression of the fetal X-zone was unaffected. Total number of nuclei in adrenal cortex, indirectly reflecting adrenal cortex size, was significantly higher in 12 weeks old male and female $Rar\alpha^{-/-}$ mice (Fig. 2C). In males, this was associated with an increased proliferation, highlighted by an increase of Ki67 positive nuclei in the adrenal cortex in $Rar\alpha^{-/-}$ mice, (Fig. 2A,D). Although the proliferative index was not changed (Fig. 2A,D) in 12 weeks old female $Rar\alpha^{-/-}$ mice, the adrenal weight was significantly increased (Table 3) in addition to ZF expansion, suggesting that increased proliferation may have occurred at an earlier time point during development.

Gene name	Control adrenal	APA	p
Retinol Binding Protein 1	56.16 ± 7.30	81.77 ± 6.934	0.0673
Stimulated by Retinoic Acid 6	1.11 ± 0.13	1.485 ± 0.1561	0.3187
Retinol Dehydrogenase 5	1.05 ± 0.22	0.8240 ± 0.0730	0.2858
Retinol Dehydrogenase 11	19.30 ± 2.21	22.07 ± 1.466	0.2179
Retinol Dehydrogenase 12 (probe 2)	1.81 ± 0.35	5.704 ± 2.323	0.6776
Retinol Dehydrogenase 14	2.54 ± 0.32	3.914 ± 0.2692	0.0058
Aldehyde dehydrogenase 1 Family Member A1	24.28 ± 2.63	22.47 ± 2.589	0.3540
Aldehyde dehydrogenase 1 Family Member A3	2.72 ± 0.33	1.098 ± 0.1432	<0.0001
Cytochrome P450 Family 26 Subfamily B Member 1	1.69 ± 0.37	0.8811 ± 0.1179	0.0189
Cellular Retinoic Acid Binding Protein 2	1.04 ± 0.31	1.076 ± 0.0825	0.2858

Table 2. Expression of enzymes responsible for retinoic acid biosynthesis in control adrenals (n = 11) and in APA (n = 48). Data are presented as mean ± SEM and are compared with the Mann-Whitney test or t-test (p significant if <0.05). Data are expressed as log2.

	Male					
	12 weeks			52 weeks		
	<i>Rarα</i> ^{+/+}	<i>Rarα</i> ^{-/-}	p	<i>Rarα</i> ^{+/+}	<i>Rarα</i> ^{-/-}	p
Total body weight (g)	28.12 ± 0.59	26.45 ± 0.70	0.0135	40.87 ± 1.31	31.53 ± 0.94	<0.0001
Left adrenal weight/total body weight (x1000)	1.531 ± 0.112	1.678 ± 0.144	0.7584	1.135 ± 0.507	1.458 ± 0.817	0.2736
Right adrenal weight/total body weight (x1000)	1.362 ± 0.099	1.161 ± 0.137	0.1112	1.173 ± 0.684	1.460 ± 0.687	0.1354
Left adrenal size (mm)	N.D.	N.D.		2.130 ± 0.124	2.080 ± 0.073	0.6663
Right adrenal size (mm)	N.D.	N.D.		2.015 ± 0.068	2.069 ± 0.066	0.7756
	Female					
Total body weight (g)	20.49 ± 0.45	19.48 ± 0.55	0.1612	29.97 ± 1.75	23.25 ± 0.71	0.0005
Left adrenal weight/total body weight (x1000)	2.044 ± 0.091	2.474 ± 0.188	0.0332	1.912 ± 0.164	1.991 ± 0.130	0.7108
Right adrenal weight/total body weight (x1000)	1.623 ± 0.097	2.116 ± 0.126	0.0036	1.506 ± 0.105	1.784 ± 0.162	0.1598
Left adrenal size (mm)	N.D.	N.D.		2.568 ± 0.173	2.463 ± 0.085	0.6095
Right adrenal size (mm)	N.D.	N.D.		2.430 ± 0.156	2.260 ± 0.132	0.3524

Table 3. Clinical characteristics of 12 and 52 weeks male and female *Rarα*^{+/+} and *Rarα*^{-/-} mice. N.D., Not Determined. Quantitative data reported as mean ± SEM and compared with the Mann-Whitney test (p significant if <0.05).

Aldosterone synthase expression was found to be restricted to the ZG in both *Rarα*^{+/+} and *Rarα*^{-/-} male and female mice (Fig. 3A). Interestingly, in both sexes, the localization of 11β-hydroxylase was not modified, despite ZF disorganization (Fig. 3A). However, the expression of genes coding for steroidogenic enzymes, including *Star* (Fig. 3B), *Cyp11a1* (Fig. 3C), and *Cyp11b1* (Fig. 3D) was reduced in 12 weeks male *Rarα*^{-/-} mice, while the expression of *Cyp11b2* was not significantly affected (Fig. 3E). Plasma aldosterone (Fig. 3F) and corticosterone levels (Fig. 3G), plasma renin concentration (Fig. 3H) and aldosterone to renin ratio (Fig. 3I) were not different in 12 weeks male *Rarα*^{-/-} mice.

Modification of vessel architecture and extra cellular matrix composition in *Rarα*^{-/-} mice. To gain mechanistic insight into the activity of *Rarα* in the adrenal cortex, microarray analysis was performed on adrenals from four *Rarα*^{+/+} and four *Rarα*^{-/-} male mice. Hierarchical clustering allowed separating three out of four *Rarα*^{-/-} mice from WT mice (Fig. 4A,B). Among the 243 statistically significantly differentially expressed genes, 184 genes (75.72%) were found to be upregulated and 59 (24.28%) down-regulated. Gene Ontology analyses allowed the identification of specific molecular functions and biological processes enriched in 12 weeks *Rarα*^{+/+} versus *Rarα*^{-/-} mice (Supplementary Fig. 2), which are mainly involved in specific metabolic processes, such as lipid, carboxylic acid or fatty acid metabolism. Interestingly, among the differentially regulated genes, *Vegfa* mRNA expression was found to be downregulated in *Rarα*^{-/-} mice compared to *Rarα*^{+/+} mice (Fig. 4B, Supplementary Table 12). The down-regulation of *Vegfa* mRNA expression was confirmed by RT-qPCR in male mice only (Fig. 4C). The expressions of *Vegfc*, involved in angiogenesis and lymphangiogenesis, and of *Hif1α*, a marker of hypoxia, were unchanged (Fig. 4D,E). Sirius red staining, performed in male mice, revealed dilatation of vessels (Fig. 4F). In addition, podocalyxin staining of endothelial cells revealed disorganization of vessel architecture in both male and female *Rarα*^{-/-} mice (Fig. 4F). Expression of fibronectin 1, microfibrillar associated protein 2 and 5 (*Mfap2* and *Mfap5*) and of collagen 3α1, all components of the extracellular matrix (ECM), was found to be up-regulated in male *Rarα*^{-/-} mice (Supplementary Table 12). To evaluate if *Rarα* inactivation alters ECM structure, laminin β1 staining was performed. Laminin β1, a component of the basement membrane of the ECM, was expressed homogeneously throughout the adrenal cortex in both male and female *Rarα*^{+/+} mice

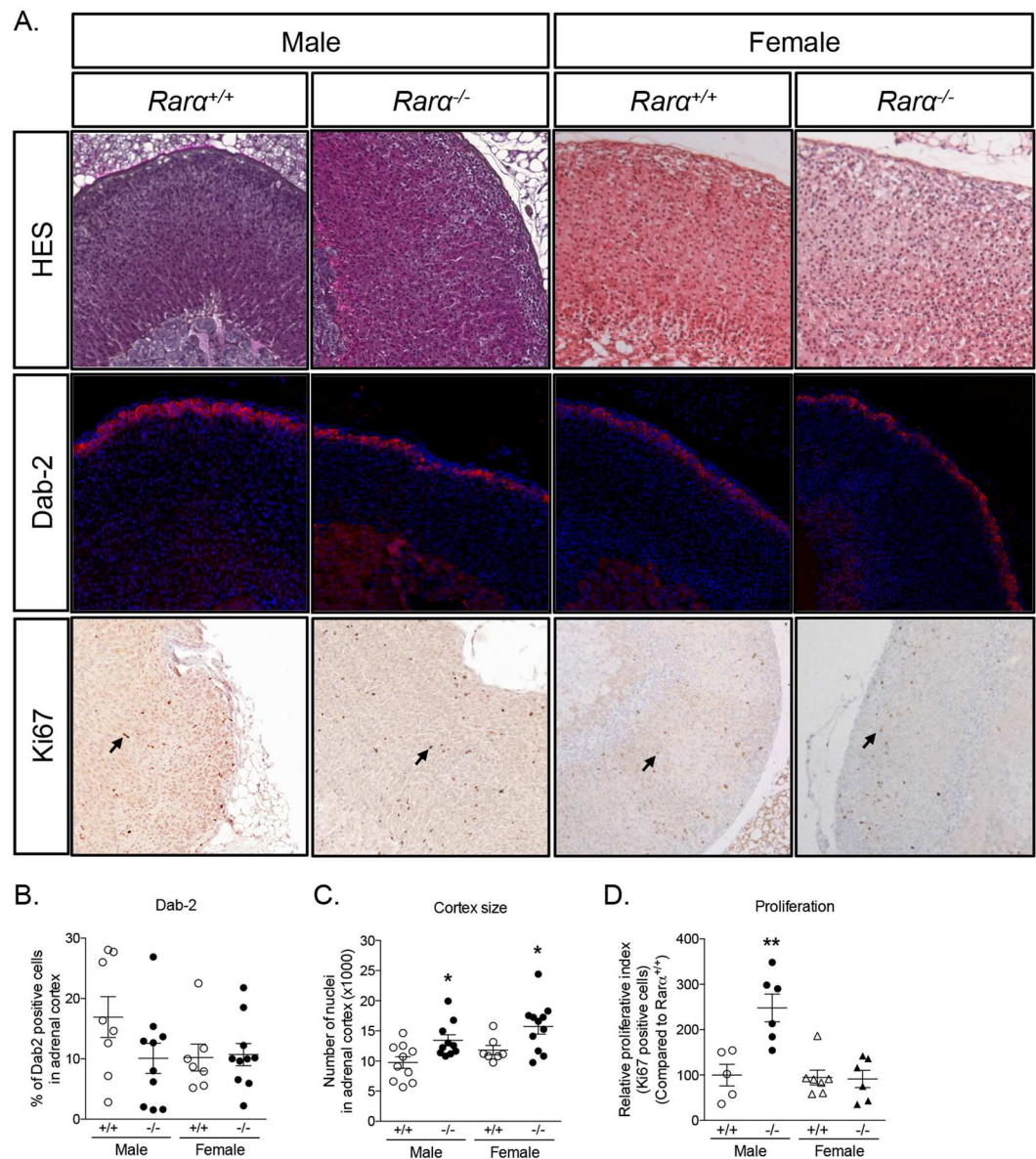


Figure 2. *Rarα* affects adrenal cortex morphology in 12 weeks old male and female mice. (A) Morphological characterization of adrenals from 12 weeks old *Rarα*^{+/+} and *Rarα*^{-/-} mice. HES staining, Dab-2 immunofluorescence and Ki67 immunohistochemistry were performed on adrenal sections from the indicated group of mice. (B) Number of Dab-2 positive cells in the cortex was determined in 7 to 11 animals of each genotype and sex using an automated molecular imaging platform (Vectra, Perkin Elmer) and is expressed as a percentage of total number of cells in the entire cortex area. (C) Number of nuclei in the adrenal cortex was determined in 7 to 11 animals of each genotype and sex using an automated molecular imaging platform (Vectra, Perkin Elmer). (D) Relative proliferative index of adrenals from male and female *Rarα*^{+/+} and *Rarα*^{-/-} mice. Ki67 positive cells were separately counted in the adrenal cortex in 5–6 animals per genotype and age. Values are presented as means ± SEM. **p*<0.05; ***p*<0.01.

(Fig. 4F). In *Rarα*^{-/-} mice, expression of laminin β1 showed a gradient, with higher expression in the ZG than in the inner part of the ZF in male but not in female mice (Fig. 4F). Since expression of laminin β1 was not modified in microarray analyses, this suggests that modification of the expression of other components of the ECM may alter proper laminin β1 localization.

Inhibition of Wnt signaling in young *Rarα*^{-/-} male mice. Wnt/β-catenin and PKA signaling are central regulators of adrenal cortex development and function, with a fine equilibrium between the activation of Wnt/β-catenin pathway in the ZG and of PKA signaling in the ZF. To gain mechanistic insight into the changes responsible for structural modifications of the adrenal cortex in *Rarα*^{-/-} mice, we thus explored Wnt/β-catenin signaling (Fig. 5). Whereas in female *Rarα*^{+/+} animals, β-catenin expression was restricted to the ZG with membranous, cytoplasmic and nuclear localization, in male mice low expression was also detected in the external part

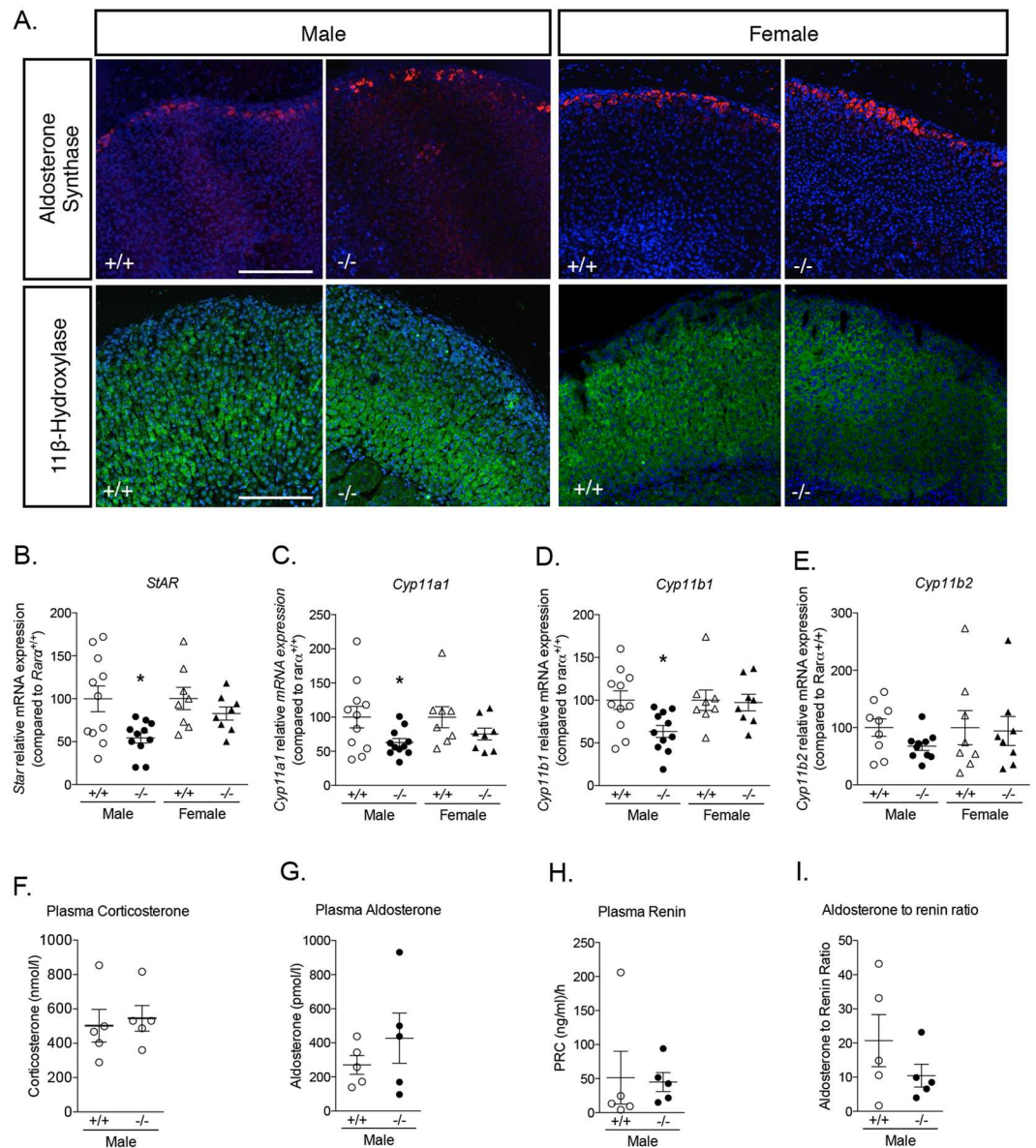


Figure 3. Impact of *Rar α* inactivation on adrenal steroidogenesis. (A) Expression of aldosterone synthase and 11 β -hydroxylase in 12 weeks old male and female *Rar α ^{+/+}* and *Rar α ^{-/-}* mice. (B–F) Expression of steroidogenic genes in male and female *Rar α ^{+/+}* and *Rar α ^{-/-}* mice. mRNA expression of *Star* (B), *Cyp11a1* (C), *Cyp11b1* (D) and *Cyp11b2* (E) was assessed by RT-qPCR. RT-qPCR were performed on mRNA extracted from 6–11 adrenals from 12 weeks old male and female *Rar α ^{+/+}* and *Rar α ^{-/-}* mice. (F,G) Measure of plasma aldosterone (F) and corticosterone (G) concentration by mass spectrometry in male mice. (H,I) Plasma renin concentration (PRC) (H) and aldosterone to renin ratio (I). Measure of plasma aldosterone, plasma corticosterone and plasma renin were done on 5–6 animals per group. Values are presented as the mean \pm SEM; p values were calculated using a Mann-Whitney test or *t*-test. For correlation, Pearson calculations were performed. *p < 0.05; **p < 0.01.

of the ZF (Fig. 5A). In male *Rar α ^{-/-}* mice, high expression of β -catenin was observed in the outer layer of the adrenal cortex, extending into the ZF (Fig. 5A); the localization of β -catenin was unaffected in female *Rar α ^{-/-}* mice (Fig. 5A). Western blot performed on whole adrenals showed that the expression of β -catenin was not significantly increased in male and female *Rar α ^{-/-}* mice compared to wild type littermates (Fig. 5B,C), although a large variation of its expression was observed in male *Rar α ^{-/-}* mice (Fig. 5C) consistent with the extended localization observed in some animals. β -catenin phosphorylation is a marker of its activation state; phosphorylation on inactivating (T41/S45) (Fig. 5D) or activating (S552, S675) (Fig. 5E,F) residues was not affected by *Rar α* expression, indicating absence of activation of the canonical Wnt/ β -catenin pathway in *Rar α ^{-/-}* mice. This was also supported by unchanged mRNA expression of *Axin2* (Fig. 5J), a target gene of the canonical Wnt/ β -catenin pathway. In contrast, mRNA expression of *Wnt4* (Fig. 5G), *Tcf3* (Fig. 5H) and *Lef1* (Fig. 5I), effectors and targets of non-canonical Wnt signaling, was reduced in adrenals from 12 weeks old *Rar α ^{-/-}* male mice. Modification of the expression of *Wnt4* (Fig. 5G), a ligand involved in non-canonical Wnt signaling, of Frizzled-2, a transmembrane

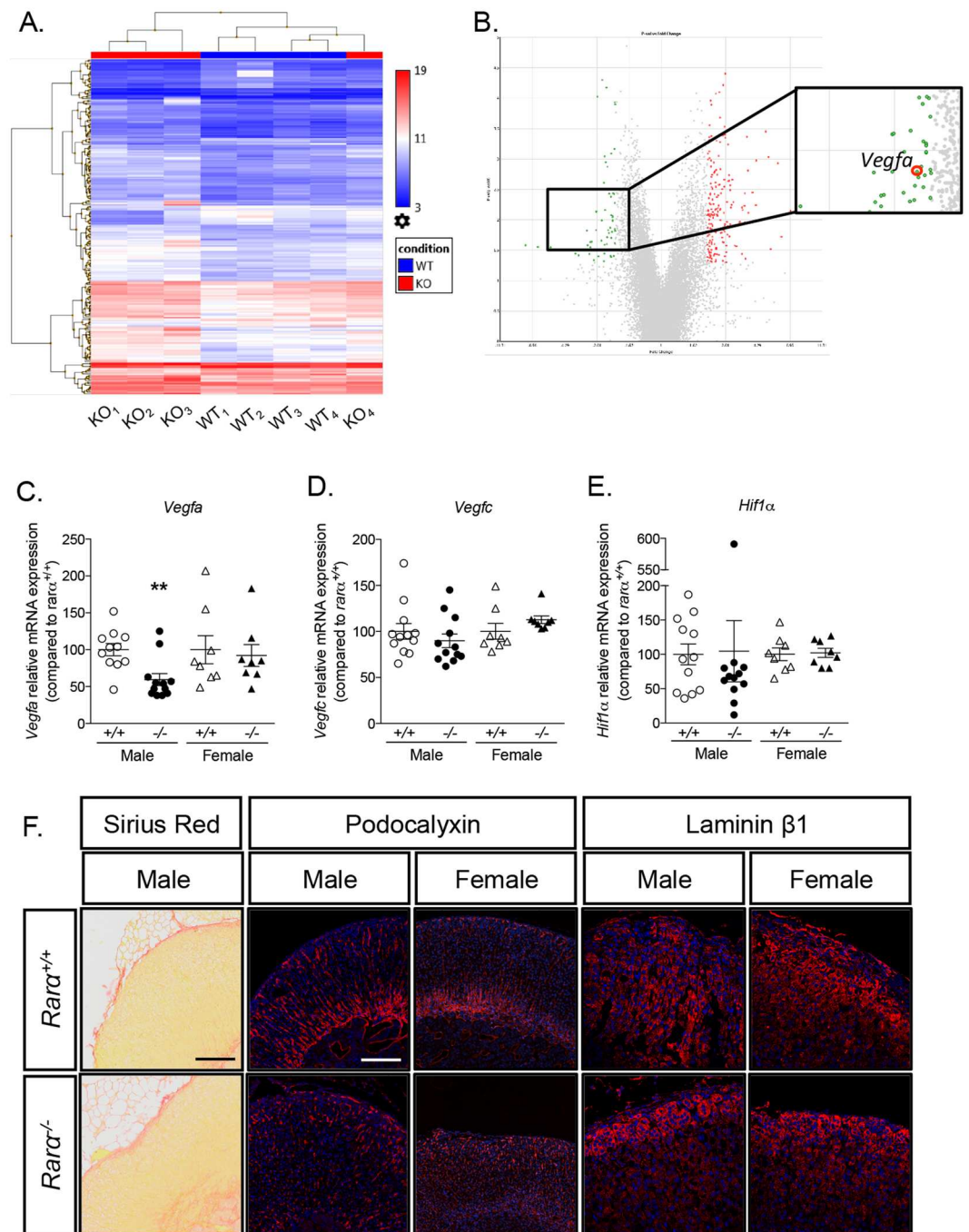


Figure 4. *Rara* affects vessel architecture and extra cellular matrix composition. **(A)** Hierarchical clustering of samples using the 243 differentially expressed genes in adrenals from 12 weeks old *Rara*^{+/+} and *Rara*^{-/-} male animals (4 animals per group). **(B)** Volcano plot showing the differentially expressed genes in 12 weeks old *Rara*^{+/+} and *Rara*^{-/-} animals. The x-axis is the fold change between the two conditions; the adjusted p value based on $-\log_{10}$ is reported on the y-axis. Genes significantly different are highlighted as green (down-regulated in *Rara*^{-/-} mice) or red (up-regulated in *Rara*^{-/-} mice) dots. **(C, D, E)** The expression of genes involved in angiogenesis, *Vegfa* **(C)** and *Vegfc* **(D)** and in hypoxia, *Hif1α* **(E)** was investigated by RT-qPCR on mRNA extracted from 6–11 adrenals from 12 weeks old male and female *Rara*^{+/+} and *Rara*^{-/-} mice. **(F)** The vascular architecture was assessed by Sirius red staining and podocalyxin immunofluorescence and extra cellular matrix integrity by laminin β1 immunofluorescence. Values are presented as the mean ± SEM; p values were calculated using a Mann-Whitney test. **p < 0.01.

receptor of the non-canonical pathway (Supplementary Table 12) and of Tcf3 (Fig. 5H), a transcription factor that can act as a transcriptional repressor independently of β-catenin binding, suggests that the non-canonical Wnt signaling pathway is altered in *Rara*^{-/-} mice.

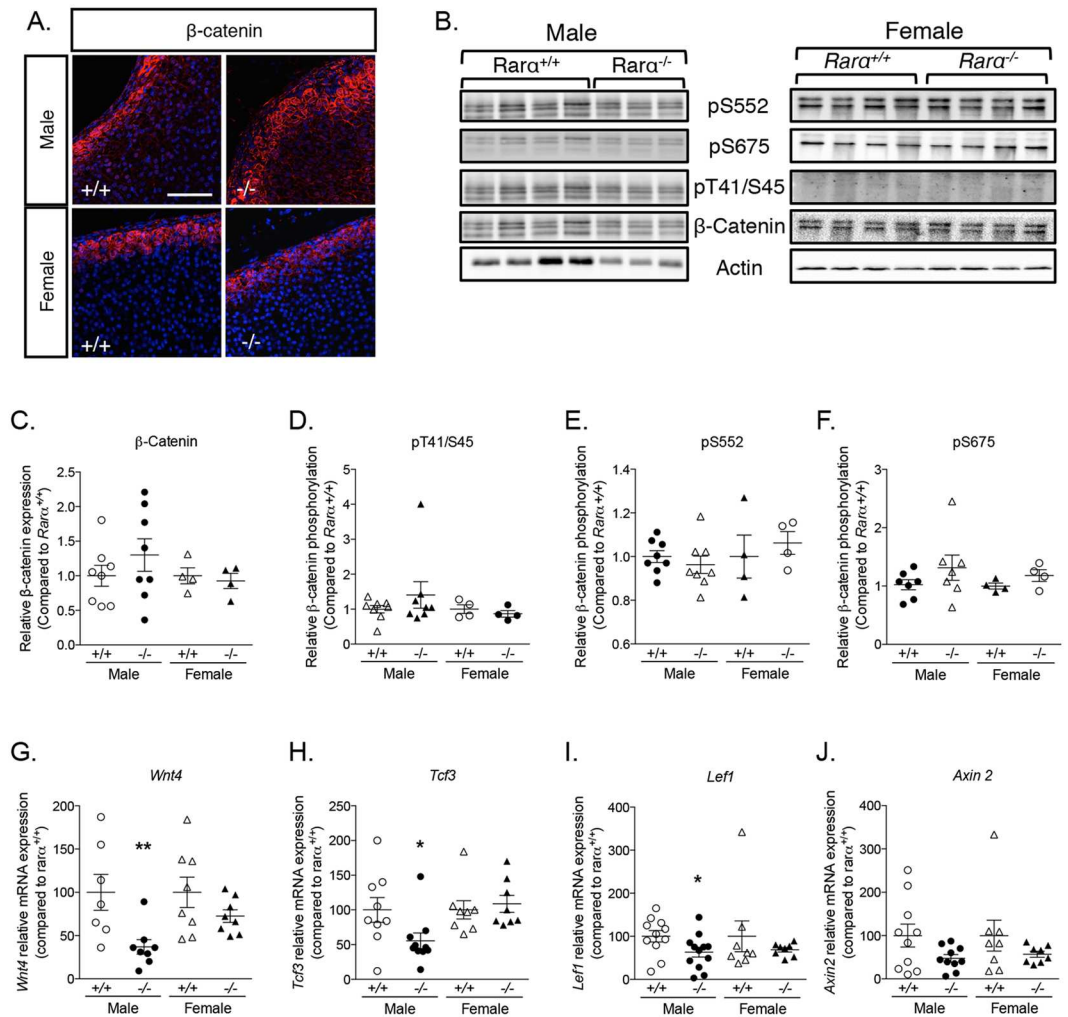


Figure 5. *Rara* inactivation alters Wnt signaling pathway in 12 weeks old male mice. (A) Expression of β -catenin was evaluated by immunofluorescence in 12 weeks old male and female $Rara^{+/+}$ and $Rara^{-/-}$ mice. (B) Expression and phosphorylation of β -catenin in response to *Rara* inactivation. Proteins were extracted from total adrenal and submitted to western blot analysis. Phosphorylation/dephosphorylation in activating (pS552 and pS675) and inactivating (pT41/S45) residues and total expression of β -catenin was investigated. (C–F) Quantification of β -catenin expression (C) and of phospho-specific signals in inactivating (D) and activating (E,F) residues was performed in $Rara^{+/+}$ and $Rara^{-/-}$ adrenal. (G–J) The expression of *Wnt4* (G), *Tcf3* (H), *Lef1* (I) and *Axin2* (J) was investigated by RT-qPCR on mRNA extracted from 6 to 11 adrenals from $Rara^{+/+}$ and $Rara^{-/-}$ male and female mice. Values are presented as mean \pm SEM; p values were calculated using a Mann-Whitney test or *t*-test. * $p < 0.05$; ** $p < 0.01$.

Since $Rara^{-/-}$ mice show significant abnormalities of the ZF, we also explored protein kinase A (PKA) activation, which plays an important role in adrenal cortex development and function. It has been proposed that PKA prevents ZG differentiation through Wnt pathway inhibition, suggesting that PKA activation in the ZF is a key driver of Wnt inhibition³⁵. PKA is the main kinase responsible for the phosphorylation of specific transcription factors including the cAMP response element-binding protein (CREB)³⁶. p-CREB was detected in the entire cortex in $Rara^{+/+}$ and $Rara^{-/-}$ (Supplementary Fig. 4), where its expression was detected in $>70\%$ of the cells (Supplementary Fig. 4). Western blot analysis of CREB and p-CREB expression, performed on whole adrenals, revealed no change in total CREB expression or in its phosphorylation status depending on genotype (Supplementary Fig. 4). mRNA expression of *Prkar1a* and *Prkaca*, the genes coding for the regulatory and catalytic subunit of PKA, was reduced in $Rara^{-/-}$ animals (Supplementary Fig. 4); given the concomitant modification of both subunits, this did not affect the expression of *S100a4*, a target gene of PKA activation in the adrenal³⁷ (Supplementary Fig. 4).

Significant disorganization of the adrenal cortex persists with aging. As *Rara* was identified as associated with abnormal cell proliferation and nodule formation in APA, we evaluated the evolution of adrenal abnormalities with aging by investigating the adrenal phenotype of $Rara^{-/-}$ animals at 52 weeks of age (Fig. 6 and Supplementary Fig. 5). Growth retardation was more pronounced in 52 weeks old compared to 12 weeks

old mice, with smaller weight for both male and female $Rar\alpha^{-/-}$ compared to $Rar\alpha^{+/+}$ mice; the relative adrenal weight was similar in both genotypes (Table 3). Adrenal morphology and size, evaluated by magnetic resonance imaging, was also similar in both genotypes and sexes (Table 3). Similar to 12 weeks old $Rar\alpha^{-/-}$, we observed loss of radial organization of the ZF with a conserved ZG in both male and female mice at 52 weeks of age (Fig. 6A,B), and absence of the fetal X-zone in both genotypes (Supplementary Fig. 3). However, at 52 weeks, the proliferation index and adrenal cortex size were similar in $Rar\alpha^{-/-}$ mice compared to their wild type littermates (Fig. 6C,D). No differences were observed in the localization of aldosterone synthase or 11 β -hydroxylase expression (Fig. 6A). Unlike 12 weeks old animals, there was no difference in the expression of steroidogenic genes such as *Star* (Fig. 6E), *Cyp11a1* (Fig. 6F), and *Cyp11b2* (Fig. 6H) in $Rar\alpha^{-/-}$ mice; a slightly decreased expression of *Cyp11b1* (Fig. 6G) was observed in female mice only. Again, plasma aldosterone (Fig. 6I) and corticosterone (Fig. 6J) levels, plasma renin concentration (Fig. 6K) and aldosterone to renin ratio (Fig. 6L) were not different in $Rar\alpha^{-/-}$ mice. However, 50% of male $Rar\alpha^{-/-}$ mice showed high plasma aldosterone concentrations (up to 1000 pmol/l), which were associated with higher plasma renin concentration (Fig. 6K).

Vessel dilatation (Sirius red) and disorganization (podocalyxin) as well as abnormal laminin β 1 localization were still present in both male and female mice (Supplementary Fig. 5), but expression of *Vegfa* was not modified in $Rar\alpha^{-/-}$ mice at 52 weeks of age (Supplementary Fig. 5). β -catenin was localized to the ZG (Supplementary Fig. 5), with an extended expression in ZF that could be observed in restricted areas in some animals (data not shown). Consistently with this observation, despite similar β -Catenin protein levels in $Rar\alpha^{+/+}$ and $Rar\alpha^{-/-}$ mice (Supplementary Fig. 5), a large variation in its expression was observed (Supplementary Fig. 5). Again, β -Catenin phosphorylation on activating (pS552 and pS675, Supplementary Fig. 5) or inactivating (pT41/S45, Supplementary Fig. 5) residues were unchanged, indicating again that canonical Wnt/ β -Catenin signaling was not modified in 52 weeks old $Rar\alpha^{-/-}$ mice. In contrast to 12 weeks old $Rar\alpha^{-/-}$ mice, only the expression of *Lef1* was reduced in 52 weeks old male $Rar\alpha^{-/-}$ mice (Supplementary Fig. 5), while the expression of *Wnt4* (Supplementary Fig. 5), *Tcf3* (Supplementary Fig. 5) and *Axin 2* (Supplementary Fig. 5) was similar in both genotypes. Similar to 12 weeks old mice, PKA signaling was unaltered at 52 weeks of age (Supplementary Fig. 4). However, p-CREB expression was reduced (<20% of cells, Supplementary Fig. 4) and found to be restricted to the external part of the adrenal cortex, mainly the ZG, in both $Rar\alpha^{+/+}$ and $Rar\alpha^{-/-}$ animals at that age (Supplementary Fig. 4).

Microarray analysis performed on adrenals from four $Rar\alpha^{+/+}$ and four $Rar\alpha^{-/-}$ 52 weeks old male mice revealed that 63 genes (60%) were upregulated and 42 (40%) down-regulated in $Rar\alpha^{-/-}$ mice. Despite the low number of differentially regulated genes, hierarchical clustering allowed separating adrenals from $Rar\alpha^{+/+}$ and $Rar\alpha^{-/-}$ mice (Supplementary Fig. 6). The expression of some genes involved in retinoic acid biosynthesis was found to be increased (alcohol dehydrogenase 1 and 7), possibly to compensate for the lack of $Rar\alpha$ expression (Supplementary Table 13). Furthermore, the expression of *Mrap* (Melanocortin 2 receptor accessory protein) was also found to be upregulated in these mice. *Mrap* has recently been shown to play a role in adrenal cortex zonation through the modulation of adrenal progenitor cell differentiation. Absence of *Mrap* was found to be associated to Wnt4/ β -Catenin pathway dysregulation in ZG cells, showing an involvement of both the canonical and the non-canonical Wnt/ β -Catenin pathway in adrenal gland zonation³⁸.

Discussion

Several germline and somatic mutations cause excessive aldosterone production in PA. Although the functional link between those mutations and aldosterone production has been clearly established, the mechanisms leading to nodule formation appear to be complex and to partly involve different mechanisms³⁹. Here we report the identification of $RAR\alpha$ as being a key element in the regulation of adrenal cortex structure and cell proliferation. Inactivation of *Rar\alpha* in mice led to significant structural disorganization of the adrenal cortex in male and female, with increased adrenal cortex size in female mice and increased cell proliferation in males, but no major modifications of aldosterone production. This disorganization is associated with modifications of the vessel architecture and ECM due to decreased *Vegfa* expression and modifications in ECM components. On the molecular level, *Rar\alpha* inactivation leads to inhibition of the non-canonical Wnt signaling, without affecting the canonical Wnt Pathway nor PKA signaling. These molecular abnormalities were detected at 12 weeks in male mice only, suggesting that in female mice they may have occurred earlier in adrenal cortex development. At 52 weeks of age, the marked disorganization of the adrenal cortex persists in absence of molecular abnormalities in Wnt or Vegf signaling.

RAR belongs to the super-family of nuclear receptors; there are three subtypes of RAR: α (*NR1B1*), β (*NR1B2*) and γ (*NR1B3*). RAR functions as a ligand dependent transcription factor, which heterodimerizes with another receptor of the same family, the retinoic X receptor (RXR). RXR/RAR heterodimers bind to specific DNA sequences, the retinoic acid (RA) response element (RAREs) composed of two direct repeats of a core hexameric motif (A/G) G [G/T] TCA located in the regulatory regions of target genes^{40,41}. In the absence of ligand, RARs are bound to RAREs in association with large protein complexes, which maintain chromatin in a condensed and repressed state⁴². Ligand binding is facilitated by association of RAR with cellular RA binding proteins. This binding induces conformational changes in the ligand-binding domain of the receptor, resulting in corepressor release and coordinated recruitment of a series of coregulator complexes.

RA mediated transcription plays critical roles in a variety of biological processes, including development, reproduction, immunity, organogenesis and homeostasis⁴³. Moreover RARs are involved in cancer development, due to mutations, fusions to other proteins, altered expression or aberrant post-translational modifications. These alterations result in modified function and disruption of homeostasis, due to the capacity of RAR to regulate growth and differentiation. In human myeloid leukemia, the $RAR\alpha$ gene is the target of chromosomal rearrangements resulting in the production of fusion proteins that allow the cells to continue to proliferate and/or prevent the terminal differentiation seen in normal myelocytes⁴⁴. Considering the associations between RAR and tumorigenesis, RAR are considered as tumor suppressor. In some cases, RAR have been correlated with survival instead

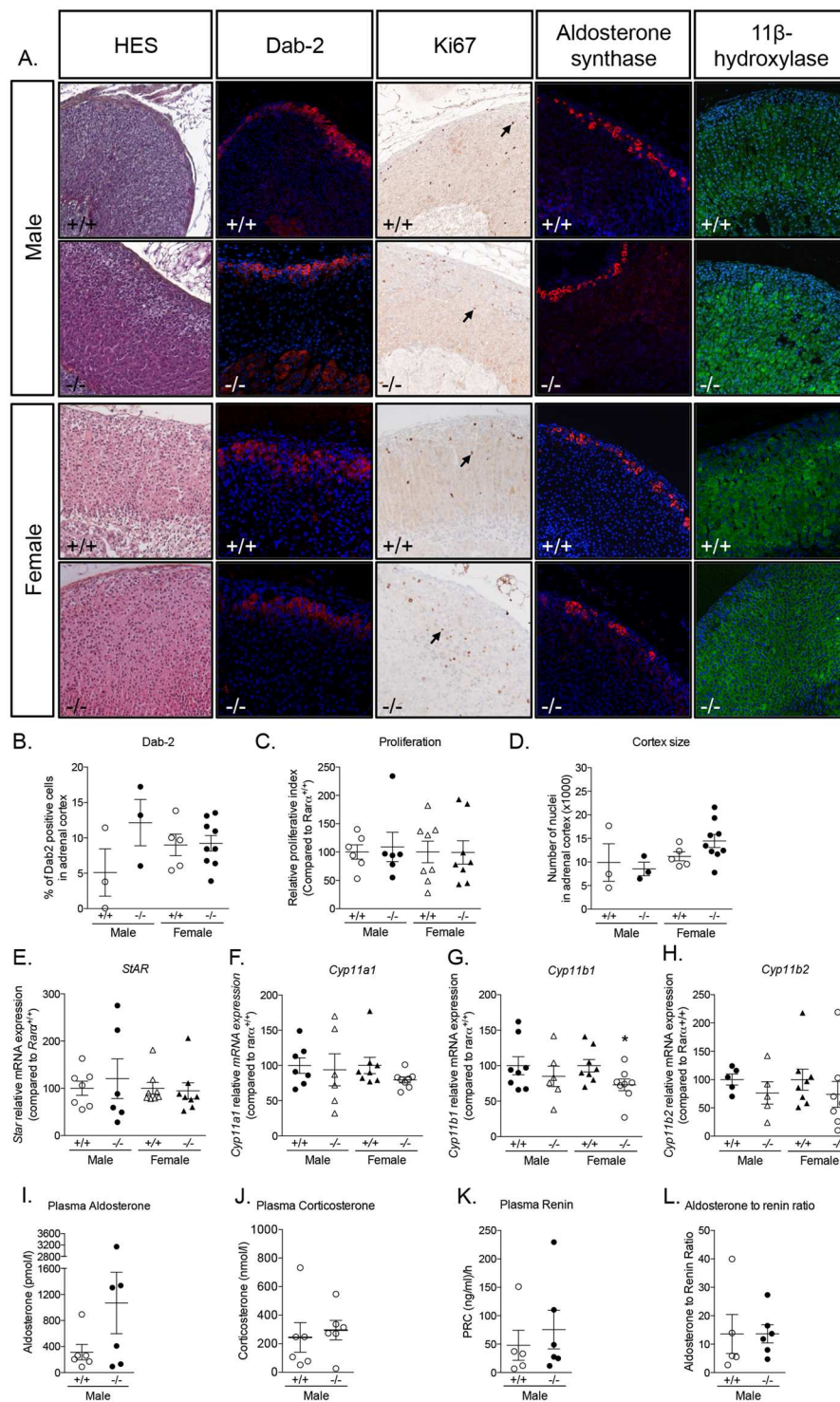


Figure 6. Adrenal cortex disorganization persists with aging in $Rara^{-/-}$ mice. **(A)** Morphological characterization of adrenals from 52 weeks old male and female $Rara^{+/+}$ and $Rara^{-/-}$ mice. HES staining, Dab-2, aldosterone synthase and 11 β -hydroxylase immunofluorescence and Ki67 immunohistochemistry were performed. **(B)** Number of Dab-2 positive cells in the cortex was determined in 3 to 9 animals of each genotype and sex using an automated molecular imaging platform (Vectra, Perkin Elmer) and is expressed as a percentage of total number of cells in the entire cortex area. **(C)** Relative proliferative index of adrenals from male and female $Rara^{+/+}$ and $Rara^{-/-}$ mice. Ki67 positive cells were separately counted in the adrenal cortex in 5–6 animals per genotype. **(D)** Number of nuclei in the adrenal cortex was determined in 3 to 9 animals of each genotype and sex using an automated molecular imaging platform (Vectra, Perkin Elmer). **(E–H)** Expression of steroidogenic genes in male and female $Rara^{+/+}$ and $Rara^{-/-}$ mice. mRNA expression of *Star* **(E)**, *Cyp11a1* **(F)**, *Cyp11b1* **(G)** and *Cyp11b2* **(H)** was assessed by RT-qPCR. RT-qPCR were performed on mRNA extracted from 6–8 adrenals from 52 weeks old male and female $Rara^{+/+}$ and $Rara^{-/-}$ mice. **(I, J)** Measure of plasma

aldosterone (I) and corticosterone (J) concentration by mass spectrometry in male mice. (K,L) Plasma renin concentration (PRC) and aldosterone to renin ratio. Measure of plasma aldosterone, plasma corticosterone and plasma renin were done on 5–6 animals per group. Values are presented as means \pm SEM. * $p < 0.05$; ** $p < 0.01$.

of growth arrest. Recently, proteomic characterization of adrenal gland embryonic development suggested a role of the retinoic acid receptor pathway in the early stages of adrenal gland development⁴⁵. Moreover, transcriptome analysis identified LPS/IL-1 mediated inhibition of RXR function pathway in the top 5 canonical pathways associated with genes differentially regulated in APA compared with adjacent ZG, suggesting a role of RXR pathways in APA⁴⁶.

RA is a derivative of vitamin A (retinol), which is provided exclusively by diet. Vitamin A is converted into retinal by the action of alcohol dehydrogenases and short chain dehydrogenases/reductases. Then retinal is oxidized into RA by retinaldehyde dehydrogenase⁴⁷. In mice, the half-life of RA has been estimated to be about 0.5 hours whereas retinol's half-life is longer (3.6 hours)⁴⁸, suggesting that RA could be produced locally in target tissues instead of being transported. All components of retinoic acid biosynthesis were identified in human and mouse adrenals in this study, supporting RA biosynthesis and action in the adrenal gland. Our data support a role for $Rar\alpha$ signaling in regulating cell proliferation: indeed, 12 weeks old male $Rar\alpha^{-/-}$ mice show an increased proliferative index compared to wild type littermates. Despite no changes in proliferative index observed in female mice at 12 weeks of age, their adrenal weight was increased, suggesting that a proliferative phase may have occurred at earlier stages of adrenal development²⁴.

The Wnt/ β -catenin pathway is known to play an important role in embryonic development, stem cell maintenance, and differentiation in many tissues^{22,49–52}. Depending on the Wnt-receptor complex formed, a β -catenin dependent (canonical pathway) or independent response (non-canonical pathway) is activated. In the canonical pathway, the binding of Wnt ligands (Wnt1, Wnt2, Wnt3a, Wnt8a, Wnt8b...) to specific frizzled – Low-density lipoprotein receptor-related protein (Lrp) complex leads to the dissociation of β -catenin from the degradation complex and its translocation into the nucleus where it induces the expression of specific target genes (*Lef1*, *Axin 2*, *c-jun*, *Cyclin D1*, *c-myc*...) through binding to specific transcription factors (T-cell factor/lymphoid enhancer factor, Tcf/Lef). The Tcf/Lef family is composed of four members Tcf1, Lef1, Tcf3 and Tcf4. Whereas Tcf1 and Lef1 mediate transcriptional activation of Wnt target genes, Tcf3 acts as a transcriptional repressor, independently of β -catenin binding⁵³. In addition to this canonical Wnt/ β -catenin pathway, a non-canonical Wnt pathway has been described involving binding of other Wnt ligands (Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b and Wnt11) to frizzled receptors (Fzd2, Fzd3, Fzd4 and Fzd6), which signals through the c-Jun N-terminal kinase (JNK) and the Ca^{2+} signaling pathways^{22,54}. Activation of the non-canonical pathway replaces Tcf1 by Tcf3 in the Tcf/Lef complex, changing the transcriptional program induced by Tcf/Lef binding to DNA⁵⁵.

In the adrenal cortex, β -catenin expression is restricted to the ZG, suggesting a role for this pathway in the development of the adrenal cortex⁵⁶. In *Wnt4*-deficient mice, a decrease in ZG cell number has been reported, resulting in a decrease of *Cyp11b2* mRNA expression and aldosterone production. These mice also show ectopic expression of adrenal-like cells in the gonads, probably due to abnormal adrenocortical progenitor cell migration during development, suggesting a role for *Wnt4* in cell sorting during development⁵⁷. In our model, the expression of *Wnt4* was highly correlated to the expression of *Cyp11b2* in both male ($r = 0.6015$, $p = 0.0064$) and female ($r = 0.5663$, $p = 0.0007$) mice, supporting the role of *Wnt4* in the regulation of *Cyp11b2* expression. Similarly, the targeted disruption of β -catenin in steroidogenic Sf-1-expressing cells causes adrenal aplasia in newborn mice, further supporting a role for the Wnt/ β -catenin pathway early in adrenal development⁵⁶.

The marked disorganization of adrenal cortex structure observed in *Rar\alpha* knockout mice, was associated to reduced expression of *Lef1*, *Tcf3*, *Wnt4* and increased expression of *Fzd2* (Supplementary Table 12), all involved in non-canonical Wnt signaling, in 12 weeks old $Rar\alpha^{-/-}$ male mice. In contrast, β -catenin phosphorylation, which reflects the activation status of the canonical Wnt/ β -catenin pathway, was unchanged. Interactions between the Wnt pathway and retinoic acid receptor signaling have been reported in some studies. Hence, in mouse embryonic stem cells, retinoic acid can concomitantly activate the non-canonical and inhibit the canonical Wnt pathway⁵⁵. Similarly, the expression of *Lef1*, known to be regulated by the canonical Wnt pathway, can also be modulated by the non-canonical pathway⁵⁸. In chondrocytes, retinoic acid, through $RAR\alpha$ /RXR α activation, plays an important role in terminal differentiation through BMP2 and *Wnt4* modulation⁵⁹. More interestingly, retinoic acid has been proposed to inhibit β -catenin/Tcf activity downstream of β -catenin by inducing expression of secreted factors such as *Wnt4* or *Wnt11*⁶⁰. Increased expression of Tcf3, concomitantly with decreased expression of Tcf1 enhances the ability of Tcf3 to bind to specific Wnt response element, inducing changes in the transcriptional program of the cells⁵⁵. Therefore, our results suggest that $Rar\alpha$ could contribute to the structural organization of the adrenal cortex through modification of the expression of *Wnt4* and Tcf3, thus modulating the non-canonical Wnt pathway. Recently, downregulation of retinoic acid signaling (liver and retinoid X receptors LXR/RXR) was reported in adrenals of *Siah1* KO mice. *Siah1* KO mice exhibit severe disorganization of adrenal glands as well as increased aldosterone and corticosterone levels and vessel dilatation. *Siah1* codes for an E3 ubiquitin-protein ligase mediating ubiquitination and subsequent proteasomal degradation of target proteins, including PIAS1. On a molecular level, the stabilisation of PIAS1 leads to the enhancing sumoylation of Sfl⁶¹ and LXR⁶². Whereas, Sfl sumoylation results in increased steroidogenesis, modification of retinoic acid signaling could contribute to adrenal cortex disorganization as well as vessel dilatation.

PKA signaling has been proposed to play a role in cell conversion from ZG to ZF and in maintenance of adrenal cortex zonation, acting through the modulation of the Wnt/ β -catenin pathway, by inhibiting *Wnt4* expression³⁵. In 12 weeks old male $Rar\alpha^{-/-}$ mice, the mRNA expression of both the regulatory and catalytic subunits of PKA (*Prkar1a* and *Prkaca* respectively) was downregulated; however CREB phosphorylation was not affected,

neither was the expression of *S100a4*, a specific adrenal target gene of PKA³⁷, suggesting that PKA activity was not affected. Therefore, the observed inhibition of the Wnt pathway does not appear to be due to modifications of PKA activity, but rather to a direct effect of Rar α .

Transcriptome analysis of adrenals from 12 and 52 weeks old Rar $\alpha^{+/+}$ and Rar $\alpha^{-/-}$ mice, combined with specific staining revealed a decrease in *Vegfa* expression as well as abnormal vascular architecture. This was associated with a differential pattern of expression of laminin β 1, which was present in the entire adrenal cortex in Rar $\alpha^{+/+}$ mice, whereas a gradient of expression was observed in Rar $\alpha^{-/-}$ mice with higher expression in the ZG than in the inner part of the ZF. The extracellular environment, in particular the vasculature and ECM, plays an important role in adrenal cortex structure and function, by modulating proliferation, migration, differentiation and survival of surrounding cells, but also steroidogenesis^{63,64}. The ECM consists of the basement membrane and the interstitial ECM. The ECM is composed of polysaccharides and extracellular proteins such as collagens, laminins and fibronectin, but also some bioactive compounds (growth factors, enzymes, chemoattractants and morphogens), that form a three-dimensional network⁶⁵. Laminins were found to be uniformly distributed in human adrenal cortex^{64,66}; they have been proposed to play a role in adrenocortical cell migration through their chemotactic and haptotactic properties⁶⁶. Interestingly, presence of RAR α response elements has been identified in the laminin β 1 promoter indicating regulation of its expression by retinoic acid⁶⁷. Different studies reported that retinoic signaling participates in the regulation of the expression of ECM proteins as well as of cell membrane ECM receptors in physiological and pathophysiological conditions⁶⁸. The modification of expression of some components of the ECM such as Fibronectin 1, MFAP2, MFAP5 and of Collagen 3 α 1, associated to the modification of the pattern of expression of laminin β 1 observed in the adrenal cortex of Rar $\alpha^{-/-}$ mice strongly suggest a rearrangement of the ECM in absence of Rar α expression. These alterations of ECM composition could contribute to the disorganization of the adrenal cortex observed in our model, by modulating adrenocortical and vascular cell migration.

The adrenal gland is a highly vascularized tissue, each cell being in contact with at least one endothelial cell, allowing rapid release of hormones into the blood stream. Blood vessels present a centripetal pattern from superficial arteries to the central vein⁶⁹, following the radial organization of the adrenal cortex⁷⁰. A characteristic feature of adrenal glands is the high expression of VEGF, which is produced by steroidogenic cells and is regulated by ACTH⁷¹. Interestingly, the Wnt pathway has also been involved in angiogenesis and vessel remodeling. It has been proposed that Wnt inhibition leads to vessel stability or regression whereas its activation could lead to angiogenesis or vessel remodeling^{72,73}. VEGF plays a central role to optimize vessel growth⁷⁴; more importantly, many studies reported an effect of retinoic acid on VEGF expression; however depending on the cell models and the experimental conditions retinoic acid inhibits or stimulates VEGF expression^{75,76}. Hence, the adrenal phenotype observed in Rar $\alpha^{-/-}$ mice could be explained by a direct effect of deficient Rar α signaling on *Vegfa* signaling and Laminin β 1 expression or by the inhibition of Wnt signaling, that could in turn also affect *Vegf* signaling. These modifications of the extracellular microenvironment in Rar α KO mice suggest that Rar α may affect the interplay between steroidogenic cell differentiation and migration, vasculature and extracellular matrix.

The development of specific mouse models in recent years has allowed a better understanding of the mechanism of male adrenal cortex development^{65,77}; however little is still known in female mice. Sex differences in adrenal physiology have been demonstrated by Heitzmann and co-workers²⁷. Invalidation of the Task1 potassium channel leads to abnormalities of adrenal zonation and PA development, which occurs only in female mice after puberty. Testosterone treatment before puberty in female Task1 $^{-/-}$ mice leads to normal adrenal zonation. Similarly, castration of male Task1 $^{-/-}$ mice leads to abnormal zonation of the adrenal cortex similar to female Task1 $^{-/-}$ mice. The comparison of gene expression profiles of male and female adrenals allowed the identification of a set of genes that are modulated according to sex and hormonal treatments²⁶. These studies highlighted the critical role for sexual hormones in adrenal gland zonation and the existence of a sexual dimorphism. Comparison of the adrenal phenotype of male and female Rar $\alpha^{-/-}$ mice revealed sex-specific molecular differences in young mice despite similar morphological abnormalities in adrenal gland development. This sexual dimorphism could be due to a different timing of the action of Rar α in adrenal development, with Rar α signaling occurring earlier in female than in male. As suggested by the adrenal phenotype of Task1 $^{-/-}$ mice, sexual hormones, such as estrogen and androgens, may play a role in adrenal development and modulate the effect of Rar α inactivation.

In summary, our study identifies RAR α as contributing to the maintenance of normal adrenal cortex structure and cell proliferation by modulating Wnt signaling. The absence of adrenal nodules in 52 weeks old mice suggests that abnormal cell proliferation induced by the absence of RAR α signaling is not enough to promote APA, but may constitute one of the elements involved in the pathogenesis of PA. We propose a model in which a homeostatic equilibrium between retinoic acid, Wnt and *Vegf* signaling pathways is required for the maintenance of normal adrenal cortex structure in a specific time-dependent manner that differs between males and females (Fig. 7). In addition to previously reported regulators and to further elements that remain to be identified, Ddysregulation of this equilibrium in adult adrenals may contribute to abnormal cell proliferation, creating the propitious environment for the emergence of specific driver mutations in PA.

Methods

Patients. Patients with primary aldosteronism were recruited between 2002 and 2006 through the COMETE (COrtico- et MEDullo-surrénale: les Tumeurs ENdocrines) network (COMETE-HEGP protocol), approved by the French Research ethics committee (Comité de Protection des Personnes, CPP) under authorization number CPP 2012-A00508-35. Methods for screening and subtype identification of PA were performed according to institutional and Endocrine Society guidelines^{78,79}. In patients diagnosed with primary aldosteronism, a thin slice CT scan or MRI of the adrenal and/or an adrenal venous sampling were performed to differentiate between unilateral and bilateral form. All patients gave written informed consent for genetic and clinical investigation.

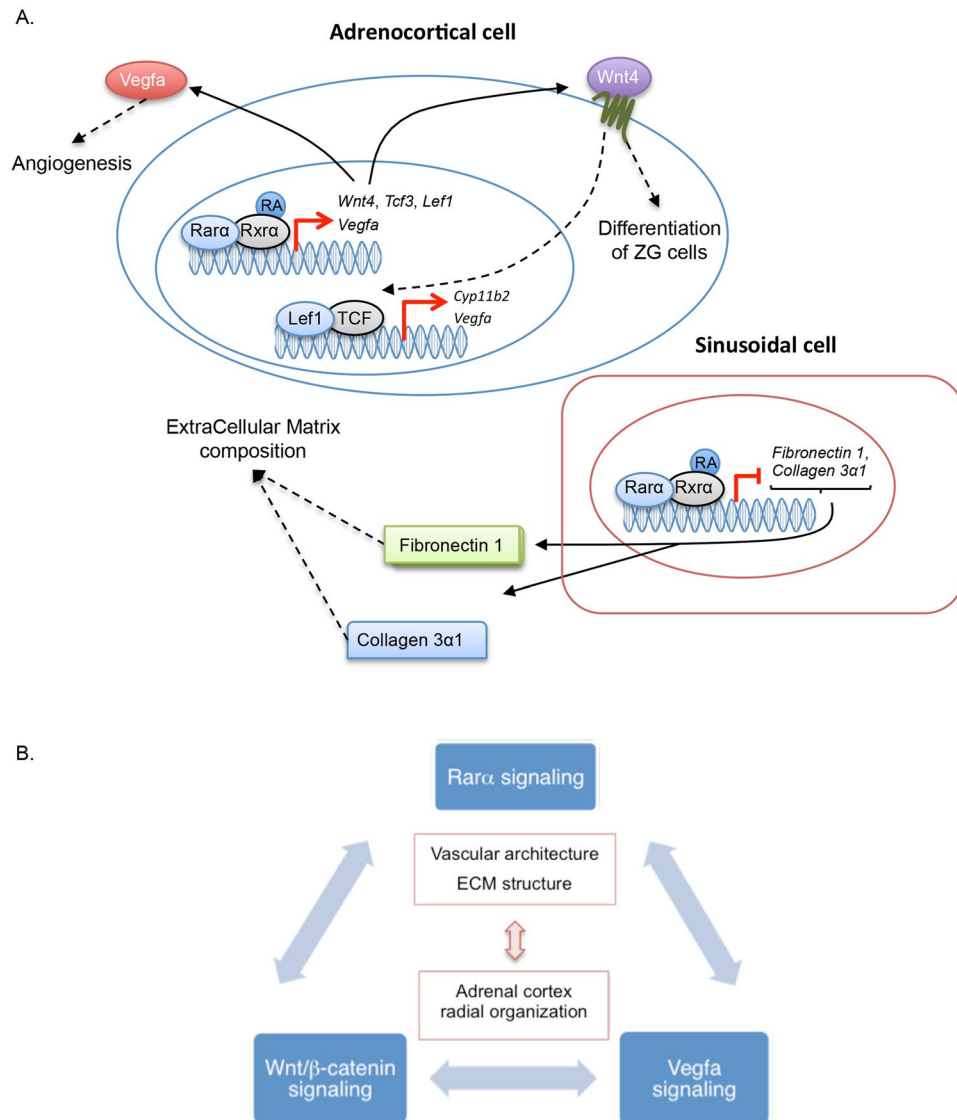


Figure 7. Proposed model for the role of $Rar\alpha$ in adrenocortical development. **(A)** $Rar\alpha$ regulates the expression of genes involved in the regulation of Wnt non-canonical pathway (*Wnt4*, *Tcf3*, *Lef1*...), angiogenesis (*Vegfa*) and Extra Cellular Matrix integrity (*Fibronectin1*, *Collagen 3 α 1*...) contributing to the organization of the adrenal cortex. *Wnt4* activated pathway contributes to the differentiation of ZG cells, *Vegfa* to normal angiogenesis and *Fibronectin1* and *Collagen 3 α 1* being components of the Extra Cellular Matrix. **(B)** Model in which a homeostatic equilibrium between $Rar\alpha$, Wnt and Vegf signaling pathways is required for the normal development of the vasculature and Extra Cellular Matrix structure, leading to normal adrenal cortex organization.

Microarray analysis on human tissues. Transcriptome analyses were performed on 48 APA samples and eleven control adrenals obtained from enlarged nephrectomies for renal carcinoma. Total RNA was isolated from frozen tissue using Trizol (Invitrogen) and then cleaned-up on silica columns using RNeasy Mini Kit (Qiagen). The integrity and quality of the RNA were systematically checked using an Agilent 2100 Bioanalyzer with the RNA6000 Nano Assay (Agilent Technologies). For APA samples, complementary RNA was synthesized from 15 μ g of total RNA using the Chemiluminescent RT Labeling Kit (Applied Biosystems). For control adrenals, complementary cRNA was synthesized from 500 ng of total RNA using the NanoAmp™ RT-IVT Labeling Kit (Applied Biosystems). Samples were hybridized to microarrays using Applied Biosystems chemiluminescence detection kit according to the manufacturer's protocol. Acquisition of the chemiluminescence images and primary data analysis were done using Applied Biosystems Expression Array System Software v1.1.1. Microarray gene expression data from the primary analysis for each sample was median centered, log₂ transformed, model-adjusted, and quality controlled⁸⁰. Mean expression profiles for each biological condition were obtained by variance-weighted averaging normalized expression values. Pairwise inter-array normalizations were carried out using the NeONORM method described previously⁸¹. Fold change values were expressed on log₂ scale. P-values were determined based on a normal distribution hypothesis of signal intensities using standard

methods⁸². Multiple probes for a single gene, cross-reactivity of a single probe to several genes, as well as the resolution of probe-ID annotations were done as defined previously⁸³. Hierarchical clustering based on Euclidean distance and complete-linkage was carried out using the R-packages *hclust* and *gplot*. Differentially expressed genes among groups were calculated using a fold-change z-test with positive false discovery rate correction. P values for over- and under-representation of pathways were calculated using a binomial distribution and a Bonferroni correction for multiple testing.

The data for the human APA transcriptome analysis have been deposited to the public data repository <http://mace.ihes.fr> under accession nos.: 2575793518 (controls) and 2293267822 (APA).

Mice. Animal studies were conducted according to the guidelines and regulations formulated by the European Commission for experimental use (Directive 2010/63/EU) and were approved by the local Ethics committee of Paris Descartes University (N°17-020) and by the French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (APAFIS authorization number #11509-201703281427107 v4). *Rara*^{-/-} mice (constitutive homozygous deletion of exon 8 of *Rara* gene, leading to inactivation of the protein) have been kindly provided by Dr Norbert Ghyselinck and Dr Pierre Chambon³². They were maintained and bred on a mixed background and littermates control animals were used in all experiments. All experiments were conducted on male and female mice between 12 and 52 weeks of age. Blood were collected in abdominal aorta in lithium-heparin blood collection tubes (Microvette CB300LH, Sarstedt). The right adrenals were frozen in liquid nitrogen and the left adrenals fixed in 4% paraformaldehyde.

Magnetic resonance imaging. Anesthesia was induced with 4% isoflurane and maintained at a 1% level during imaging. Magnetic resonance imaging was performed with a dedicated small-animal 4.7 Tesla MR system (Biospec 47/40 USR Bruker). The animals were placed in a supine position on a four-channel phased array receive coil inside a volume transmit coil (BrukerBiospin, Ettlingen, Germany). We performed two anatomic sequences (TurboRARA T2 and FISP) in two directions (coronal and sagittal) to optimized measurement of the size of the adrenal. The thickness of the slices was 0.7 mm and the resolution was 175 × 175 μm and 156 × 126 μm respectively.

Aldosterone, corticosterone and plasma renin concentration measurement in murine plasma samples. For steroids quantification, 150 μl of heparinized murine plasma was spiked with 500 ng of stable isotope labeled internal standards for aldosterone and corticosterone. Following C18-based solid-phase-extraction, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC® C18, Waters, Milford, MA, USA) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters Xevo TQ/S, Milford, MA, USA) in multiple reaction monitoring mode. Internal standards were used to correct for analyte recovery across the sample preparation procedure in each sample. Analyte concentrations were calculated from integrated chromatograms considering the corresponding responses determined in appropriate calibration curves in plasma matrix.

The plasma renin concentration was determined in murine plasma samples by determining the Ang I formation rate in the presence of excess of angiotensinogen, where it becomes solely dependent on the concentration of active renin. Therefore, diluted plasma was supplemented with an excess of recombinant murine angiotensinogen. Samples were incubated at 37°C in the presence of an Ang I stabilizing inhibitor cocktail for 60 minutes at 37°C. Following LC-MS/MS based quantification of Ang I as described⁸⁴, the Ang I formation rate was calculated by subtracting Ang I values obtained in non-incubated control samples and expressed in [(ng Ang I/ml)/h].

RNA extraction and RT-qPCR analysis. Total RNA was extracted using Janke and Kunkel's Ultra-Turrax T25 (IKA technologies, Staufen DE) in Trizol reagent (Ambion Life Technologies, Carlsbad CA) according to the manufacturer's recommendations. After deoxyribonuclease I treatment (Life Technologies, Calsbad CA), 500 ng of total RNA were retro-transcribed (iScript reverse transcriptase, Biorad, Hercules, CA). The quantitative qPCR was performed using SYBRgreen (Sso advanced universal SyBr Green Supermix, Biorad, Hercules, CA) on a C1000 touch thermal cycler of Biorad (CFX96 Real Time System) according to the manufacturer's instructions. Controls without template were included to verify that fluorescence was not overestimated from primer dimer formation or PCR contaminations. RT-qPCR products were analyzed in a post amplification fusion curve to ensure that a single amplicon was obtained. Normalization for RNA quantity and reverse transcriptase efficiency was performed against three reference genes (geometric mean of the expression of Ribosomal 18S RNA, β2-microglobulin and Ubiquitin C for mouse samples and 18S RNA, GAPDH and HPRT for human samples), in accordance with the MIQE guidelines⁸⁵. Quantification was performed using the standard curve method. Standard curves were generated using serial dilutions from a cDNA pool of all samples. The following primers were used: 5'-CCCTGCCCTTTGTACACACC-3' and 5'-CGATCCGAGGGCCTCACTA-3' for 18S; 5'-ATTCACCCC CACTGAGACTG-3' and 5'-TGCTATTTCTTTCTGCGTGC-3' for *mβ2-microglobulin*; 5'-AGCCCAGTGTTA CCACCAAG-3' and 5'-ACCCAAGAACAAGCACAAG-3' for *mUbiquitinC*; 5'-GTGCTTCATCCACTG GCTGGAA-3' and 5'-GTCTGCGATAGGACCTGGTTGA-3' for *mStar*; 5'-TGCTCAACCTGCCTCCA GACTT-3' and 5'-ACTGGCTGAAGTCTCGTTCTG-3' for *mCyp11a1*; 5'-TGATCGAGAGCTGGCAGAG-3' and 5'-CCTGGATGGCATCCATTGAC-3' for *mCyp11b1*; 5'-ACCTACAGTGGCATTGTG-3' and 5'-GATTGCTGTCGTGTC AAC-3' for *mCyp11b2*; 5'-CCCTGTCTTTGGGAAGGTGGTG-3' and 5'-CACCTGC TGAAGATGGCGTATAC-3' for *mWnt4*; 5'-CAGATGGTGGCCTGGATACT-3' and 5'-CATCCCT GCTGTAGCTGTCA-3' for *mTcf3*; 5'-AAATGGGTCCCTTTCTCCAC-3' and 5'-TCGTGCTG TAGGTGATGAG-3' for *mLef1*; 5'-AGCCTAAAGTCTTATGTGG-3' and 5'-ATGGAATCGTCGGTCAGT-3' for *mAxin2*; 5'-CCTGGTGGACATCTCCAGGAGTACC-3' and 5'-GAAGCTCATCTCCTATGTGCTGGC-3' for *mVegfa*; 5'-GGGAAGAAGTCCACCATCA-3' and 5'-ATGTGGCCTTTTCCAATACG-3' for *mVegfc*;

5'-TCAAGTCAGCAACGTGGAAG-3' and 5'-TATCGAGGCTGTGTCGACTG-3' for *mHif1 α* ; 5'-CGGGAATGC GAGCTCTATGT-3' and 5'-CTCGAGTCAGTACGGATGCC-3' for *mPrkar1a*; 5'-CCCAGATTATCCT GAGCAA-3' and 5'-ATAGGCTGGTCAGCGAAGAA-3' for *mPrkaca*; 5'-TCCACAAATACTCAGGC AAAGAG-3' and 5'-GCAGCTCCCTGGTCAGTAG-3' for *mS100a4*; 5'-GTCTCCTCTGACTTCAACAGCG-3' and 5'-ACCACCCTGTTGCTGTAGCCAA-3' for *hGAPDH*; 5'-CTCAACTTAACTGGAAAGAATGTC-3' and 5'-TCCTTTTCACCAGCAAGCT-3' for *hHPRT*; 5'-GACCAGATCACCCCTCCTCAA-3' and 5'-GTCCGAGAAGGTCATGGTGT-3' for *hRAR α* .

Immunostainings. Immunohistochemistry and immunofluorescence were performed on Formalin-Fixed Paraffin-Embedded mouse adrenals from 12 and 52 week-old WT and KO male mice. Adrenal sections were deparaffinized in xylene and rehydrated through graded ethanol. To unmask the antigen, the slides were incubated in antigen unmasking solution (Vector laboratories Ltd) for 30 minutes at 98°C.

Immunofluorescence was performed for Dab2 (1/100, sc-13982, Santa Cruz Biotechnology), Laminin β 1 (1/500, ab69633, Abcam), β -catenin (1/1000, 61054, BD Transduction Laboratories), p-Creb (1/800, 9198, Cell Signaling Technology), mouse Cyp11b2 (mCyp11b2, 1/100, generous gift from CE Gomez-Sanchez), mouse Cyp11b1 (mCyp11b1, 1/50, generous gift from CE Gomez-Sanchez) and mouse podocalyxin (1/100, AF1556, R&D Systems). Tissues were permeabilized by a treatment of 15 min with TBS 0.1% Triton X100. The sections were then blocked with 5% BSA diluted in TBS for Dab2, laminin β 1, β -catenin and p-Creb or with 10% normal donkey serum diluted in TBS 0.1% Triton X100 for mCyp11b2 and mCyp11b1. Primary antibodies were diluted in 3% BSA diluted in TBS (for Dab2, laminin β 1, β -catenin and p-Creb) or in 5% normal donkey serum and 10% normal rat serum diluted in TBS 0.1% Triton X100 (for mCyp11b2 and mCyp11b1) and incubated overnight at 4°C (for laminin β 1, β -catenin, p-Creb, mCyp11b2 and mCyp11b1) or 1 h at room temperature (Dab2). Primary antibodies were detected with donkey anti-rabbit Alexa 594 (1/400 for Dab2, laminin β 1 and p-Creb, 1/500 for mCyp11b2, A21207, Thermo Fisher), donkey anti-mouse Alexa 594 (1/400 for β -catenin, A21203, Thermo Fisher), Donkey anti-goat Alexa 594 (1/400 for mouse podocalyxin, A11058, Thermo Fisher) and Donkey anti-sheep Alexa 488 (1/500 for mCyp11b1, A11015, Thermo Fisher). Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) (1/5000, Roche Diagnostics GmbH).

Immunohistochemistry was performed for mouse Ki67 (1/200, RM-9106, Thermo Fisher), mouse 20- α HSD (1/5000, generous gift from Y Weinstein), human RAR α (1/500, ab28767, Abcam) and human CYP11B2 (hCYP11B2, 1/100, generous gift from CE Gomez-Sanchez). Sections were incubated in 3% hydrogen peroxide (Sigma-Aldrich) for 10 min to inhibit endogenous peroxidases. Non-specific staining was blocked by incubating sections 30 min with 10% normal goat serum diluted in PBS for Ki67, 20- α HSD and RAR α or 10% horse serum (HS) and 0.5% SDS diluted in Tris 0.1 M pH 7.4 for hCYP11B2. Primary antibodies were incubated overnight at 4°C: Ki67, 20- α HSD and RAR α were diluted in 1% NGS or HS in PBS and CYP11B2 was diluted in 10% horse serum and 0.2% Tween 20 in Tris 0.1 M pH 7.4. Primary antibodies were detected by incubation for 30 min with affinity-purified goat anti-rabbit (1/400 for Ki67 and 20- α HSD; Vector Laboratories) or rabbit anti-goat (1/400 for RAR α ; Vector Laboratories) diluted in 1% normal goat serum in PBS or horse anti mouse (1/400 for hCYP11B2; Vector Laboratories) diluted in 10% horse serum and 0.2% Tween 20 in Tris 0.1 M pH 7.4. After incubation for 30 min with an avidin-biotin-peroxidase complex (Vectastain ABC Elite; Vector Laboratories), the slides were developed using diaminobenzidine (Vector Laboratories) and counter-stained using Hematoxylin Harris Hematoxylin, RAL Diagnostics).

All microscopic examinations were done with a x10 and x20 objectives lens using Leica confocal or Vectra™ system.

Multispectral images were acquired using a Vectra® automated imaging system and automatically quantified with InForm® software (both Perkin Elmer). Results of quantification are expressed in percentage of Dab2 or Ki67 positive cells over total adrenal cortex cells. Statistical significance was assessed among groups using *t* test or Mann-Whitney.

Histological examination. Histological examination was performed on 6 μ m sections stained with hematoxylin-eosin safran (HES) and with Sirius red. Microscopic examinations were done with a x10 and x20 objectives lens using Vectra™ system or with a x40 objective lens using confocal SP8 Leica microscope.

Western blot. Western Blot analysis was performed on mouse adrenals from 12 and 52 week-old WT and KO male mice. Total proteins were extracted using RIPA buffer (RB 4475, BioBasic Canada) with EDTA-free protease and phosphatase inhibitor (Bimake), proteins were then spun on a rotor for 30 min and centrifuged at 13000 rpm for 15 min, the proteins were then recovered and concentration determined using Bradford protein assay (Biorad). 10 μ g of total proteins were loaded on 10% Acrylamide SDS-PAGE gel, transferred onto nitrocellulose membrane and tagged with the following antibodies: phospho- β -Catenin (Thr41/Ser45; 1/1000, 9565, Cell Signaling Technology), phospho- β -Catenin (Ser552; 1/1000, 5651, Cell Signaling Technology), phospho- β -Catenin (Ser675; 1/1000, 4176, Cell Signaling Technology), β -Catenin (1/1000, 8480, Cell Signaling Technology), phospho-CREB (Ser133; 1/1000, 9198, Cell Signaling Technology), CREB (1/1000, 9197, Cell Signaling Technology) anti-rabbit IgG, HRP-linked antibody (1/2000, 7074, Cell Signaling technology), β -actin (1/5000, 2228, Sigma-Aldrich) and anti-mouse IgG, HRP-linked antibody (1/5000, A90-146P, Bethyl Laboratories). The signals were developed by Clarity Max™ Western ECL substrate (Biorad, Hercules, CA) and detected by Fujifilm Las-4000 mini Luminescent image analyzer (Fujifilm, Tokyo-Japan) and quantified by Multi gauge software (Fujifilm, Tokyo-Japan). Expression of the phosphoprotein was normalized to the expression of the corresponding total protein and the expression of total proteins was normalized to the expression of the housekeeping protein β -actin.

Microarray analysis of mouse adrenal gene expression. Adrenal gene expression profiles for 16 mice (4 *Rara*^{+/+} and 4 *Rara*^{-/-} 12 weeks old mice and 4 *Rara*^{+/+} and 4 *Rara*^{-/-} 52 weeks old mice) were analyzed using Affymetrix Clariom S Mouse array. After validation of the RNA quality with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit), 100 ng of total RNA was reverse transcribed following the GeneChip® WT Plus Reagent Kit (Affymetrix). Briefly, the resulting double strand cDNA was used for *in vitro* transcription with T7 RNA polymerase (all these steps are included in the WT cDNA synthesis and amplification kit of Affymetrix). After purification according to Affymetrix protocol, 5.5 µg of Sens Target DNA were fragmented and biotin labeled. After control of fragmentation using Bioanalyzer 2100, cDNA was then hybridized to GeneChip® Clariom S Mouse (Affymetrix) at 45°C for 17 hours. After overnight hybridization, chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images were then analyzed with Expression Console software (Affymetrix) to obtain raw data (cel files) and metrix for Quality Controls.

Analyses were performed using TAC (Transcriptome Analysis Console) software. Genes with adjusted P value <0.05 and log₂ fold changes <-1 or >1 were considered to be down- or up-regulated.

Molecular function and biological process enrichment analyses were performed using Gene Ontology (www.geneontology.org). For the analyses, we used the list of differentially expressed genes in 12 or 52 weeks old *Rara*^{+/+} and *Rara*^{-/-} mice. The analysis criteria were kept as default values. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE136801 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136801>).

Statistics. Data are shown in mean ± SEM. Statistical significance was assessed between the groups using unpaired t-test or Mann-Whitney test (Prism, GraphPad Software, USA). A *p*-value <0.05 was considered statistically significant.

References

- Hannemann, A. & Wallaschofski, H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies – a review of the current literature. *Horm Metab Res* **44**, 157–162, <https://doi.org/10.1055/s-0031-1295438> (2012).
- Monticone, S. *et al.* Cardiovascular events and target organ damage in primary aldosteronism compared with essential hypertension: a systematic review and meta-analysis. *The lancet. Diabetes & endocrinology* **6**, 41–50, [https://doi.org/10.1016/S2213-8587\(17\)30319-4](https://doi.org/10.1016/S2213-8587(17)30319-4) (2018).
- Calhoun, D. A., Zaman, M. A. & Nishizaka, M. K. Resistant hypertension. *Curr Hypertens Rep* **4**, 221–228 (2002).
- Douma, S. *et al.* Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet* **371**, 1921–1926, [https://doi.org/10.1016/S0140-6736\(08\)60834-X](https://doi.org/10.1016/S0140-6736(08)60834-X) (2008).
- Milliez, P. *et al.* Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology* **45**, 1243–1248 (2005).
- Rossi, G., Boscaro, M., Ronconi, V. & Funder, J. W. Aldosterone as a cardiovascular risk factor. *Trends in endocrinology and metabolism: TEM* **16**, 104–107 (2005).
- Rossi, G. P. *et al.* Primary aldosteronism: cardiovascular, renal and metabolic implications. *Trends in endocrinology and metabolism: TEM* **19**, 88–90, <https://doi.org/10.1016/j.tem.2008.01.006> (2008).
- Rossi, G. P. *et al.* A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *Journal of the American College of Cardiology* **48**, 2293–2300 (2006).
- Savard, S., Amar, L., Plouin, P. F. & Steichen, O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension* **62**, 331–336, <https://doi.org/10.1161/HYPERTENSIONAHA.113.01060> (2013).
- Amar, L., Plouin, P. F. & Steichen, O. Aldosterone-producing adenoma and other surgically correctable forms of primary aldosteronism. *Orphanet journal of rare diseases* **5**, 9, <https://doi.org/10.1186/1750-1172-5-9> (2010).
- Funder, J. W. *et al.* The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* **101**, 1889–1916, <https://doi.org/10.1210/jc.2015-4061> (2016).
- Choi, M. *et al.* K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* **331**, 768–772, <https://doi.org/10.1126/science.1198785> (2011).
- Beuschlein, F. *et al.* Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet* **45**(440–444), 444e441–442, <https://doi.org/10.1038/ng.2550> (2013).
- Azizan, E. A. *et al.* Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nat Genet* **45**, 1055–1060, <https://doi.org/10.1038/ng.2716> (2013).
- Scholl, U. I. *et al.* Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet* **45**, 1050–1054, <https://doi.org/10.1038/ng.2695> (2013).
- Fernandes-Rosa, F. L. *et al.* Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* **64**, 354–361, <https://doi.org/10.1161/HYPERTENSIONAHA.114.03419> (2014).
- Nanba, K. *et al.* Targeted Molecular Characterization of Aldosterone-Producing Adenomas in White Americans. *J Clin Endocrinol Metab* **103**, 3869–3876, <https://doi.org/10.1210/jc.2018-01004> (2018).
- Akerstrom, T. *et al.* Activating mutations in CTNNB1 in aldosterone producing adenomas. *Sci Rep* **6**, 19546, <https://doi.org/10.1038/srep19546> (2016).
- Scholl, U. I. *et al.* Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype. *Clin Endocrinol (Oxf)* **83**, 779–789, <https://doi.org/10.1111/cen.12873> (2015).
- Boulikroun, S. *et al.* Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. *Endocrinology* **152**, 4753–4763, <https://doi.org/10.1210/en.2011-1205> (2011).
- Berthon, A. *et al.* WNT/beta-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Human molecular genetics* **23**, 889–905, <https://doi.org/10.1093/hmg/ddt484> (2014).
- El Wakil, A. & Lalli, E. The Wnt/beta-catenin pathway in adrenocortical development and cancer. *Molecular and cellular endocrinology* **332**, 32–37, <https://doi.org/10.1016/j.mce.2010.11.014> (2011).
- Fernandes-Rosa, F. L. *et al.* Functional histopathological markers of aldosterone producing adenoma and somatic KCNJ5 mutations. *Molecular and cellular endocrinology* **408**, 220–226, <https://doi.org/10.1016/j.mce.2015.01.020> (2015).
- Vouillarmet, J. *et al.* Aldosterone-Producing Adenoma With a Somatic KCNJ5 Mutation Revealing APC-Dependent Familial Adenomatous Polyposis. *J Clin Endocrinol Metab* **101**, 3874–3878, <https://doi.org/10.1210/jc.2016-1874> (2016).
- Pihlajoki, M., Dorner, J., Cochran, R. S., Heikinheimo, M. & Wilson, D. B. Adrenocortical zonation, renewal, and remodeling. *Front Endocrinol (Lausanne)* **6**, 27, <https://doi.org/10.3389/fendo.2015.00027> (2015).

26. El Wakil, A., Mari, B., Barhanin, J. & Lalli, E. Genomic analysis of sexual dimorphism of gene expression in the mouse adrenal gland. *Horm Metab Res* **45**, 870–873, <https://doi.org/10.1055/s-0033-1349881> (2013).
27. Heitzmann, D. *et al.* Invalidation of TASK1 potassium channels disrupts adrenal gland zonation and mineralocorticoid homeostasis. *The EMBO journal* **27**, 179–187 (2008).
28. Levasseur, A., St-Jean, G., Paquet, M., Boerboom, D. & Boyer, A. Targeted Disruption of YAP and TAZ Impairs the Maintenance of the Adrenal Cortex. *Endocrinology* **158**, 3738–3753, <https://doi.org/10.1210/en.2017-00098> (2017).
29. Dumontet, T. *et al.* PKA signaling drives reticularis differentiation and sexually dimorphic adrenal cortex renewal. *JCI Insight* **3**, <https://doi.org/10.1172/jci.insight.98394> (2018).
30. Grabek, A. *et al.* The Adult Adrenal Cortex Undergoes Rapid Tissue Renewal in a Sex-Specific Manner. *Cell Stem Cell*, <https://doi.org/10.1016/j.stem.2019.04.012> (2019).
31. Balmer, J. E. & Blomhoff, R. Gene expression regulation by retinoic acid. *J Lipid Res* **43**, 1773–1808, <https://doi.org/10.1194/jlr.r100015-jlr200> (2002).
32. Chapellier, B. *et al.* A conditional floxed (loxP-flanked) allele for the retinoic acid receptor alpha (RARalpha) gene. *Genesis* **32**, 87–90 (2002).
33. Romero, D. G. *et al.* Disabled-2 is expressed in adrenal zona glomerulosa and is involved in aldosterone secretion. *Endocrinology* **148**, 2644–2652 (2007).
34. Hershkovitz, L., Beuschlein, F., Klammer, S., Krup, M. & Weinstein, Y. Adrenal 20alpha-hydroxysteroid dehydrogenase in the mouse catabolizes progesterone and 11-deoxycorticosterone and is restricted to the X-zone. *Endocrinology* **148**, 976–988, <https://doi.org/10.1210/en.2006-1100> (2007).
35. Drelon, C. *et al.* PKA inhibits WNT signalling in adrenal cortex zonation and prevents malignant tumour development. *Nat Commun* **7**, 12751, <https://doi.org/10.1038/ncomms12751> (2016).
36. Ruggiero, C. & Lalli, E. Impact of ACTH Signaling on Transcriptional Regulation of Steroidogenic Genes. *Front Endocrinol (Lausanne)* **7**, 24, <https://doi.org/10.3389/fendo.2016.00024> (2016).
37. Cao, Y. *et al.* Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science* **344**, 913–917, <https://doi.org/10.1126/science.1249480> (2014).
38. Novoselova, T. V. *et al.* MRAP deficiency impairs adrenal progenitor cell differentiation and gland zonation. *FASEB J*, [fj201701274RR](https://doi.org/10.1096/fj.201701274RR), <https://doi.org/10.1096/fj.201701274RR> (2018).
39. Zennaro, M. C., Boulkroun, S. & Fernandes-Rosa, F. Genetic Causes of Functional Adrenocortical Adenomas. *Endocr Rev* **38**, 516–537, <https://doi.org/10.1210/er.2017-00189> (2017).
40. Leid, M., Kastner, P. & Chambon, P. Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends in biochemical sciences* **17**, 427–433 (1992).
41. Bushue, N. & Wan, Y. J. Retinoid pathway and cancer therapeutics. *Adv Drug Deliv Rev* **62**, 1285–1298, <https://doi.org/10.1016/j.addr.2010.07.003> (2010).
42. Perissi, V., Jepsen, K., Glass, C. K. & Rosenfeld, M. G. Deconstructing repression: evolving models of co-repressor action. *Nature reviews. Genetics* **11**, 109–123, <https://doi.org/10.1038/nrg2736> (2010).
43. Mark, M., Ghyselinck, N. B. & Chambon, P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annual review of pharmacology and toxicology* **46**, 451–480, <https://doi.org/10.1146/annurev.pharmtox.46.120604.141156> (2006).
44. Sell, S. Leukemia: stem cells, maturation arrest, and differentiation therapy. *Stem cell reviews* **1**, 197–205, <https://doi.org/10.1385/SCR.1.3:197> (2005).
45. Dihazi, G. H. *et al.* Proteomic characterization of adrenal gland embryonic development reveals early initiation of steroid metabolism and reduction of the retinoic acid pathway. *Proteome Sci* **13**, 6, <https://doi.org/10.1186/s12953-015-0063-8> (2015).
46. Zhou, J. *et al.* Transcriptome Pathway Analysis of Pathological and Physiological Aldosterone-Producing Human Tissues. *Hypertension* **68**, 1424–1431, <https://doi.org/10.1161/HYPERTENSIONAHA.116.08033> (2016).
47. Blomhoff, R. Transport and metabolism of vitamin A. *Nutr Rev* **52**, S13–23 (1994).
48. McPhillips, D. M., Kalin, J. R. & Hill, D. L. The pharmacokinetics of all-trans-retinoic acid and N-(2-hydroxyethyl)retinamide in mice as determined with a sensitive and convenient procedure. Solid-phase extraction and reverse-phase high performance liquid chromatography. *Drug Metab Dispos* **15**, 207–211 (1987).
49. Jordan, B. K., Shen, J. H., Olaso, R., Ingraham, H. A. & Vilain, E. Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. *Proc Natl Acad Sci USA* **100**, 10866–10871, <https://doi.org/10.1073/pnas.1834480100> (2003).
50. Vainio, S., Heikkila, M., Kispert, A., Chin, N. & McMahon, A. P. Female development in mammals is regulated by Wnt-4 signalling. *Nature* **397**, 405–409, <https://doi.org/10.1038/17068> (1999).
51. Briskin, C. *et al.* Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes & development* **14**, 650–654 (2000).
52. Mandel, H. *et al.* SERKAL syndrome: an autosomal-recessive disorder caused by a loss-of-function mutation in WNT4. *American journal of human genetics* **82**, 39–47, <https://doi.org/10.1016/j.ajhg.2007.08.005> (2008).
53. Kuwahara, A. *et al.* Tcf3 represses Wnt-beta-catenin signaling and maintains neural stem cell population during neocortical development. *PLoS One* **9**, e94408, <https://doi.org/10.1371/journal.pone.0094408> (2014).
54. Slusarski, D. C., Corces, V. G. & Moon, R. T. Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410–413, <https://doi.org/10.1038/37138> (1997).
55. Osei-Sarfo, K. & Gudas, L. J. Retinoic acid suppresses the canonical Wnt signaling pathway in embryonic stem cells and activates the noncanonical Wnt signaling pathway. *Stem cells*, <https://doi.org/10.1002/stem.1706> (2014).
56. Kim, A. C. *et al.* Targeted disruption of beta-catenin in Sf1-expressing cells impairs development and maintenance of the adrenal cortex. *Development* **135**, 2593–2602 (2008).
57. Heikkila, M. *et al.* Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology* **143**, 4358–4365 (2002).
58. He, L. *et al.* Gsdma3 regulates hair follicle differentiation via Wnt5a-mediated non-canonical Wnt signaling pathway. *Oncotarget* **8**, 100269–100279, <https://doi.org/10.18632/oncotarget.22212> (2017).
59. Zhang, H. L. *et al.* WNT4 acts downstream of BMP2 to mediate the regulation of ATRA signaling on RUNX1 expression: Implications for terminal differentiation of antler chondrocytes. *J Cell Physiol* **233**, 1129–1145, <https://doi.org/10.1002/jcp.25972> (2018).
60. Elizalde, C. *et al.* Distinct roles for Wnt-4 and Wnt-11 during retinoic acid-induced neuronal differentiation. *Stem cells* **29**, 141–153, <https://doi.org/10.1002/stem.562> (2011).
61. Scortegagna, M. *et al.* The E3 ubiquitin ligase Siah1 regulates adrenal gland organization and aldosterone secretion. *JCI Insight* **2**, <https://doi.org/10.1172/jci.insight.97128> (2017).
62. Lee, J. H. *et al.* Small heterodimer partner SHP mediates liver X receptor (LXR)-dependent suppression of inflammatory signaling by promoting LXR SUMOylation specifically in astrocytes. *Sci Signal* **9**, ra78, <https://doi.org/10.1126/scisignal.aaf4850> (2016).
63. Otis, M., Campbell, S., Payet, M. D. & Gallo-Payet, N. Expression of extracellular matrix proteins and integrins in rat adrenal gland: importance for ACTH-associated functions. *The Journal of endocrinology* **193**, 331–347, <https://doi.org/10.1677/JOE-07-0055> (2007).

64. Chamoux, E., Bolduc, L., Lehoux, J. G. & Gallo-Payet, N. Identification of extracellular matrix components and their integrin receptors in the human fetal adrenal gland. *J Clin Endocrinol Metab* **86**, 2090–2098, <https://doi.org/10.1210/jcem.86.5.7462> (2001).
65. Lerario, A. M., Finco, I., LaPensee, C. & Hammer, G. D. Molecular Mechanisms of Stem/Progenitor Cell Maintenance in the Adrenal Cortex. *Front Endocrinol (Lausanne)* **8**, 52, <https://doi.org/10.3389/fendo.2017.00052> (2017).
66. Pellerin, S., Keramidas, M., Chambaz, E. M. & Feige, J. J. Expression of laminin and its possible role in adrenal cortex homeostasis. *Endocrinology* **138**, 1321–1327, <https://doi.org/10.1210/endo.138.3.4962> (1997).
67. Matsui, T. Differential activation of the murine laminin B1 gene promoter by RAR alpha, ROR alpha, and AP-1. *Biochemical and biophysical research communications* **220**, 405–410, <https://doi.org/10.1006/bbrc.1996.0418> (1996).
68. Barber, T., Esteban-Pretel, G., Marin, M. P. & Timoneda, J. Vitamin A deficiency and alterations in the extracellular matrix. *Nutrients* **6**, 4984–5017, <https://doi.org/10.3390/nu6114984> (2014).
69. Pitynski, K., Litwin, J. A., Nowogrodzka-Zagorska, M. & Miodonski, A. J. Vascular architecture of the human fetal adrenal gland: a SEM study of corrosion casts. *Ann Anat* **178**, 215–222, [https://doi.org/10.1016/S0940-9602\(96\)80050-1](https://doi.org/10.1016/S0940-9602(96)80050-1) (1996).
70. Murakami, T., Oukouchi, H., Uno, Y., Ohtsuka, A. & Taguchi, T. Blood vascular beds of rat adrenal and accessory adrenal glands, with special reference to the corticomedullary portal system: a further scanning electron microscopic study of corrosion casts and tissue specimens. *Arch Histol Cytol* **52**, 461–476 (1989).
71. Feige, J. J. Angiogenesis in adrenocortical physiology and tumor development. *Ann Endocrinol (Paris)* **70**, 153–155, <https://doi.org/10.1016/j.ando.2009.02.005> (2009).
72. Goodwin, A. M. & D'Amore, P. A. Wnt signaling in the vasculature. *Angiogenesis* **5**, 1–9 (2002).
73. Olsen, J. J. *et al.* The Role of Wnt Signalling in Angiogenesis. *Clin Biochem Rev* **38**, 131–142 (2017).
74. Karaman, S., Leppanen, V. M. & Alitalo, K. Vascular endothelial growth factor signaling in development and disease. *Development* **145**, <https://doi.org/10.1242/dev.151019> (2018).
75. Pourjafar, M. *et al.* All-trans retinoic acid preconditioning enhances proliferation, angiogenesis and migration of mesenchymal stem cell *in vitro* and enhances wound repair *in vivo*. *Cell Prolif* **50**, <https://doi.org/10.1111/cpr.12315> (2017).
76. Liang, C., Guo, S. & Yang, L. Effects of alltrans retinoic acid on VEGF and HIF1alpha expression in glioma cells under normoxia and hypoxia and its antiangiogenic effect in an intracerebral glioma model. *Mol Med Rep* **10**, 2713–2719, <https://doi.org/10.3892/mmr.2014.2543> (2014).
77. Freedman, B. D. *et al.* Adrenocortical zonation results from lineage conversion of differentiated zona glomerulosa cells. *Developmental cell* **26**, 666–673, <https://doi.org/10.1016/j.devcel.2013.07.016> (2013).
78. Funder, J. W. *et al.* Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* **93**, 3266–3281, <https://doi.org/10.1210/jc.2008-0104> (2008).
79. Letavernier, E. *et al.* Blood pressure outcome of adrenalectomy in patients with primary hyperaldosteronism with or without unilateral adenoma. *J Hypertens* **26**, 1816–1823 (2008).
80. Brysbaert, G., Pellay, F. X., Noth, S. & Benecke, A. Quality assessment of transcriptome data using intrinsic statistical properties. *Genomics Proteomics Bioinformatics* **8**, 57–71, [https://doi.org/10.1016/S1672-0229\(10\)60006-X](https://doi.org/10.1016/S1672-0229(10)60006-X) (2010).
81. Noth, S., Brysbaert, G. & Benecke, A. Normalization using weighted negative second order exponential error functions (NeONORM) provides robustness against asymmetries in comparative transcriptome profiles and avoids false calls. *Genomics Proteomics Bioinformatics* **4**, 90–109, [https://doi.org/10.1016/S1672-0229\(06\)60021-1](https://doi.org/10.1016/S1672-0229(06)60021-1) (2006).
82. Wilhelm, E. *et al.* TAF6delta orchestrates an apoptotic transcriptome profile and interacts functionally with p53. *BMC Mol Biol* **11**, 10, <https://doi.org/10.1186/1471-2199-11-10> (2010).
83. Noth, S. & Benecke, A. Avoiding inconsistencies over time and tracking difficulties in Applied Biosystems AB1700/Panther probe-to-gene annotations. *BMC Bioinformatics* **6**, 307, <https://doi.org/10.1186/1471-2105-6-307> (2005).
84. Basu, R. *et al.* Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. *Journal of the American College of Cardiology* **69**, 805–819, <https://doi.org/10.1016/j.jacc.2016.11.064> (2017).
85. Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**, 611–622, <https://doi.org/10.1373/clinchem.2008.112797> (2009).
86. Becavin, C. & Benecke, A. New dimensionality reduction methods for the representation of high dimensional 'omics' data. *Expert Rev Mol Diagn* **11**, 27–34, <https://doi.org/10.1586/erm.10.95> (2011).
87. Becavin, C., Tchitchek, N., Mintsia-Eya, C., Lesne, A. & Benecke, A. Improving the efficiency of multidimensional scaling in the analysis of high-dimensional data using singular value decomposition. *Bioinformatics* **27**, 1413–1421, <https://doi.org/10.1093/bioinformatics/btr143> (2011).

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Author Contributions

M.-C. Zennaro and S. Boulkroun designed research; R.M. El Zein, AH Soria, J.F. Golib-Dzib, F.L. Fernandes-Rosa, A. Benecke, M. Poglitsch, M.-C. Zennaro and S. Boulkroun analyzed data; R.M. El-Zein, A.H. Soria, A.J. Rickard, B. Samson-Couterie, I. Giscos-Douriez, A. Rocha, M. Poglitsch, M.-C. Zennaro and S. Boulkroun performed research; R.M. El Zein, M.-C. Zennaro and S. Boulkroun wrote the paper; C.E. Gomez-Sanchez, N. Ghyselink and L. Amar contributed new reagents, mice or tissue samples.

Additional Information

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Online Supplementary Information

Retinoic acid receptor α as a novel contributor to adrenal cortex structure and function through interactions with Wnt and Vegfa signalling

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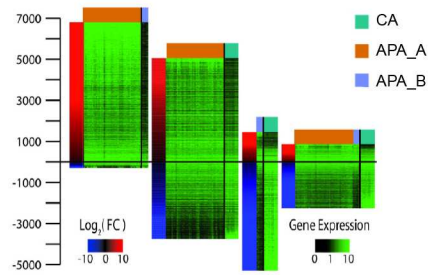
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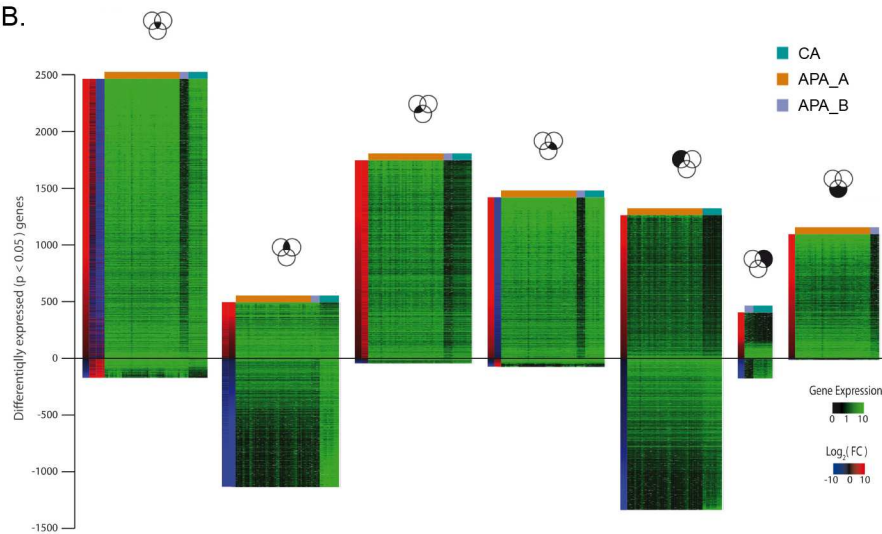
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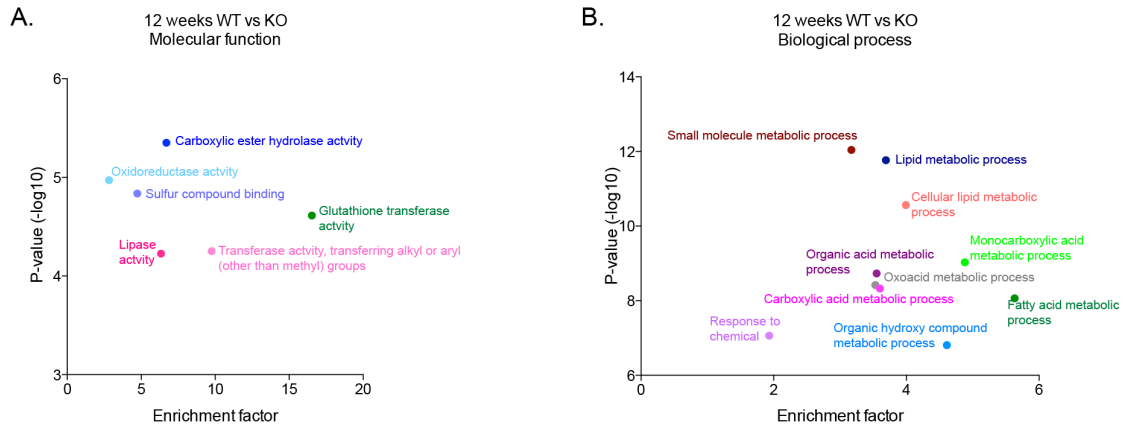
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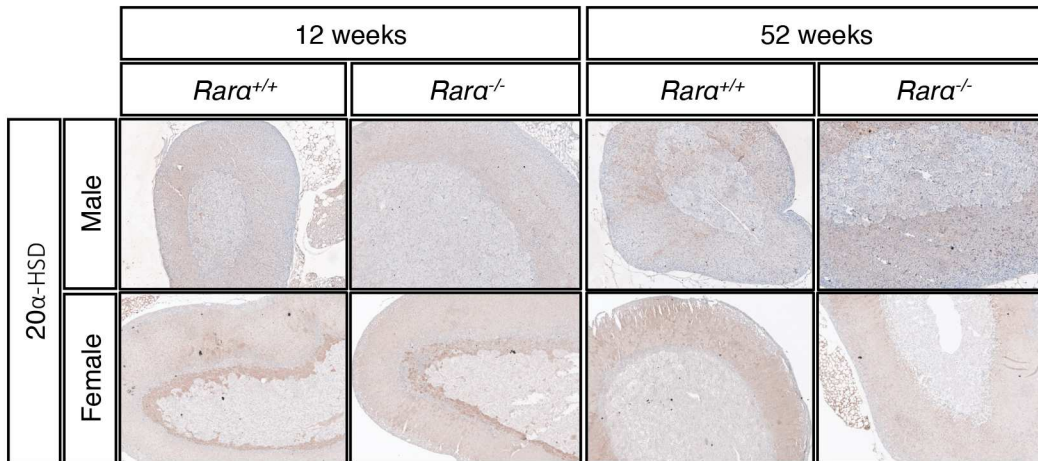
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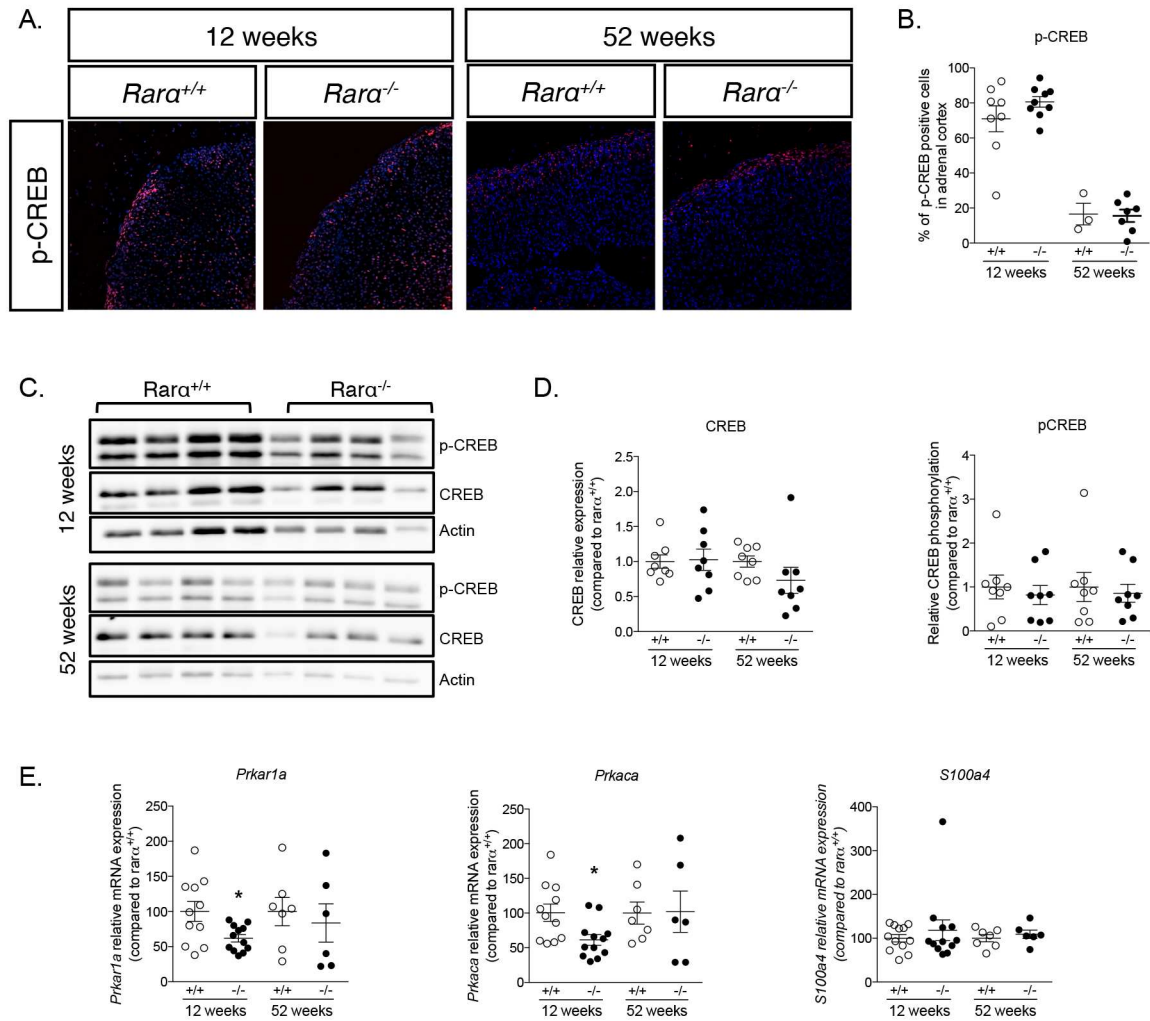
Supplementary Figure 1: Heat-maps of the seven sets of differentially expressed genes from pairwise comparisons among two subpopulations of APA and controls. (A) Heat-maps of the statistically significantly (post-hoc $P < 0.01$) differentially expressed genes between control adrenals (cyan), APA_A (orange) and APA_B (purple). (B) Heat-maps of the statistically significantly (post-hoc $p < 0.05$) differentially expressed genes between the seven groups of samples identified. Samples separated into three different groups are depicted as control adrenals (CA), APA Group A (APA_A) and APA Group B. The seven sets of overlapping and paired-wise-comparison-specific genes are delineated at the bottom. (C) Expression of *RAR α* was investigated by RT-qPCR on mRNA extracted from 6 control adrenals and 11 APA. Values are presented as the mean \pm SEM; p values were calculated using a two-sided Mann-Whitney test. *, $p < 0.05$.



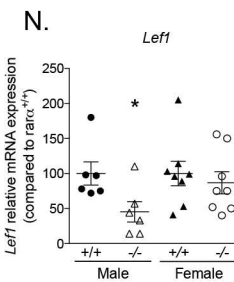
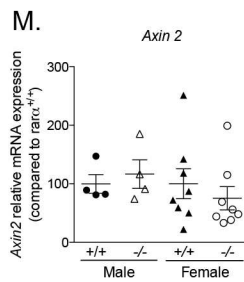
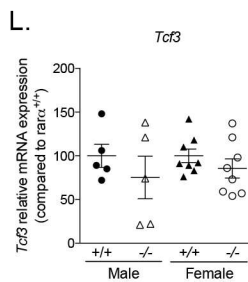
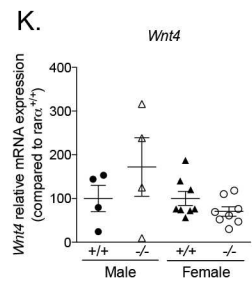
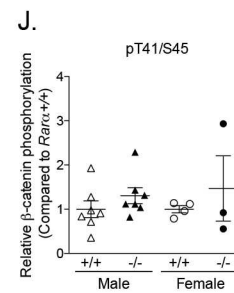
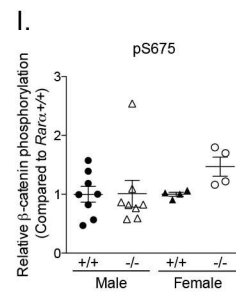
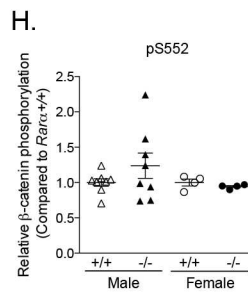
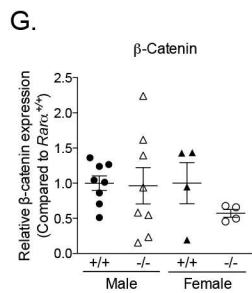
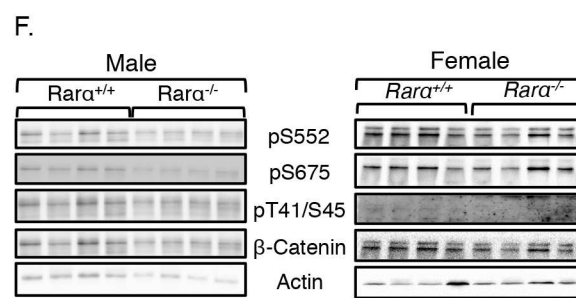
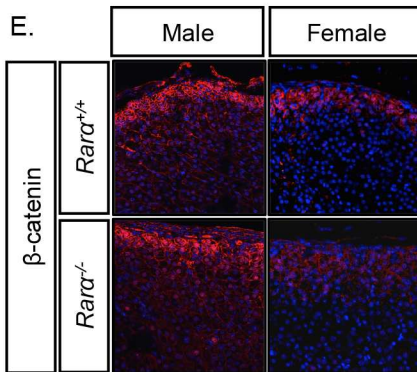
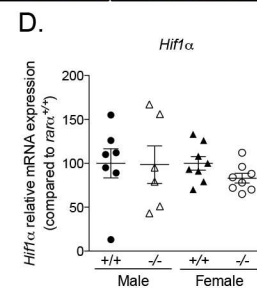
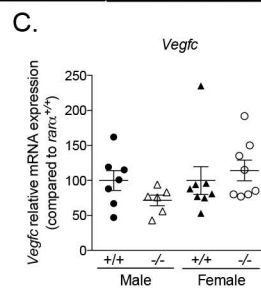
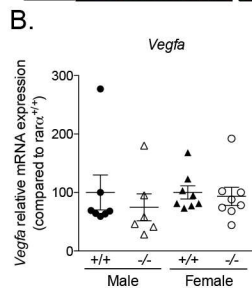
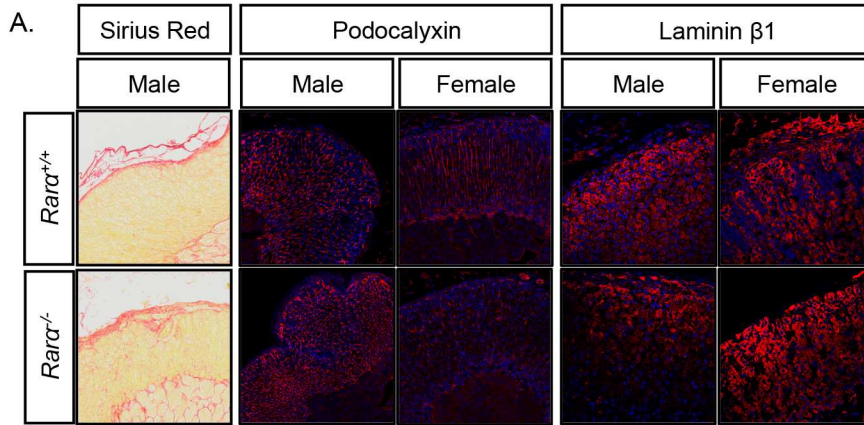
Supplementary Figure 2: Molecular function and biological process enrichment analyses in *Rara*^{-/-} mice compared to *Rara*^{+/+}. Molecular function and biological process enrichment analyses were performed using Gene Ontology. (A) Molecular function and (B) biological process enrichments were determined using the list of differentially expressed genes in adrenal from 12 weeks old *Rara*^{+/+} and *Rara*^{-/-} mice.



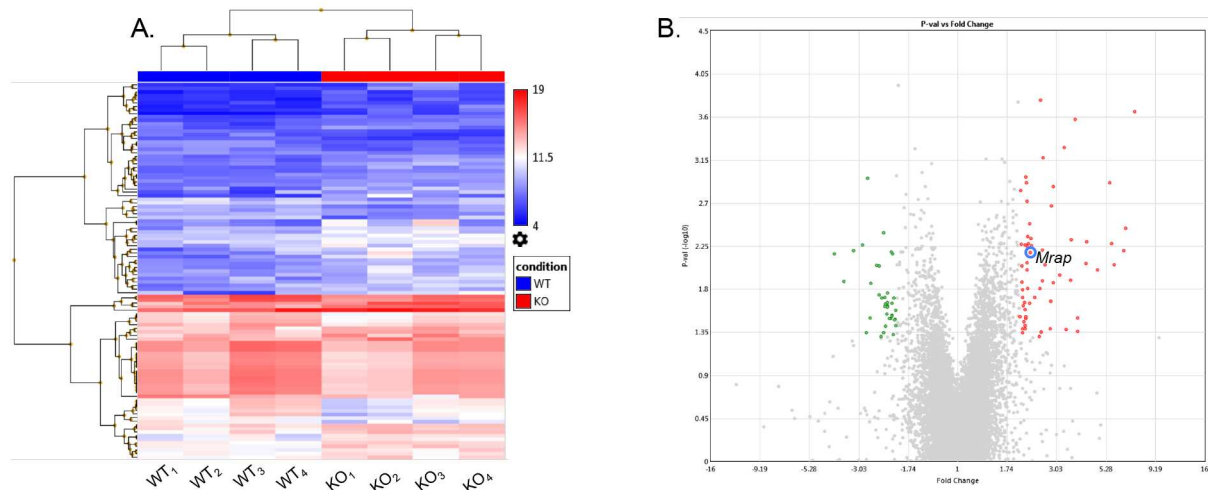
Supplementary Figure 3: *Rara* does not affect X-zone. 20- α HSD expression in adrenals from male and female 12 and 52 weeks old *Rara*^{+/+} and *Rara*^{-/-} mice detected by immunohistochemistry.



Supplementary Figure 4: *Rara* does not affect PKA signaling pathway in male mice. (A) p-CREB expression in adrenals from 12 and 52 weeks old *Rara*^{+/+} and *Rara*^{-/-} mice detected by immunofluorescence. (B) Percentage of p-CREB positive cells was quantified using an automated imaging platform. (C) Expression of CREB and p-CREB in response to *Rara* invalidation in adrenal was evaluated by western blot in eight *Rara*^{+/+} and *Rara*^{-/-} adrenal from 12 and 52 weeks old mice. (D) Quantification of CREB and p-CREB expression detected by western blot. (E) The expression of *Prkar1a*, *Prkaca* and *S100a4* was investigated by RT-qPCR. Values are presented as mean \pm SEM; p values were calculated using a Mann-Whitney test or *t*-test. *, p < 0.05.



Supplementary Figure 5: *Rara* inactivation does not alter Wnt/ β -catenin pathway in 52 weeks old male and female mice. (A) Expression of β -catenin was evaluated by immunofluorescence in 12 weeks old male and female *Rara*^{+/+} and *Rara*^{-/-} mice. (B) Expression and phosphorylation of β -catenin in response to *Rara* inactivation. Proteins were extracted from total adrenal and submitted to western blot analysis. Phosphorylation/dephosphorylation in activating (pS552 and pS675) and inactivating (pT41/S45) residues and total expression of β -catenin was investigated. (C-F) Quantification of β -catenin expression (C) and of phospho-specific signals in inactivating (D) and activating (E, F) residues was performed in *Rara*^{+/+} and *Rara*^{-/-} adrenal. (G-J) The expression of *Wnt4* (G), *Tcf3* (H), *Lef1* (I) and *Axin2* (J) was investigated by RT-qPCR on mRNA extracted from 4 to 8 adrenals from *Rara*^{+/+} and *Rara*^{-/-} male and female mice. Values are presented as mean \pm SEM; p values were calculated using a Mann-Whitney test or *t*-test. *, p<0.05



Supplemental Figure 6: *Rara* modifies adrenal transcriptome profile at 52 weeks of age.

(A) Hierarchical clustering of samples using the 243 genes differentially expressed genes in adrenals from 52 weeks old *Rara*^{+/+} and *Rara*^{-/-} male animals (4 animals per group). (B) Volcano plot showing the differentially expressed genes in 52 weeks old *Rara*^{+/+} and *Rara*^{-/-} animals. The x-axis is the fold change between the two conditions; the adjusted p value based on $-\log_{10}$ is reported on the y-axis. Genes significantly different are highlighted as green (down-regulated in *Rara*^{-/-} mice) or red (up-regulated in *Rara*^{-/-} mice) dots.

6.4 Discussion and perspectives

In summary, we have shown for the first time that Rar α is directly implicated in adrenal cortex development and functioning by interacting with Wnt and Vegfa signaling. We show that knock-out of Rar α in mice leads to a disorganization of the adrenal cortex, with increased adrenal cortex size in female mice, and increased cell proliferation in male adrenal cortex. This is associated to a disorganized vessel architecture and extracellular matrix composition. On a molecular level, non-canonical Wnt signaling and Vegfa were downregulated in young KO male mice, this was associated with a decrease in the expression of steroidogenic genes. PKA signaling was not affected.

The particularity of RAR α compared with other isoforms is its expression in different tissues from embryonic life to adulthood, while RAR β and RAR γ are restricted to different time points during human life (266, 267). Decreased expression of RAR α in APA could either be a secondary effect, or be the trigger to disrupted cell proliferation and adrenocortical microenvironment homeostasis. Based on our results, Rar α seems to be essential for normal adrenal gland development. Any molecular disruption, despite not being visible at an older age, induce irreversible phenotypic alterations. Remarkably, female and male mice do not exhibit the same molecular phenotypes at the ages that were analyzed (12 and 52 weeks). This could be due to the faster rate of cell renewal in female mice, female mice adrenal glands might have expressed the molecular alterations at a younger age.

Disorganization of vessel architecture may lead to a disruption of normal adrenal cortex function and cell homeostasis. The adrenal gland is one of the most highly vascularized organs, allowing to maintain cellular homeostasis and metabolism and steroidogenesis, as well as the diffusion of hormones into the blood stream. Interestingly, our group has previously reported reduced vascularization in the adrenal cortex adjacent to APA (187). Steroidogenesis is regulated through different signals such as Ang II and changes in extracellular K⁺ levels in ZG cells, and ACTH in ZF cells. Vascular abnormalities, by impairing correct distribution of stimulatory signals to cells, may lead to a disruption of steroidogenesis in the adrenal cortex. This could explain the reduction in steroidogenic gene expression that is observed in KO mice, in particular *Cyp11b1* and other steroidogenic genes, as the ZF exhibits the most severe disorganization in Rar α KO. The decreased Mrap expression in Rar α KO mice suggests a role for ACTH in the observed adrenal gland

phenotype. Moreover, alterations in vessel architecture could induce changes in the metabolism of the cells. This could lead to significant deregulation, which could eventually lead to dysfunction of methylation, epigenetic abnormalities and tumorigenesis.

Retinoic acid signaling has been shown to activate and stabilize PKA signaling in the human leukemia cell line HL60 during cell differentiation (268). This occurs by stabilization of the regulatory RII α subunit of PKA and increase in the protein levels of both regulatory and catalytic subunits of PKA, increasing the overall activity of PKA signaling and thus the phosphorylating activity of PKA. Consistently, in our mouse model, KO of Rar α reduced mRNA levels of both the catalytic and regulatory subunits of PKA. Yet, this reduction did not affect the activity of PKA signaling, with no differences in p-CREB between mouse groups. PKA activity is necessary for ZF cell differentiation from ZG cells and maintenance of normal adrenal cortex zonation (81). RAR α is a direct target of PKA and phosphorylation by PKA affects its nuclear localization and transcriptional activity (269). Disruptions in this signaling could also contribute to the disorganization of the ZF in Rar α KO mice.

Furthermore, changes in the extracellular matrix could affect cell migration and stability which could be a direct effect of the KO of Rar α . Laminin β 1 has been shown to have retinoic acid response elements in its promoter region (270). ECM components have also been shown to affect fetal adrenal cell proliferation, apoptosis, morphology and steroidogenic profile (33).

mTOR signaling has been shown to be involved in APA (115) and its inhibition decreased cell proliferation and aldosterone production in the adrenocortical cell line H295R (116). Interactions between retinoic acid signaling and mTOR signaling have been reported in many studies. Interestingly, RAR α has been recently shown to interact with mTOR signaling in the stabilization and synaptic plasticity in the hippocampus where KO of Rar α leads to increased activity of mTOR (271). Beside its role in regulating Vegfa expression (111), mTOR could also interact with Rar α in regulating cell proliferation leading to increased cell proliferation and adrenal cortex size in Rar α KO mice. Upregulation of mTOR signaling in APA could be a secondary event to reduced expression of RAR α in APA.

Although we did not find any alterations in β -catenin activity in Rar α KO mice, abnormalities in this signaling pathway could occur at a younger age during development and induce anomalies in the cellular organization of the adrenal cortex. This could involve dysregulation of cadherin junctions, which is one of the functions of β -catenin. Interactions

between ECM and Wnt/ β -catenin have been reported in many studies and alterations in these interactions may affect cellular migration into the ZF. Although it has not been studied yet, adrenal cells may require cadherin for proper migration into the ZF and radial organization of the cells in the adrenal cortex. Moreover, Wnt4 deficiency in these adrenal glands could also induce the disorganization of the ZF due to its role in cell sorting during cell migration from the adrenal capsule (79). Wnt4 could also contribute to the disorganization of the adrenal cortex through the Wnt/PCP pathway, which is involved in coordinated cell migration (272).

The adrenal phenotype of Rar α KO mice could also result from indirect paracrine, endocrine or systemic regulations. ACTH stimulates the expression of VEGF mRNA through a post-transcriptional mechanism (90). ACTH is also required for the maintenance of a normal adrenal vasculature (91). It has been proposed that VEGF reduction is a secondary effect of ACTH deprivation. VEGF dysregulation itself can induce abnormalities in the basal membrane by causing its degradation by metalloproteases (273, 274). Although we have not measured plasma levels of ACTH in Rar α KO mice, slight modifications of ACTH may contribute to the adrenal phenotype of our KO mouse model.

Finally, retinoic acid signaling is a recognized therapeutic target in different diseases. Unpublished work from our laboratory on H295R cells using 9-cis RA or ATRA showed a significant decrease in viable cells when treated with either compound in a time and dose dependent manner. 9-cis RA significantly decreased cell viability and steroid hormone secretion in a concentration- and time-dependent manner in the H295R cells and reduced tumour growth in an *in vivo* xenograft model (275). Whether RAR α targeting would be of beneficial value to treat patients with APA is yet to be determined.

PART 2: ROLE OF THE MINERALOCORTICOID RECEPTOR IN
THE PHYSIOLOGY OF THE ADRENAL CORTEX AND THE
DEVELOPMENT OF APA

7 THESIS WORK

7.1 Aldosterone and the mineralocorticoid receptor

Despite changes in salt intake, the kidney is capable of maintaining sodium and water levels within very narrow margins. By doing so, it controls blood volume and pressure. One of the major hormones contributing to this homeostasis is aldosterone, which is the most potent sodium-retaining factor in the organism.

As discussed previously, aldosterone production is stimulated either by the increases in extracellular K^+ levels or by Ang II, which both have synergistic effects in the aim of normalizing K^+ levels and BP respectively. It is also induced acutely by ACTH while chronic exposure represses aldosterone production possibly due to reduction in AT1 receptor expression in these cells following ACTH treatment (52, 276). Aldosterone levels follow a circadian rhythm and vary throughout the day with highest plasmatic levels in the morning and lower levels during the day (277). Aldosterone is produced in a daily quantity of 100 to 150 μg .

Aldosterone acts mainly by binding to the MR. The MR is a hormone-dependent transcription factor, which upon ligand binding, translocates into the nucleus of the cell and binds to hormone response elements (MRE, mineralocorticoid response element, or GRE, glucocorticoid response element) in the regulatory regions of target genes to either induce or inhibit their transcription.

The MR is expressed in a variety of tissues. In the kidney, the MR is expressed specifically in the aldosterone sensitive distal nephron (ASDN), which comprises the distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting duct (CD), where aldosterone exerts its physiologic activity. It is important to note that MR is not only a receptor for aldosterone, but binds aldosterone and cortisol with the same affinity. As cortisol is much more abundant in the circulation than aldosterone (100 to 1000 fold more), in aldosterone-responsive cells of the kidney and colon, the expression of the enzyme 11- β hydroxysteroid dehydrogenase type 2 (11- β HSD2) allows hormone selectivity. Indeed, 11- β HSD2 catalyzes the conversion of cortisol (in man) and corticosterone (in rats and mice) to their inactive keto-derivatives cortisone and 11-dehydrocorticosterone, which are unable to bind the MR. This prevents the illicit occupation of MR by glucocorticoids, allowing aldosterone to exert its

mineralocorticoid effect. Moreover, unlike aldosterone, which circulates in a free form, corticosteroid-binding globulins bind cortisol and corticosterone with high affinity and capacity, reducing the levels of free glucocorticoids to ~10% (278). In other tissues, such as the brain and adipose tissue, MR is mostly occupied by glucocorticoids, as 11- β HSD2 is not expressed. It is interesting to note that despite having the same binding affinity, the kinetics of aldosterone and glucocorticoid binding to MR are not exactly the same, the rate of dissociation of glucocorticoids is almost 5 times faster than that of aldosterone (279).

In the kidney, the MR regulates sodium reabsorption and potassium secretion, and indirectly increases water reabsorption, which is an osmotic effect related to increased sodium reabsorption. Thus, aldosterone regulates blood volume and blood pressure by maintaining electrolyte and fluid homeostasis. MR is also largely expressed in blood vessels, in both smooth muscular and endothelial cells, where it affects arterial stiffness. Among known MR target genes are genes coding for proteins involved in transepithelial sodium transport, such as the amiloride sensitive epithelial sodium channel (ENaC) and the Na⁺/K⁺ -ATPase, as well as their regulatory proteins such as SGK1 (serum and glucocorticoid regulated kinase 1) and GILZ (glucocorticoid induced leucine zipper) (280) (Fig. 12).

Heterozygous mutations of the MR lead to pseudohypoaldosteronism type 1, a rare disease of mineralocorticoid resistance (281). The disease presents in the new-born with weight loss, failure to thrive, vomiting and dehydration, associated with hyperkalemia and metabolic acidosis, despite elevated plasma renin and aldosterone levels. Homozygous MR KO mice present with symptoms resembling pseudohypoaldosteronism type 1, and die within 10 days after birth due to sodium and water loss. This is due to reduced ENaC expression in the kidneys and colon. They also show hyperkalemia and increased levels of renin, Ang II and aldosterone (282). Remarkably, these mice can be rescued by NaCl supplementation (283), similar to what is observed in the human disease.

7.2 Transcriptional regulation by the mineralocorticoid receptor

The MR is encoded by the *NR3C2* gene and is part of the steroid receptor subfamily of nuclear receptors. These are ligand-activated transcription factors that regulate transcription by binding to hormone responsive elements located in the regulatory regions of target genes (284). MR has a very high degree of homology with the GR, both in their ligand-binding

domain (57% homology) and their DNA-binding domain (94% homology) (285). Similar to other nuclear receptors, the MR is composed of 3 distinct domains. The N-terminal region encompasses an autonomous activation function 1 (AF1), which is thought to be constitutively active and facilitates the interaction between MR and transcriptional coregulators. A central DBD is composed of two zinc-finger structures that are involved in DNA binding and receptor dimerization (286). The LBD encompasses an activation function 2 (AF2) and is involved in ligand binding and interaction with heat shock protein 90 (HSP90), dimerization, as well as interaction with transcriptional coregulators.

In the absence of hormone, the MR is located in the cytoplasm of the cell, where it is associated to chaperone proteins such as Hsp90 (heat shock protein 90) and FKBP51 and also interacts with HSP70, p23 and p48 proteins (287-289). These chaperone proteins stabilize the MR and facilitate hormone binding. Following aldosterone binding, a conformational change occurs in the receptor, FKBP51 dissociates from the complex and is replaced by FKBP52, the complex then translocates into the nucleus, where MR dissociates from the complex (290). MR then binds to GRE or MRE on regulatory regions of target genes, where it associates with the co-activator complex SRC-1/P300/CBP (steroid receptor co-activator 1, binding protein 300 and CREB binding protein respectively) and chromatin remodeling proteins, allowing recruitment of RNA polymerase II and transcriptional regulation. The analysis of genomic binding of MR in a kidney cell line (HK eGFP-hMR) using chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing, showed that most MR binding sites (MBS) occur at around 10 kilo base pairs (kbs) from the transcription start site of the gene (291). MR can bind to its MBS as soon as 15 minutes following aldosterone treatment and this binding is dynamic with oscillating cycles of MR binding and release and a cycle rhythm between 10 to 25 minutes. Interestingly, the RNA-polymerase II enzyme closely follows MR binding cycles with a delay of 8 minutes (292).

MR regulates different genes in a tissue-specific manner. In contrast to renal cells, where MR regulates the expression of *SCNN1A*, *ATP1A1*, *SGK1*, *NDRG2*, *GILZ* and other genes implicated directly or indirectly in sodium ion transport, in vascular smooth muscle cells Jaffe and Mendelson have shown that most of the MR-regulated genes consist of pro-fibrotic, vascular calcification and inflammatory immune response genes (293). Aldosterone also acts on the central nervous system, notably the hippocampus and the amygdala, where MR binding and activation regulates stress response and salt appetite (294-296). When infused intracerebroventricularly, aldosterone increases blood pressure in rats (297). Aldosterone has

also been shown to increase salt appetite and intake by its effects on the amygdala, inducing hypertension and organ damage (298). In the hippocampus, MR-regulated genes include Jun dimerization protein 2 (Jdp2), Nitric oxide synthase 1 adaptor protein (Nos1ap) and Suv3 like RNA helicase (Supv3 11) (299).

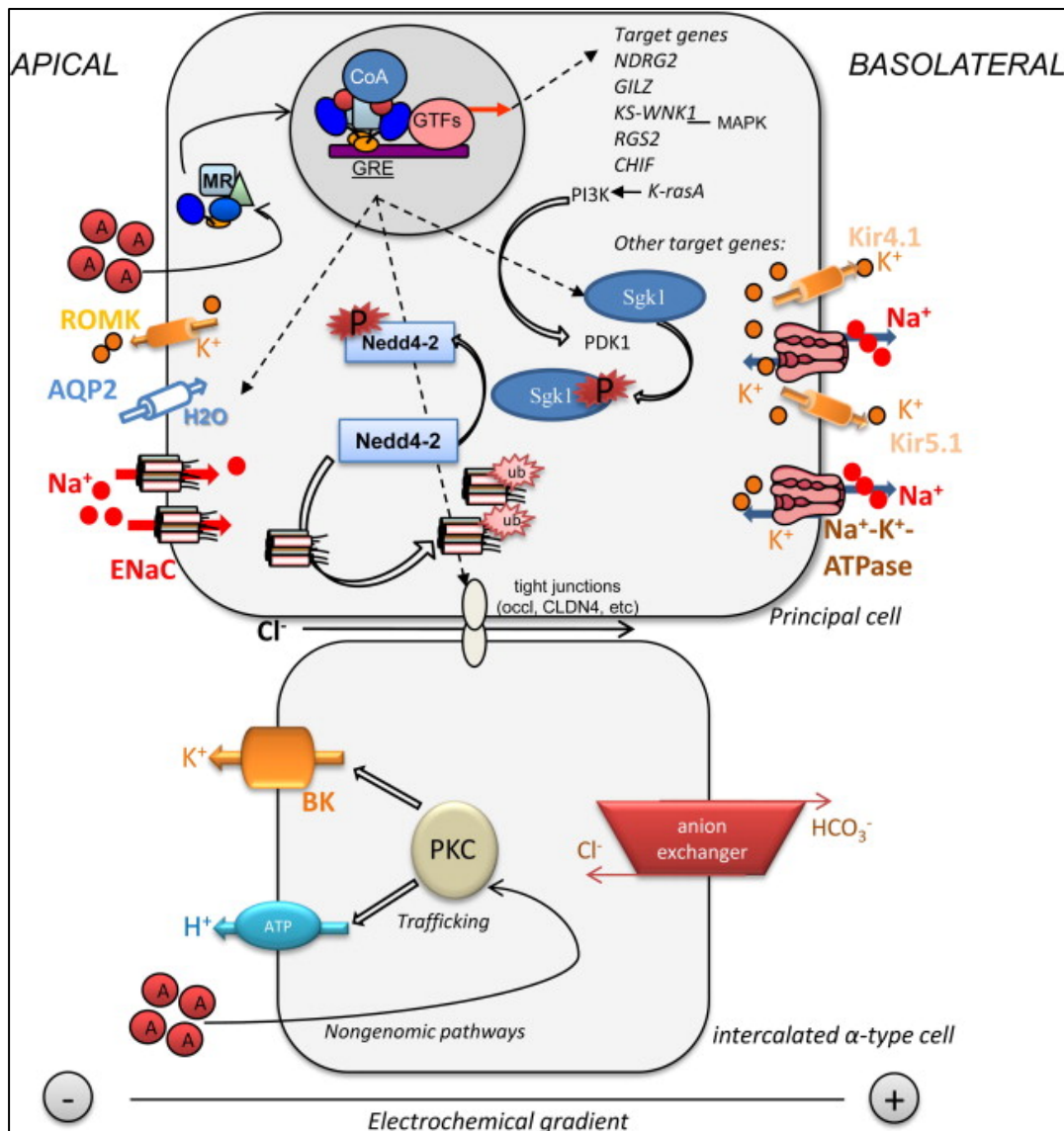


Figure 12. MR effects in kidney ASDN cells in regulating sodium and water balance (300). In ASDN cells, following the activation of MR by aldosterone, MR translocates to the nucleus and binds to regulatory regions of hormone response elements. This activation involves the recruitment of transcriptional coregulators in order to activate the transcription machinery. This leads to the expression of aldosterone target genes, notably, genes involved in sodium ion transport. Those genes include the ENaC sodium channel, the Na⁺-K⁺-ATPase and regulatory proteins such as SGK1, NDRG2 and GILZ. In the absence of aldosterone, Nedd4-2 a ubiquitin ligase, will ubiquitinate ENaC leading to its degradation by the proteasome. Aldosterone stimulation will induce the phosphorylation of sgk1, which will phosphorylate Nedd4-2. This phosphorylation inhibits the interaction between Nedd4-2 and ENaC, thus increasing its localization on the apical cell membrane. Na⁺ enters the cell through ENaC at the apical side of the cell and exits into the interstitial fluid through the Na⁺-K⁺-ATPase on the basolateral side of the cell. By non-genomic pathways, aldosterone rapidly increases the activity of the large conductance potassium channel. Aldosterone also induces hydrogen ion excretion into urine via the H⁺-ATPase in intercalated cells. This leads to a release of bicarbonate into the bloodstream through the chloride/bicarbonate exchanger at the basolateral membrane.

In the ASDN, aldosterone via the MR, regulates the rapid transcription of genes encoding proteins that are involved in the regulation of ENaC trafficking and function. These proteins include SGK1, GILZ, NDRG2 and 14-3-3 proteins (301-303). Indeed, ENaC channels undergo a constant turnover by an endocytosis/exocytosis process. When ENaC channels are synthesized at the endoplasmic reticulum (ER), they are transported to the cell surface via Golgi apparatus. Conversely, ENaC channels which are already at the cell surface undergo endocytosis by the action of Nedd4-2, which is part of the epithelial sodium channel regulatory complex (ERC), an E3 ubiquitin protein ligase that induces the removal of ENaC from the cell surface. This mechanism involves binding of Nedd4-2 to ENaC. Furthermore, Nedd4-2 ubiquitinates ENaC, leading to channel internalization where it is recycled or degraded after monoubiquitination or polyubiquitination (304, 305). SGK1 on the other hand increases the expression of ENaC at the cell surface, by phosphorylating Nedd4-2. Phosphorylation of Nedd4-2 promotes its binding to 14-3-3 proteins and inhibits Nedd4-2's ability to bind to ENaC. GILZ induces the association of SGK1 with the ERC, thus increasing the phosphorylation of Nedd4-2 (306, 307). The net result of these effects is the increase of the abundance of ENaC on the cellular membrane.

7.3 Cardiovascular effects of aldosterone and MR

Patients with PA have a higher risk of developing myocardial infarction, stroke, atrial fibrillation, renal impairment than patients with essential hypertension. They also suffer from severe left ventricular hypertrophy and increased wall thickness and diastolic dysfunction (19). Aldosterone has also been shown to be responsible for endothelial dysfunction and atherosclerosis by modulating ICAM-1 expression and leucocyte adhesion in endothelial cells in type 2 diabetes mellitus, metabolic syndrome and hypercholesterolemia (151, 308-311).

In 1942 it was reported that desoxycorticosterone (a precursor of aldosterone) induces nephrosclerosis and cardiac hypertrophy (312) and that treatment with spironolactone, an MR antagonist (MRA), protected rats from aldosterone-induced cardiac necrosis (313). Spironolactone or eplerenone, another MRA, added on top of standard therapy, substantially reduce the risk of both morbidity and death in severe heart failure (314) and in heart failure post myocardial infarction (315). In addition, MR blockade with eplerenone reduces both the risk of death and the risk of hospitalization and the incidence of new onset atrial fibrillation or

flutter in patients with systolic heart failure and mild symptoms (316, 317). MR activation by aldosterone in endothelial cells results in increased oxidative stress and reduced nitric oxide activity (318). In patients with heart failure, MR antagonism by spironolactone increases nitric oxide bioactivity and improves endothelium-dependent vasodilation (319). MR also seems to play an important role in proteinuric kidney disease (320), and treatment with MRA reduces the proteinuria in patients with chronic kidney disease (321). In animals, MR blockade reduced obesity-related alterations in the expression of pro-inflammatory, prothrombotic and insulin sensitizing factors in adipose tissue in obese diabetic mice; MR blockade also increased the expression of adiponectin in the heart and adipose tissue of these mice, possibly contributing to the cardioprotective effect of MR blockade (322). MR knockout in vascular smooth muscle cells leads to decreased blood pressure in aging mice, due to reduced vascular myogenic tone, agonist-dependent contraction and expression and activity of L-type calcium channels (323), suggesting that the MR may affect blood pressure not only by its renal effects but also by modulating vascular contractility.

7.4 MR and tissue remodeling

It has been shown that aldosterone has an effect on collagen synthesis in intra-myocardial coronary arteries in rats (324). Moreover, it has been demonstrated that vascular inflammation could be one of the mechanisms leading to aldosterone-mediated myocardial injury following chronic aldosterone treatment in rats (325). The role of MR along with GR has also been described in inflammation, tissue remodeling, atherosclerosis and inflammation via monocytes and macrophages, where it has been shown that aldosterone via MR induces an increase in oxidative stress in macrophages (326, 327). Moreover, mice lacking *Nr3c2* in macrophages were protected from DOCA-salt induced inflammation and fibrosis, suggesting that these mice had a phenotype that limits tissue remodeling (328). Similar results were shown in mice lacking MR expression in myeloid cells when treated with L-NAME (N(gamma)-nitro-L-arginine methyl ester)/Ang II (329). MR deletion from cardiomyocytes blocked reactive fibrosis in the heart. After myocardial infarction, cardiomyocyte MR KO mice exhibited a better cardiac repair in the infarcted zone and showed better revascularization and improved cardiac function. This phenotype was associated with a better response to ischemic events and lower inflammatory and profibrotic marker expression (330).

Furthermore, endothelial cell MR null mice were protected from cardiovascular inflammation and remodeling (331). Vascular smooth muscle cell conditional knockout of MR in mice showed similar protective effects following myocardial infarction, with reduced tissue fibrosis and preserved coronary vascular reserve in comparison to WT mice (332). A study showed that *MR* expression is similar between patients with atrial fibrillation and those with sinus rhythm, however, the expression of 11- β HSD2 was increased in the left atrium of patients with atrial fibrillation, thus suggesting a role of aldosterone via MR in left atrial remodeling (333). Aldosterone induced an increase in connective tissue growth factor in cardiomyocytes. Aldosterone also increased the expression of miRNA-21, which is a known to be implicated in heart diseases and different types of cancers, in cardiac fibroblasts through MR (333).

In summary, MR not only plays a key role in the regulation of water and electrolyte balance, it also promotes cardiovascular remodeling, affects endothelial functioning, induces inflammation, plays a role in adipocyte functioning and metabolism, and modulates centrally regulated functions in the brain (Fig. 13).

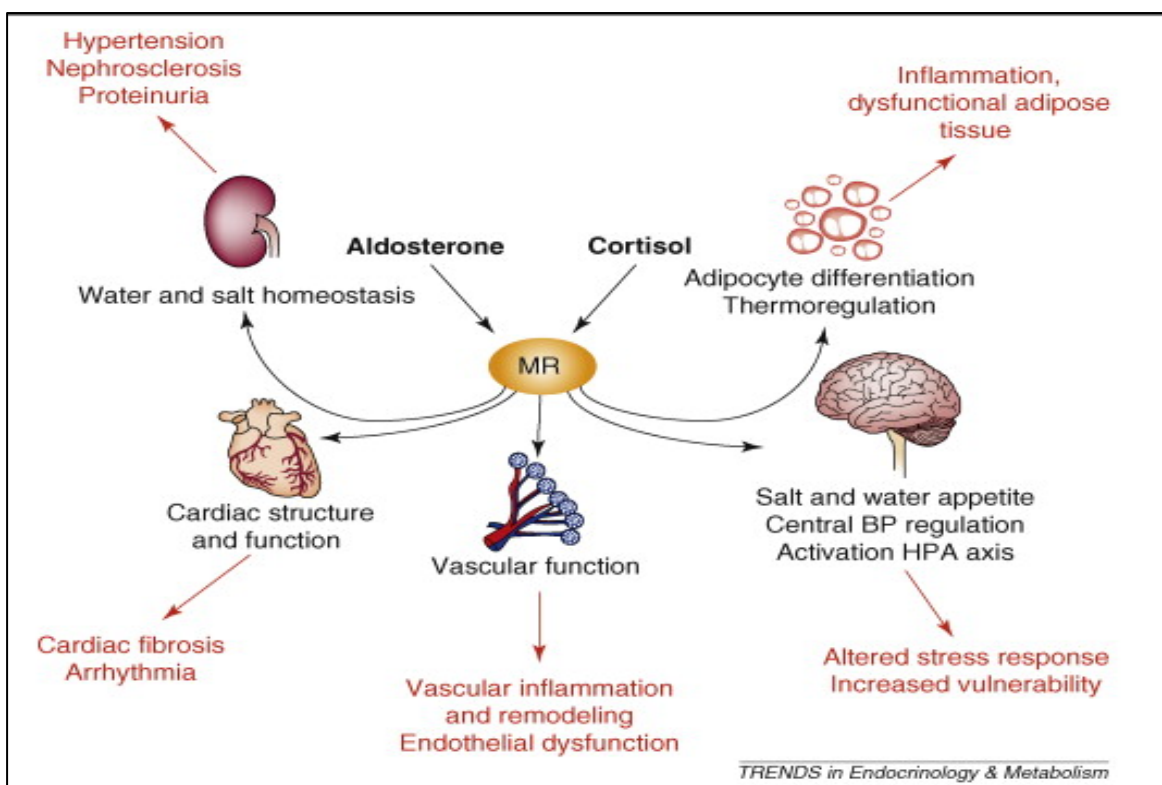


Figure 13. MR deleterious effects on various organs. Inappropriate activation of MR by aldosterone, cortisol or by genetic factors triggers deleterious complications across different tissues (334).

8 General conclusion

My PhD work has allowed the discovery of a direct implication of RAR α as a novel contributor to adrenal cortex development and normal function. [REDACTED]

[REDACTED] Finally, I have contributed to the identification, for the first time, of the role of the chloride channel CLC-2 in aldosterone production and aldosterone producing adenoma (APA).

My work has shown that RAR α is downregulated in APA in comparison to control adrenals. Furthermore, I have shown that mice lacking the expression of Rar α exhibit severe disorganization of the adrenal cortex, characterized by a loss of the radial structure of the zona fasciculata, abnormalities in the extracellular matrix, increased proliferation and downregulation in steroidogenic gene expression, Wnt signaling and Vegfa signaling. I have shown that, although the molecular abnormalities disappear with aging, the structural disorganization persists in older mice. These results indicate that loss of Rar α in the adrenal cortex leads to irreversible structural and functional disorganization. Translated to human disease, reduced function of RAR α in the adrenal cortex, due to genetic or inherited factors, may modify cell proliferation and create a propitious environment for the emergence of specific driver mutations in APA.

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Finally, my work has helped to decipher for the first time the role of RAR α , CLC-2 and MR in the adrenal cortex and APA. These results could open new perspectives in our understanding of the molecular mechanisms involved in nodule formation and autonomous aldosterone production in the adrenal gland. More importantly, these results could help in identifying new therapeutic targets in primary aldosteronism for improved patient care and prevention of cardiovascular complications.

9 References

1. Collaboration NCDRF. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *Lancet*. 2017;389(10064):37-55.
2. Collaborators GBDRF, Forouzanfar MH, Alexander L, Anderson HR, Bachman VF, Biryukov S, et al. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015;386(10010):2287-323.
3. Chow CK, Teo KK, Rangarajan S, Islam S, Gupta R, Avezum A, et al. Prevalence, awareness, treatment, and control of hypertension in rural and urban communities in high-, middle-, and low-income countries. *JAMA*. 2013;310(9):959-68.
4. Van Epps HL. Harry Goldblatt and the discovery of renin. *J Exp Med*. 2005;201(9):1351.
5. Goldblatt H, Lynch J, Hanzal RF, Summerville WW. Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia. *J Exp Med*. 1934;59(3):347-79.
6. Centers for Disease C, Prevention. Vital signs: prevalence, treatment, and control of hypertension--United States, 1999-2002 and 2005-2008. *MMWR Morb Mortal Wkly Rep*. 2011;60(4):103-8.
7. King JA, Lush DJ, Fray JC. Regulation of renin processing and secretion: chemiosmotic control and novel secretory pathway. *Am J Physiol*. 1993;265(2 Pt 1):C305-20.
8. Gembardt F, Heringer-Walther S, van Esch JH, Sterner-Kock A, van Veghel R, Le TH, et al. Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors. *FASEB J*. 2008;22(8):3068-77.
9. Lavoie JL, Sigmund CD. Minireview: overview of the renin-angiotensin system--an endocrine and paracrine system. *Endocrinology*. 2003;144(6):2179-83.
10. Pearce D, Bhargava A, Cole TJ. Aldosterone: its receptor, target genes, and actions. *Vitam Horm*. 2003;66:29-76.
11. Conn JW, Louis LH. Primary aldosteronism: a new clinical entity. *Trans Assoc Am Physicians*. 1955;68:215-31; discussion, 31-3.
12. Hannemann A, Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies--a review of the current literature. *Horm Metab Res*. 2012;44(3):157-62.
13. Monticone S, Burrello J, Tizzani D, Bertello C, Viola A, Buffolo F, et al. Prevalence and Clinical Manifestations of Primary Aldosteronism Encountered in Primary Care Practice. *J Am Coll Cardiol*. 2017;69(14):1811-20.
14. Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, et al. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol*. 2006;48(11):2293-300.
15. Calhoun DA, Nishizaka MK, Zaman MA, Thakkar RB, Weissmann P. Hyperaldosteronism among black and white subjects with resistant hypertension. *Hypertension*. 2002;40(6):892-6.
16. Douma S, Petidis K, Doumas M, Papaefthimiou P, Triantafyllou A, Kartali N, et al. Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet*. 2008;371(9628):1921-6.
17. Fagugli RM, Taglioni C. Changes in the perceived epidemiology of primary hyperaldosteronism. *Int J Hypertens*. 2011;2011:162804.

18. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* 2016;101(5):1889-916.
19. Savard S, Amar L, Plouin PF, Steichen O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension.* 2013;62(2):331-6.
20. Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, et al. Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2008;93(9):3266-81.
21. Williams TA, Lenders JWM, Mulatero P, Burrello J, Rottenkolber M, Adolf C, et al. Outcomes after adrenalectomy for unilateral primary aldosteronism: an international consensus on outcome measures and analysis of remission rates in an international cohort. *Lancet Diabetes Endocrinol.* 2017.
22. Eguchi K, Hoshida S, Nagashima S, Maekawa T, Sasano H, Kario K. An adverse pregnancy-associated outcome due to overlooked primary aldosteronism. *Intern Med.* 2014;53(21):2499-504.
23. Mulatero P, Monticone S, Bertello C, Viola A, Tizzani D, Iannaccone A, et al. Long-term cardio- and cerebrovascular events in patients with primary aldosteronism. *J Clin Endocrinol Metab.* 2013;98(12):4826-33.
24. Letavernier E, Peyrard S, Amar L, Zinzindohoue F, Fiquet B, Plouin PF. Blood pressure outcome of adrenalectomy in patients with primary hyperaldosteronism with or without unilateral adenoma. *J Hypertens.* 2008;26(9):1816-23.
25. Rossi GP, Bolognesi M, Rizzoni D, Seccia TM, Piva A, Porteri E, et al. Vascular remodeling and duration of hypertension predict outcome of adrenalectomy in primary aldosteronism patients. *Hypertension.* 2008;51(5):1366-71.
26. Sparrow RA, Coupland RE. Blood flow to the adrenal gland of the rat: its distribution between the cortex and the medulla before and after haemorrhage. *J Anat.* 1987;155:51-61.
27. Gagnon R. The arterial supply of the human adrenal gland. *Rev Can Biol.* 1957;16(4):421-33.
28. Murakami T, Yamamoto C, Ohtsuka A, Kikuta A, Taguchi T. Blood vascular architecture of the rat extra-adrenal cortical body: a scanning electron microscopic study of corrosion casts. *Arch Histol Cytol.* 1988;51(3):263-70.
29. Unsicker K. On the innervation of the rat and pig adrenal cortex. *Z Zellforsch Mikrosk Anat.* 1971;116(1):151-6.
30. Parker TL, Kesse WK, Mohamed AA, Afework M. The innervation of the mammalian adrenal gland. *J Anat.* 1993;183 (Pt 2):265-76.
31. Itcho K, Oki K, Kobuke K, Yoshii Y, Ohno H, Yoneda M, et al. Aberrant G protein-receptor expression is associated with DNA methylation in aldosterone-producing adenoma. *Mol Cell Endocrinol.* 2018;461:100-4.
32. Chamoux E, Bolduc L, Lehoux JG, Gallo-Payet N. Identification of extracellular matrix components and their integrin receptors in the human fetal adrenal gland. *J Clin Endocrinol Metab.* 2001;86(5):2090-8.
33. Chamoux E, Nancy A, Lehoux JG, Gallo-Payet N. Fibronectin, laminin, and collagen IV as modulators of cell behavior during adrenal gland development in the human fetus. *J Clin Endocrinol Metab.* 2002;87(4):1819-28.
34. Rege J, Rainey WE. The steroid metabolome of adrenarcho. *J Endocrinol.* 2012;214(2):133-43.
35. Kim AC, Barlaskar FM, Heaton JH, Else T, Kelly VR, Krill KT, et al. In search of adrenocortical stem and progenitor cells. *Endocr Rev.* 2009;30(3):241-63.

36. Sancho-Martinez I, Baek SH, Izpisua Belmonte JC. Lineage conversion methodologies meet the reprogramming toolbox. *Nat Cell Biol.* 2012;14(9):892-9.
37. Sisakhtnezhad S, Matin MM. Transdifferentiation: a cell and molecular reprogramming process. *Cell Tissue Res.* 2012;348(3):379-96.
38. Luo X, Ikeda Y, Parker KL. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell.* 1994;77(4):481-90.
39. Beuschlein F, Mutch C, Bavers DL, Ulrich-Lai YM, Engeland WC, Keegan C, et al. Steroidogenic factor-1 is essential for compensatory adrenal growth following unilateral adrenalectomy. *Endocrinology.* 2002;143(8):3122-35.
40. Crawford PA, Sadovsky Y, Milbrandt J. Nuclear receptor steroidogenic factor 1 directs embryonic stem cells toward the steroidogenic lineage. *Mol Cell Biol.* 1997;17(7):3997-4006.
41. Sakai N, Terami H, Suzuki S, Haga M, Nomoto K, Tsuchida N, et al. Identification of NR5A1 (SF-1/AD4BP) gene expression modulators by large-scale gain and loss of function studies. *J Endocrinol.* 2008;198(3):489-97.
42. Freedman BD, Kempna PB, Carlone DL, Shah MS, Guagliardo NA, Barrett PQ, et al. Adrenocortical zonation results from lineage conversion of differentiated zona glomerulosa cells. *Dev Cell.* 2013;26(6):666-73.
43. Ishimoto H, Jaffe RB. Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit. *Endocr Rev.* 2011;32(3):317-55.
44. Jones IC. The disappearance of the X zone of the mouse adrenal cortex during first pregnancy. *Proc R Soc Lond B Biol Sci.* 1952;139(896):398-410.
45. Deacon CF, Mosley W, Jones IC. The X zone of the mouse adrenal cortex of the Swiss albino strain. *Gen Comp Endocrinol.* 1986;61(1):87-99.
46. Wright N, Voncina D. Studies on the postnatal growth of the rat adrenal cortex. *J Anat.* 1977;123(Pt 1):147-56.
47. Stachowiak A, Nussdorfer GG, Malendowicz LK. Proliferation and distribution of adrenocortical cells in the gland of ACTH- or dexamethasone-treated rats. *Histol Histopathol.* 1990;5(1):25-9.
48. McNeill H, Puddefoot JR, Vinson GP. MAP Kinase in the rat adrenal gland. *Endocr Res.* 1998;24(3-4):373-80.
49. El Wakil A, Mari B, Barhanin J, Lalli E. Genomic analysis of sexual dimorphism of gene expression in the mouse adrenal gland. *Horm Metab Res.* 2013;45(12):870-3.
50. Grabek A, Dolfi B, Klein B, Jian-Motamedi F, Chaboissier MC, Schedl A. The Adult Adrenal Cortex Undergoes Rapid Tissue Renewal in a Sex-Specific Manner. *Cell Stem Cell.* 2019;25(2):290-6 e2.
51. Spat A, Hunyady L, Szanda G. Signaling Interactions in the Adrenal Cortex. *Front Endocrinol (Lausanne).* 2016;7:17.
52. Spat A, Hunyady L. Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol Rev.* 2004;84(2):489-539.
53. Aragao-Santiago L, Gomez-Sanchez CE, Mulatero P, Spyroglou A, Reincke M, Williams TA. Mouse Models of Primary Aldosteronism: From Physiology to Pathophysiology. *Endocrinology.* 2017;158(12):4129-38.
54. Bassett MH, White PC, Rainey WE. The regulation of aldosterone synthase expression. *Mol Cell Endocrinol.* 2004;217(1-2):67-74.
55. Cooke BA. Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Mol Cell Endocrinol.* 1999;151(1-2):25-35.

56. Fuchs-Hammoser R, Schweiger M, Oelkers W. The effect of chronic low-dose infusion of ACTH (1-24) on renin, renin-substrate, aldosterone and other corticosteroids in sodium replete and deplete man. *Acta Endocrinol (Copenh)*. 1980;95(2):198-206.
57. Holland OB, Carr B. Modulation of aldosterone synthase messenger ribonucleic acid levels by dietary sodium and potassium and by adrenocorticotropin. *Endocrinology*. 1993;132(6):2666-73.
58. Aguilera G, Fujita K, Catt KJ. Mechanisms of inhibition of aldosterone secretion by adrenocorticotropin. *Endocrinology*. 1981;108(2):522-8.
59. Aguilera G, Kiss A. Regulation of the hypothalamic-pituitary-adrenal axis and vasopressin secretion. Role of angiotensin II. *Adv Exp Med Biol*. 1996;396:105-12.
60. Crivello JF, Gill GN. Induction of cultured bovine adrenocortical zona glomerulosa cell 17-hydroxylase activity by ACTH. *Mol Cell Endocrinol*. 1983;30(1):97-107.
61. El Ghorayeb N, Bourdeau I, Lacroix A. Role of ACTH and Other Hormones in the Regulation of Aldosterone Production in Primary Aldosteronism. *Front Endocrinol (Lausanne)*. 2016;7:72.
62. Lefebvre H, Contesse V, Delarue C, Soubrane C, Legrand A, Kuhn JM, et al. Effect of the serotonin-4 receptor agonist zacopride on aldosterone secretion from the human adrenal cortex: in vivo and in vitro studies. *J Clin Endocrinol Metab*. 1993;77(6):1662-6.
63. Lefebvre H, Compagnon P, Contesse V, Delarue C, Thuillez C, Vaudry H, et al. Production and metabolism of serotonin (5-HT) by the human adrenal cortex: paracrine stimulation of aldosterone secretion by 5-HT. *J Clin Endocrinol Metab*. 2001;86(10):5001-7.
64. Louiset E, Duparc C, Lenglet S, Gomez-Sanchez CE, Lefebvre H. Role of cAMP/PKA pathway and T-type calcium channels in the mechanism of action of serotonin in human adrenocortical cells. *Mol Cell Endocrinol*. 2017;441:99-107.
65. Lefebvre H, Cartier D, Duparc C, Contesse V, Lihmann I, Delarue C, et al. Effect of serotonin4 (5-HT4) receptor agonists on aldosterone secretion in idiopathic hyperaldosteronism. *Endocr Res*. 2000;26(4):583-7.
66. Kraan GP, Dullaart RP, Pratt JJ, Wolthers BG, Drayer NM, De Bruin R. The daily cortisol production reinvestigated in healthy men. The serum and urinary cortisol production rates are not significantly different. *J Clin Endocrinol Metab*. 1998;83(4):1247-52.
67. Dekloet ER. Steroids, stability and stress. *Front Neuroendocrinol*. 1995;16(4):416-25.
68. Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, et al. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat Genet*. 2005;37(2):166-70.
69. Cooray SN, Almiro Do Vale I, Leung KY, Webb TR, Chapple JP, Egertova M, et al. The melanocortin 2 receptor accessory protein exists as a homodimer and is essential for the function of the melanocortin 2 receptor in the mouse y1 cell line. *Endocrinology*. 2008;149(4):1935-41.
70. Clark AJ, McLoughlin L, Grossman A. Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet*. 1993;341(8843):461-2.
71. Calebiro D, Di Dalmazi G, Bathon K, Ronchi CL, Beuschlein F. cAMP signaling in cortisol-producing adrenal adenoma. *Eur J Endocrinol*. 2015;173(4):M99-106.
72. El Wakil A, Lalli E. The Wnt/beta-catenin pathway in adrenocortical development and cancer. *Mol Cell Endocrinol*. 2011;332(1-2):32-7.
73. Slusarski DC, Yang-Snyder J, Busa WB, Moon RT. Modulation of embryonic intracellular Ca²⁺ signaling by Wnt-5A. *Dev Biol*. 1997;182(1):114-20.
74. Yuzugullu H, Benhaj K, Ozturk N, Senturk S, Celik E, Toyly A, et al. Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. *Mol Cancer*. 2009;8:90.

75. Jordan BK, Shen JH, Olaso R, Ingraham HA, Vilain E. Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. *Proc Natl Acad Sci U S A*. 2003;100(19):10866-71.
76. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. *Nature*. 1999;397(6718):405-9.
77. Brisken C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, et al. Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev*. 2000;14(6):650-4.
78. Mandel H, Shemer R, Borochowitz ZU, Okopnik M, Knopf C, Indelman M, et al. SERKAL syndrome: an autosomal-recessive disorder caused by a loss-of-function mutation in WNT4. *Am J Hum Genet*. 2008;82(1):39-47.
79. Heikkila M, Peltoketo H, Leppaluoto J, Ilves M, Vuolteenaho O, Vainio S. Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology*. 2002;143(11):4358-65.
80. L'Episcopo F, Tirolo C, Caniglia S, Testa N, Morale MC, Serapide MF, et al. Targeting Wnt signaling at the neuroimmune interface for dopaminergic neuroprotection/repair in Parkinson's disease. *J Mol Cell Biol*. 2014;6(1):13-26.
81. Drelon C, Berthon A, Sahut-Barnola I, Mathieu M, Dumontet T, Rodriguez S, et al. PKA inhibits WNT signalling in adrenal cortex zonation and prevents malignant tumour development. *Nat Commun*. 2016;7:12751.
82. Taylor SS, Knighton DR, Zheng J, Ten Eyck LF, Sowadski JM. Structural framework for the protein kinase family. *Annu Rev Cell Biol*. 1992;8:429-62.
83. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol*. 2001;2(8):599-609.
84. Sahut-Barnola I, de Jossineau C, Val P, Lambert-Langlais S, Damon C, Lefrancois-Martinez AM, et al. Cushing's syndrome and fetal features resurgence in adrenal cortex-specific Prkar1a knockout mice. *PLoS Genet*. 2010;6(6):e1000980.
85. Dumontet T, Sahut-Barnola I, Septier A, Montanier N, Plotton I, Roucher-Boulez F, et al. PKA signaling drives reticularis differentiation and sexually dimorphic adrenal cortex renewal. *JCI Insight*. 2018;3(2).
86. Zilbermint M, Stratakis CA. Protein kinase A defects and cortisol-producing adrenal tumors. *Curr Opin Endocrinol Diabetes Obes*. 2015;22(3):157-62.
87. Stewart GN. A Note on Some Obvious Consequences of the High Rate of Blood Flow through the Adrenals. *American Journal of Physiology-Legacy Content*. 1917;45(1):92-5.
88. Mariniello B, Rosato A, Zuccolotto G, Rubin B, Cicala MV, Finco I, et al. Combination of sorafenib and everolimus impacts therapeutically on adrenocortical tumor models. *Endocr Relat Cancer*. 2012;19(4):527-39.
89. Gennari-Moser C, Khankin EV, Escher G, Burkhard F, Frey BM, Karumanchi SA, et al. Vascular endothelial growth factor-A and aldosterone: relevance to normal pregnancy and preeclampsia. *Hypertension*. 2013;61(5):1111-7.
90. Cherradi N, Lejczak C, Desroches-Castan A, Feige JJ. Antagonistic functions of tetradecanoyl phorbol acetate-inducible-sequence 11b and HuR in the hormonal regulation of vascular endothelial growth factor messenger ribonucleic acid stability by adrenocorticotropin. *Mol Endocrinol*. 2006;20(4):916-30.
91. Thomas M, Keramidas M, Monchaux E, Feige JJ. Dual hormonal regulation of endocrine tissue mass and vasculature by adrenocorticotropin in the adrenal cortex. *Endocrinology*. 2004;145(9):4320-9.
92. Williams TA, Monticone S, Morello F, Liew CC, Mengozzi G, Pilon C, et al. Teratocarcinoma-derived growth factor-1 is upregulated in aldosterone-producing adenomas

and increases aldosterone secretion and inhibits apoptosis in vitro. *Hypertension*. 2010;55(6):1468-75.

93. Parker KL, Rice DA, Lala DS, Ikeda Y, Luo X, Wong M, et al. Steroidogenic factor 1: an essential mediator of endocrine development. *Recent Prog Horm Res*. 2002;57:19-36.

94. Val P, Lefrancois-Martinez AM, Veyssiere G, Martinez A. SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl Recept*. 2003;1(1):8.

95. Ikeda Y, Shen WH, Ingraham HA, Parker KL. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol Endocrinol*. 1994;8(5):654-62.

96. Gondo S, Yanase T, Okabe T, Tanaka T, Morinaga H, Nomura M, et al. SF-1/Ad4BP transforms primary long-term cultured bone marrow cells into ACTH-responsive steroidogenic cells. *Genes Cells*. 2004;9(12):1239-47.

97. Bassett MH, Zhang Y, Clyne C, White PC, Rainey WE. Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. *J Mol Endocrinol*. 2002;28(2):125-35.

98. Ye P, Nakamura Y, Lalli E, Rainey WE. Differential effects of high and low steroidogenic factor-1 expression on CYP11B2 expression and aldosterone production in adrenocortical cells. *Endocrinology*. 2009;150(3):1303-9.

99. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, et al. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc Natl Acad Sci U S A*. 1995;92(24):10939-43.

100. Kurihara I, Shibata H, Kobayashi S, Suda N, Ikeda Y, Yokota K, et al. Ubc9 and Protein Inhibitor of Activated STAT 1 Activate Chicken Ovalbumin Upstream Promoter-Transcription Factor I-mediated Human CYP11B2 Gene Transcription. *J Biol Chem*. 2005;280(8):6721-30.

101. Boulkroun S, Samson-Couterie B, Golib-Dzib JF, Amar L, Plouin PF, Sibony M, et al. Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. *Endocrinology*. 2011;152(12):4753-63.

102. Lentjes MH, Niessen HE, Akiyama Y, de Bruine AP, Melotte V, van Engeland M. The emerging role of GATA transcription factors in development and disease. *Expert Rev Mol Med*. 2016;18:e3.

103. Kiiveri S, Liu J, Westerholm-Ormio M, Narita N, Wilson DB, Voutilainen R, et al. Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. *Endocrinology*. 2002;143(8):3136-43.

104. Tremblay JJ, Viger RS. Novel roles for GATA transcription factors in the regulation of steroidogenesis. *J Steroid Biochem Mol Biol*. 2003;85(2-5):291-8.

105. Jimenez P, Saner K, Mayhew B, Rainey WE. GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. *Endocrinology*. 2003;144(10):4285-8.

106. Kiiveri S, Liu J, Arola J, Heikkila P, Kuulasmaa T, Lehtonen E, et al. Transcription factors GATA-6, SF-1, and cell proliferation in human adrenocortical tumors. *Mol Cell Endocrinol*. 2005;233(1-2):47-56.

107. Hatano O, Takakusu A, Nomura M, Morohashi K. Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes Cells*. 1996;1(7):663-71.

108. Keegan CE, Hammer GD. Recent insights into organogenesis of the adrenal cortex. *Trends Endocrinol Metab*. 2002;13(5):200-8.

109. Haber DA, Housman DE. The genetics of Wilms' tumor. *Adv Cancer Res*. 1992;59:41-68.

110. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development*. 1999;126(9):1845-57.
111. Takekoshi K, Isobe K, Yashiro T, Hara H, Ishii K, Kawakami Y, et al. Expression of vascular endothelial growth factor (VEGF) and its cognate receptors in human pheochromocytomas. *Life Sci*. 2004;74(7):863-71.
112. Fottner C, Minnemann T, Kalmbach S, Weber MM. Overexpression of the insulin-like growth factor I receptor in human pheochromocytomas. *J Mol Endocrinol*. 2006;36(2):279-87.
113. Fassnacht M, Weismann D, Ebert S, Adam P, Zink M, Beuschlein F, et al. AKT is highly phosphorylated in pheochromocytomas but not in benign adrenocortical tumors. *J Clin Endocrinol Metab*. 2005;90(7):4366-70.
114. De Martino MC, van Koetsveld PM, Pivonello R, Hofland LJ. Role of the mTOR pathway in normal and tumoral adrenal cells. *Neuroendocrinology*. 2010;92 Suppl 1:28-34.
115. Swierczynska MM, Betz MJ, Colombi M, Dazert E, Jenö P, Moes S, et al. Proteomic Landscape of Aldosterone-Producing Adenoma. *Hypertension*. 2019;73(2):469-80.
116. Su H, Gu Y, Li F, Wang Q, Huang B, Jin X, et al. The PI3K/AKT/mTOR signaling pathway is overactivated in primary aldosteronism. *PLoS One*. 2013;8(4):e62399.
117. Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, et al. K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011;331(6018):768-72.
118. Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP, Gomez-Sanchez CE. Potassium Channel Mutant KCNJ5 T158A Expression in HAC-15 Cells Increases Aldosterone Synthesis. *Endocrinology*. 2012;153(4):1774-82.
119. Cheng CJ, Sung CC, Wu ST, Lin YC, Sytwu HK, Huang CL, et al. Novel KCNJ5 mutations in sporadic aldosterone-producing adenoma reduce Kir3.4 membrane abundance. *J Clin Endocrinol Metab*. 2015;100(1):E155-63.
120. Scholl UI, Nelson-Williams C, Yue P, Grekin R, Wyatt RJ, Dillon MJ, et al. Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. *Proc Natl Acad Sci U S A*. 2012;109(7):2533-8.
121. Hardege I, Long L, Al Maskari R, Figg N, O'Shaughnessy KM. Targeted disruption of the *Kcnj5* gene in the female mouse lowers aldosterone levels. *Clin Sci (Lond)*. 2018;132(1):145-56.
122. Catterall WA. Signaling complexes of voltage-gated sodium and calcium channels. *Neurosci Lett*. 2010;486(2):107-16.
123. Dutta RK, Soderkvist P, Gimm O. Genetics of primary hyperaldosteronism. *Endocr Relat Cancer*. 2016;23(10):R437-54.
124. Azizan EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, et al. Somatic mutations in *ATP1A1* and *CACNA1D* underlie a common subtype of adrenal hypertension. *Nat Genet*. 2013;45(9):1055-60.
125. Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, Boulkroun S, et al. Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension*. 2014;64(2):354-61.
126. Zennaro MC, Boulkroun S, Fernandes-Rosa F. Genetic causes of functional adrenocortical adenomas. *Endocr Rev*. 2017.
127. Daniil G, Fernandes-Rosa FL, Chemin J, Blesneac I, Beltrand J, Polak M, et al. *CACNA1H* Mutations Are Associated With Different Forms of Primary Aldosteronism. *EBioMedicine*. 2016;13:225-36.

128. Scholl UI, Stolting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, et al. Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. *Elife*. 2015;4:e06315.
129. Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45(4):440-4.
130. Stindl J, Tauber P, Sterner C, Tegtmeier I, Warth R, Bandulik S. Pathogenesis of Adrenal Aldosterone-Producing Adenomas Carrying Mutations of the Na(+)/K(+)-ATPase. *Endocrinology*. 2015;156(12):4582-91.
131. Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, et al. Cellular Pathophysiology of an Adrenal Adenoma-Associated Mutant of the Plasma Membrane Ca(2+)-ATPase ATP2B3. *Endocrinology*. 2016;157(6):2489-99.
132. Williams TA, Peitzsch M, Dietz AS, Dekkers T, Bidlingmaier M, Riester A, et al. Genotype-Specific Steroid Profiles Associated With Aldosterone-Producing Adenomas. *Hypertension*. 2016;67(1):139-45.
133. El Zein RM, Boulkroun S, Fernandes-Rosa FL, Zennaro MC. Molecular genetics of Conn adenomas in the era of exome analysis. *Presse Med*. 2018;47(7-8 Pt 2):e151-e8.
134. Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, et al. WNT/beta-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Hum Mol Genet*. 2014;23(4):889-905.
135. Akerstrom T, Maharjan R, Sven Willenberg H, Cupisti K, Ip J, Moser A, et al. Activating mutations in CTNNB1 in aldosterone producing adenomas. *Sci Rep*. 2016;6:19546.
136. Scholl UI, Healy JM, Thiel A, Fonseca AL, Brown TC, Kunstman JW, et al. Novel Somatic Mutations in Primary Hyperaldosteronism are related to the Clinical, Radiological and Pathological Phenotype. *Clin Endocrinol (Oxf)*. 2015.
137. Teo AE, Garg S, Shaikh LH, Zhou J, Karet Frankl FE, Gurnell M, et al. Pregnancy, Primary Aldosteronism, and Adrenal CTNNB1 Mutations. *N Engl J Med*. 2015;373(15):1429-36.
138. Rhayem Y, Perez-Rivas LG, Dietz A, Bathon K, Gebhard C, Riester A, et al. PRKACA Somatic Mutations Are Rare Findings in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab*. 2016;101(8):3010-7.
139. Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, et al. Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nat Genet*. 2014;46(6):613-7.
140. Cao Y, He M, Gao Z, Peng Y, Li Y, Li L, et al. Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science*. 2014;344(6186):913-7.
141. Beuschlein F, Fassnacht M, Assie G, Calebiro D, Stratakis CA, Osswald A, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. *N Engl J Med*. 2014;370(11):1019-28.
142. Arlt W, Lang K, Sitch AJ, Dietz AS, Rhayem Y, Bancos I, et al. Steroid metabolome analysis reveals prevalent glucocorticoid excess in primary aldosteronism. *JCI Insight*. 2017;2(8).
143. Medeau V, Assie G, Zennaro MC, Clauser E, Plouin PF, Jeunemaitre X. [Familial aspect of primary hyperaldosteronism: analysis of families compatible with primary hyperaldosteronism type 2]. *Ann Endocrinol (Paris)*. 2005;66(3):240-6.
144. Mulatero P, Tizzani D, Viola A, Bertello C, Monticone S, Mengozzi G, et al. Prevalence and characteristics of familial hyperaldosteronism: the PATOGEN study (Primary Aldosteronism in TORino-GENetic forms). *Hypertension*. 2011;58(5):797-803.

145. Pallauf A, Schirpenbach C, Zwermann O, Fischer E, Morak M, Holinski-Feder E, et al. The prevalence of familial hyperaldosteronism in apparently sporadic primary aldosteronism in Germany: a single center experience. *Horm Metab Res.* 2012;44(3):215-20.
146. Aglony M, Martinez-Aguayo A, Carvajal CA, Campino C, Garcia H, Bancalari R, et al. Frequency of familial hyperaldosteronism type 1 in a hypertensive pediatric population: clinical and biochemical presentation. *Hypertension.* 2011;57(6):1117-21.
147. Mulatero P, Williams TA, Monticone S, Veglio F. Is familial hyperaldosteronism underdiagnosed in hypertensive children? *Hypertension.* 2011;57(6):1053-5.
148. Sutherland DJ, Ruse JL, Laidlaw JC. Hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone. *Can Med Assoc J.* 1966;95(22):1109-19.
149. Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S, et al. A chimaeric 11beta-hydroxylase aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature.* 1992;355:262-5.
150. Pascoe L, Curnow KM, Slutsker L, Connell JM, Speiser PW, New MI, et al. Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. *Proc Natl Acad Sci U S A.* 1992;89(17):8327-31.
151. Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D, et al. Evidence for abnormal left ventricular structure and function in normotensive individuals with familial hyperaldosteronism type I. *J Clin Endocrinol Metab.* 2005;90(9):5070-6.
152. Litchfield WR, Anderson BF, Weiss RJ, Lifton RP, Dluhy RG. Intracranial aneurysm and hemorrhagic stroke in glucocorticoid-remediable aldosteronism. *Hypertension.* 1998;31(1 Pt 2):445-50.
153. Stowasser M, Gordon RD. Familial hyperaldosteronism. *J Steroid Biochem Mol Biol.* 2001;78(3):215-29.
154. Lafferty AR, Torpy DJ, Stowasser M, Taymans SE, Lin JP, Huggard P, et al. A novel genetic locus for low renin hypertension: familial hyperaldosteronism type II maps to chromosome 7 (7p22). *J Med Genet.* 2000;37(11):831-5.
155. Fernandes-Rosa FL, Daniil G, Orozco IJ, Goppner C, El Zein R, Jain V, et al. A gain-of-function mutation in the CLCN2 chloride channel gene causes primary aldosteronism. *Nat Genet.* 2018;50(3):355-61.
156. Grunder S, Thiemann A, Pusch M, Jentsch TJ. Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. *Nature.* 1992;360(6406):759-62.
157. Stowasser M, Gordon RD, Tunny TJ, Klemm SA, Finn WL, Krek AL. Familial hyperaldosteronism type II: five families with a new variety of primary aldosteronism. *Clin Exp Pharmacol Physiol.* 1992;19(5):319-22.
158. Scholl UI, Stolting G, Schewe J, Thiel A, Tan H, Nelson-Williams C, et al. CLCN2 chloride channel mutations in familial hyperaldosteronism type II. *Nat Genet.* 2018;50(3):349-54.
159. Geller DS, Zhang J, Wisgerhof MV, Shackleton C, Kashgarian M, Lifton RP. A novel form of human mendelian hypertension featuring nonglucocorticoid-remediable aldosteronism. *J Clin Endocrinol Metab.* 2008;93(8):3117-23.
160. Mulatero P, Monticone S, Rainey WE, Veglio F, Williams TA. Role of KCNJ5 in familial and sporadic primary aldosteronism. *Nat Rev Endocrinol.* 2013;9(2):104-12.
161. Splawski I, Yoo DS, Stotz SC, Cherry A, Clapham DE, Keating MT. CACNA1H mutations in autism spectrum disorders. *J Biol Chem.* 2006;281(31):22085-91.
162. Chen Y, Lu J, Pan H, Zhang Y, Wu H, Xu K, et al. Association between genetic variation of CACNA1H and childhood absence epilepsy. *Ann Neurol.* 2003;54(2):239-43.

163. Steinberg KM, Yu B, Koboldt DC, Mardis ER, Pamphlett R. Exome sequencing of case-affected-parents trios reveals recessive and de novo genetic variants in sporadic ALS. *Sci Rep.* 2015;5:9124.
164. Reimer EN, Walenda G, Seidel E, Scholl UI. CACNA1H M1549V mutant calcium channel causes autonomous aldosterone production in HAC15 cells and is inhibited by Mibefradil. *Endocrinology.* 2016;en20161170.
165. Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, et al. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet.* 2013;45(9):1050-4.
166. Korah HE, Scholl UI. An Update on Familial Hyperaldosteronism. *Horm Metab Res.* 2015;47(13):941-6.
167. Zilbermint M, Xekouki P, Faucz FR, Berthon A, Gkourogiani A, Schernthaner-Reiter MH, et al. Primary Aldosteronism and ARMC5 Variants. *J Clin Endocrinol Metab.* 2015;100(6):E900-9.
168. Assie G, Libe R, Espiard S, Rizk-Rabin M, Guimier A, Luscap W, et al. ARMC5 mutations in macronodular adrenal hyperplasia with Cushing's syndrome. *N Engl J Med.* 2013;369(22):2105-14.
169. Espiard S, Drougat L, Libe R, Assie G, Perlemoine K, Guignat L, et al. ARMC5 Mutations in a Large Cohort of Primary Macronodular Adrenal Hyperplasia: Clinical and Functional Consequences. *J Clin Endocrinol Metab.* 2015;100(6):E926-35.
170. Mulatero P, Schiavi F, Williams TA, Monticone S, Barbon G, Opocher G, et al. ARMC5 mutation analysis in patients with primary aldosteronism and bilateral adrenal lesions. *J Hum Hypertens.* 2016;30(6):374-8.
171. Black VH, Sanjay A, van Leyen K, Luring B, Kreibich G. Cholesterol and steroid synthesizing smooth endoplasmic reticulum of adrenocortical cells contains high levels of proteins associated with the translocation channel. *Endocrinology.* 2005;146(10):4234-49.
172. Mishunina TM, Kononenko VY. Specific GABA binding in the adrenals and blood corticosteroid levels in stress in intact rats and rats with changes in the functional activity of the hypothalamo-pituitary-adrenal system. *Neurosci Behav Physiol.* 2002;32(2):109-12.
173. Ye P, Mariniello B, Mantero F, Shibata H, Rainey WE. G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism. *J Endocrinol.* 2007;195(1):39-48.
174. Campbell WB, Brady MT, Gomez-Sanchez CE. Effects of angiotensin, prostaglandin E2 and indomethacin on the early and late steps of aldosterone biosynthesis in isolated adrenal cells. *J Steroid Biochem.* 1986;24(4):865-70.
175. Yoshii Y, Oki K, Gomez-Sanchez CE, Ohno H, Itcho K, Kobuke K, et al. Hypomethylation of CYP11B2 in Aldosterone-Producing Adenoma. *Hypertension.* 2016;68(6):1432-7.
176. Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Mol Endocrinol.* 2004;18(2):279-90.
177. Bassett MH, Mayhew B, Rehman K, White PC, Mantero F, Arnaldi G, et al. Expression profiles for steroidogenic enzymes in adrenocortical disease. *J Clin Endocrinol Metab.* 2005;90(9):5446-55.
178. Williams TA, Monticone S, Crudo V, Warth R, Veglio F, Mulatero P. Visinin-like 1 is upregulated in aldosterone-producing adenomas with KCNJ5 mutations and protects from calcium-induced apoptosis. *Hypertension.* 2012;59(4):833-9.
179. Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, et al. Microarray, qPCR, and KCNJ5 Sequencing of Aldosterone-Producing Adenomas Reveal Differences in

- Genotype and Phenotype between Zona Glomerulosa- and Zona Fasciculata-Like Tumors. *J Clin Endocrinol Metab.* 2012;97(5):E819-29.
180. Tan GC, Negro G, Pinggera A, Tizen Laim NMS, Mohamed Rose I, Ceral J, et al. Aldosterone-Producing Adenomas: Histopathology-Genotype Correlation and Identification of a Novel CACNA1D Mutation. *Hypertension.* 2017;70(1):129-36.
181. Dekkers T, ter Meer M, Lenders JW, Hermus AR, Schultze Kool L, Langenhuijsen JF, et al. Adrenal nodularity and somatic mutations in primary aldosteronism: one node is the culprit? *J Clin Endocrinol Metab.* 2014;99(7):E1341-51.
182. Fernandes-Rosa FL, Giscos-Douriez I, Amar L, Gomez-Sanchez CE, Meatchi T, Boulkroun S, et al. Different Somatic Mutations in Multinodular Adrenals With Aldosterone-Producing Adenoma. *Hypertension.* 2015;66(5):1014-22.
183. Nanba K, Omata K, Tomlins SA, Giordano TJ, Hammer GD, Rainey WE, et al. Double adrenocortical adenomas harboring independent KCNJ5 and PRKACA somatic mutations. *Eur J Endocrinol.* 2016;175(2):K1-6.
184. Nanba K, Chen AX, Omata K, Vinco M, Giordano TJ, Else T, et al. Molecular Heterogeneity in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab.* 2016;101(3):999-1007.
185. Vouillarmet J, Fernandes-Rosa F, Graeppe-Dulac J, Lantelme P, Decaussin-Petrucci M, Thivolet C, et al. Aldosterone-Producing Adenoma With a Somatic KCNJ5 Mutation Revealing APC-Dependent Familial Adenomatous Polyposis. *J Clin Endocrinol Metab.* 2016;101(11):3874-8.
186. Nishimoto K, Nakagawa K, Li D, Kosaka T, Oya M, Mikami S, et al. Adrenocortical zonation in humans under normal and pathological conditions. *J Clin Endocrinol Metab.* 2010;95(5):2296-305.
187. Boulkroun S, Samson-Couterie B, Dzib JF, Lefebvre H, Louiset E, Amar L, et al. Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. *Hypertension.* 2010;56(5):885-92.
188. Omata K, Anand SK, Hovelson DH, Liu CJ, Yamazaki Y, Nakamura Y, et al. Aldosterone-Producing Cell Clusters Frequently Harbor Somatic Mutations and Accumulate With Age in Normal Adrenals. *J Endocr Soc.* 2017;1(7):787-99.
189. Nishimoto K, Tomlins SA, Kuick R, Cani AK, Giordano TJ, Hovelson DH, et al. Aldosterone-stimulating somatic gene mutations are common in normal adrenal glands. *Proc Natl Acad Sci U S A.* 2015;112(33):E4591-9.
190. Nishimoto K, Seki T, Kurihara I, Yokota K, Omura M, Nishikawa T, et al. Case Report: Nodule Development From Subcapsular Aldosterone-Producing Cell Clusters Causes Hyperaldosteronism. *J Clin Endocrinol Metab.* 2016;101(1):6-9.
191. Omata K, Satoh F, Morimoto R, Ito S, Yamazaki Y, Nakamura Y, et al. Cellular and Genetic Causes of Idiopathic Hyperaldosteronism. *Hypertension.* 2018;72(4):874-80.
192. Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW, Rossi GP. A Meta-Analysis of Somatic KCNJ5 K(+) Channel Mutations In 1636 Patients With an Aldosterone-Producing Adenoma. *J Clin Endocrinol Metab.* 2015;100(8):E1089-95.
193. Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, et al. Prevalence, Clinical, and Molecular Correlates of KCNJ5 Mutations in Primary Aldosteronism. *Hypertension.* 2012;59(3):592-8.
194. Rossi GP, Cesari M, Letizia C, Seccia TM, Cicala MV, Zinamosca L, et al. KCNJ5 gene somatic mutations affect cardiac remodelling but do not preclude cure of high blood pressure and regression of left ventricular hypertrophy in primary aldosteronism. *J Hypertens.* 2014;32(7):1514-21; discussion 22.

195. Nanba K, Omata K, Else T, Beck PCC, Nanba AT, Turcu AF, et al. Targeted Molecular Characterization of Aldosterone-Producing Adenomas in White Americans. *J Clin Endocrinol Metab.* 2018;103(10):3869-76.
196. Wu VC, Huang KH, Peng KY, Tsai YC, Wu CH, Wang SM, et al. Prevalence and clinical correlates of somatic mutation in aldosterone producing adenoma-Taiwanese population. *Sci Rep.* 2015;5:11396.
197. Kitamoto T, Omura M, Suematsu S, Saito J, Nishikawa T. KCNJ5 mutation as a predictor for resolution of hypertension after surgical treatment of aldosterone-producing adenoma. *J Hypertens.* 2018;36(3):619-27.
198. Calhoun DA, Zaman MA, Nishizaka MK. Resistant hypertension. *Curr Hypertens Rep.* 2002;4(3):221-8.
199. Dooley R, Harvey BJ, Thomas W. The regulation of cell growth and survival by aldosterone. *Front Biosci (Landmark Ed).* 2011;16:440-57.
200. Escriva H, Delaunay F, Laudet V. Ligand binding and nuclear receptor evolution. *Bioessays.* 2000;22(8):717-27.
201. Pawlak M, Lefebvre P, Staels B. General molecular biology and architecture of nuclear receptors. *Curr Top Med Chem.* 2012;12(6):486-504.
202. Freedman LP, Luisi BP, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature.* 1988;334:543-6.
203. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature.* 1991;352(6335):497-505.
204. Umesono K, Evans RM. Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell.* 1989;57:1139-46.
205. Borngraeber S, Budny MJ, Chiellini G, Cunha-Lima ST, Togashi M, Webb P, et al. Ligand selectivity by seeking hydrophobicity in thyroid hormone receptor. *Proc Natl Acad Sci U S A.* 2003;100(26):15358-63.
206. Westin S, Kurokawa R, Nolte RT, Wisely GB, McInerney EM, Rose DW, et al. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature.* 1998;395(6698):199-202.
207. Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, et al. Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J Steroid Biochem Mol Biol.* 2001;76(1-5):23-30.
208. Ceraline J, Cruchant MD, Erdmann E, Erbs P, Kurtz JE, Duclos B, et al. Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int J Cancer.* 2004;108(1):152-7.
209. Schaarschmidt J, Huth S, Meier R, Paschke R, Jaeschke H. Influence of the hinge region and its adjacent domains on binding and signaling patterns of the thyrotropin and follitropin receptor. *PLoS One.* 2014;9(10):e111570.
210. Kastner P, Mark M, Ghyselinck N, Krezel W, Dupe V, Grondona JM, et al. Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development.* 1997;124(2):313-26.
211. Zechel C, Shen XQ, Chen JY, Chen ZP, Chambon P, Gronemeyer H. The dimerization interfaces formed between the DNA binding domains of RXR, RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. *EMBO J.* 1994;13(6):1425-33.
212. Moras D, Gronemeyer H. The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol.* 1998;10(3):384-91.

213. Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* 2000;14(2):121-41.
214. Egea PF, Rochel N, Birck C, Vachette P, Timmins PA, Moras D. Effects of ligand binding on the association properties and conformation in solution of retinoic acid receptors RXR and RAR. *J Mol Biol.* 2001;307(2):557-76.
215. Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. *Oncogene.* 2001;20(24):3047-54.
216. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, et al. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell.* 1992;68(2):397-406.
217. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene.* 2004;328:1-16.
218. Ross SA, McCaffery PJ, Drager UC, De Luca LM. Retinoids in embryonal development. *Physiol Rev.* 2000;80(3):1021-54.
219. Wilson JG, Roth CB, Warkany J. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am J Anat.* 1953;92(2):189-217.
220. McPhillips DM, Kalin JR, Hill DL. The pharmacokinetics of all-trans-retinoic acid and N-(2-hydroxyethyl)retinamide in mice as determined with a sensitive and convenient procedure. Solid-phase extraction and reverse-phase high performance liquid chromatography. *Drug Metab Dispos.* 1987;15(2):207-11.
221. Bushue N, Wan YJ. Retinoid pathway and cancer therapeutics. *Adv Drug Deliv Rev.* 2010;62(13):1285-98.
222. Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, et al. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature.* 1994;372(6501):107-11.
223. Austenaa LM, Carlsen H, Ertesvag A, Alexander G, Blomhoff HK, Blomhoff R. Vitamin A status significantly alters nuclear factor-kappaB activity assessed by in vivo imaging. *FASEB J.* 2004;18(11):1255-7.
224. Liao YP, Ho SY, Liou JC. Non-genomic regulation of transmitter release by retinoic acid at developing motoneurons in *Xenopus* cell culture. *J Cell Sci.* 2004;117(Pt 14):2917-24.
225. Masia S, Alvarez S, de Lera AR, Baretino D. Rapid, nongenomic actions of retinoic acid on phosphatidylinositol-3-kinase signaling pathway mediated by the retinoic acid receptor. *Mol Endocrinol.* 2007;21(10):2391-402.
226. Aggarwal S, Kim SW, Cheon K, Tabassam FH, Yoon JH, Koo JS. Nonclassical action of retinoic acid on the activation of the cAMP response element-binding protein in normal human bronchial epithelial cells. *Mol Biol Cell.* 2006;17(2):566-75.
227. Lu TY, Li WC, Chen RY, Fan QX, Wang LX, Wang RL, et al. Inhibition effects of all trans-retinoic acid on the growth and angiogenesis of esophageal squamous cell carcinoma in nude mice. *Chin Med J (Engl).* 2011;124(17):2708-14.
228. Saito A, Sugawara A, Uruno A, Kudo M, Kagechika H, Sato Y, et al. All-trans retinoic acid induces in vitro angiogenesis via retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling. *Endocrinology.* 2007;148(3):1412-23.
229. Easwaran V, Pishvaian M, Salimuddin, Byers S. Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. *Curr Biol.* 1999;9(23):1415-8.
230. Flamant F, Samarut J. Thyroid hormone receptors: lessons from knockout and knock-in mutant mice. *Trends Endocrinol Metab.* 2003;14(2):85-90.
231. Kato S. The function of vitamin D receptor in vitamin D action. *J Biochem.* 2000;127(5):717-22.

232. Lee SS, Gonzalez FJ. Targeted disruption of the peroxisome proliferator-activated receptor alpha gene, PPAR alpha. *Ann N Y Acad Sci.* 1996;804:524-9.
233. Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. *Curr Opin Genet Dev.* 1998;8(5):571-5.
234. Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, Everett C, et al. Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). *Mol Cell Biol.* 2000;20(14):5119-28.
235. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell.* 2000;102(6):731-44.
236. Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature.* 2000;407(6806):920-3.
237. Ghyselinck NB, Dupe V, Dierich A, Messaddeq N, Garnier JM, Rochette-Egly C, et al. Role of the retinoic acid receptor beta (RARbeta) during mouse development. *Int J Dev Biol.* 1997;41(3):425-47.
238. Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. *Cell.* 1993;73(4):643-58.
239. Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM. RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev.* 1994;8(9):1007-18.
240. Sucov HM, Izpisua-Belmonte JC, Ganam Y, Evans RM. Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. *Development.* 1995;121(12):3997-4003.
241. Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, et al. Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev.* 1996;10(1):80-92.
242. Mascres B, Ghyselinck NB, Watanabe M, Annicotte JS, Chambon P, Auwerx J, et al. Ligand-dependent contribution of RXRbeta to cholesterol homeostasis in Sertoli cells. *EMBO Rep.* 2004;5(3):285-90.
243. Brown NS, Smart A, Sharma V, Brinkmeier ML, Greenlee L, Camper SA, et al. Thyroid hormone resistance and increased metabolic rate in the RXR-gamma-deficient mouse. *J Clin Invest.* 2000;106(1):73-9.
244. Giguere V, Ong ES, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. *Nature.* 1987;330(6149):624-9.
245. Leroy P, Krust A, Zelent A, Mendelsohn C, Garnier JM, Kastner P, et al. Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. *EMBO J.* 1991;10(1):59-69.
246. Dolle P, Ruberte E, Leroy P, Morriss-Kay G, Chambon P. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development.* 1990;110(4):1133-51.
247. Ruberte E, Dolle P, Chambon P, Morriss-Kay G. Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development.* 1991;111(1):45-60.
248. Leroy P, Nakshatri H, Chambon P. Mouse retinoic acid receptor alpha 2 isoform is transcribed from a promoter that contains a retinoic acid response element. *Proc Natl Acad Sci U S A.* 1991;88(22):10138-42.
249. Zelent A, Krust A, Petkovich M, Kastner P, Chambon P. Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature.* 1989;339(6227):714-7.

250. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, et al. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci U S A*. 1993;90(15):7225-9.
251. Dawson MI, Chao WR, Pine P, Jong L, Hobbs PD, Rudd CK, et al. Correlation of retinoid binding affinity to retinoic acid receptor alpha with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. *Cancer Res*. 1995;55(19):4446-51.
252. Ross-Innes CS, Stark R, Holmes KA, Schmidt D, Spyrou C, Russell R, et al. Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. *Genes Dev*. 2010;24(2):171-82.
253. Chen N, Napoli JL. All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *FASEB J*. 2008;22(1):236-45.
254. Piskunov A, Rochette-Egly C. A retinoic acid receptor RARalpha pool present in membrane lipid rafts forms complexes with G protein alphaQ to activate p38MAPK. *Oncogene*. 2012;31(28):3333-45.
255. Darwiche N, Celli G, Tennenbaum T, Glick AB, Yuspa SH, De Luca LM. Mouse skin tumor progression results in differential expression of retinoic acid and retinoid X receptors. *Cancer Res*. 1995;55(13):2774-82.
256. Lotan R. Retinoids in cancer chemoprevention. *FASEB J*. 1996;10(9):1031-9.
257. de The H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor a gene to a novel transcribe locus. *Nature*. 1990;347:558-61.
258. De Braekeleer E, Douet-Guilbert N, De Braekeleer M. RARA fusion genes in acute promyelocytic leukemia: a review. *Expert Rev Hematol*. 2014;7(3):347-57.
259. Fenaux P, Chomienne C, Degos L. Treatment of acute promyelocytic leukaemia. *Best Pract Res Clin Haematol*. 2001;14(1):153-74.
260. Tallman MS, Rowe JM. Long-term follow-up and potential for cure in acute promyelocytic leukaemia. *Best Pract Res Clin Haematol*. 2003;16(3):535-43.
261. Osei-Sarfo K, Gudas LJ. Retinoic acid suppresses the canonical Wnt signaling pathway in embryonic stem cells and activates the noncanonical Wnt signaling pathway. *Stem Cells*. 2014;32(8):2061-71.
262. Mao XM, Li H, Zhang XY, Zhou P, Fu QR, Chen QE, et al. Retinoic Acid Receptor alpha Knockdown Suppresses the Tumorigenicity of Esophageal Carcinoma via Wnt/beta-catenin Pathway. *Dig Dis Sci*. 2018;63(12):3348-58.
263. Gupta K, Gulen F, Sun L, Aguilera R, Chakrabarti A, Kiselar J, et al. GSK3 is a regulator of RAR-mediated differentiation. *Leukemia*. 2012;26(6):1277-85.
264. Si J, Mueller L, Collins SJ. GSK3 inhibitors enhance retinoic acid receptor activity and induce the differentiation of retinoic acid-sensitive myeloid leukemia cells. *Leukemia*. 2011;25(12):1914-8.
265. Dihazi GH, Mueller GA, Asif AR, Eltoweissy M, Wessels JT, Dihazi H. Proteomic characterization of adrenal gland embryonic development reveals early initiation of steroid metabolism and reduction of the retinoic acid pathway. *Proteome Sci*. 2015;13:6.
266. Ruberte E, Dolle P, Krust A, Zelent A, Morriss-Kay G, Chambon P. Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development*. 1990;108(2):213-22.
267. Dolle P, Ruberte E, Kastner P, Petkovich M, Stoner CM, Gudas LJ, et al. Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature*. 1989;342(6250):702-5.

268. Sakai A, Imai M, Takahashi K, Hasegawa S, Yamasaki M, Ohba T, et al. Protein kinase A activation by retinoic acid in the nuclei of HL60 cells. *Biochim Biophys Acta Gen Subj*. 2017;1861(2):276-85.
269. Santos NC, Kim KH. Activity of retinoic acid receptor-alpha is directly regulated at its protein kinase A sites in response to follicle-stimulating hormone signaling. *Endocrinology*. 2010;151(5):2361-72.
270. Matsui T. Differential activation of the murine laminin B1 gene promoter by RAR alpha, ROR alpha, and AP-1. *Biochem Biophys Res Commun*. 1996;220(2):405-10.
271. Hsu YT, Li J, Wu D, Sudhof TC, Chen L. Synaptic retinoic acid receptor signaling mediates mTOR-dependent metaplasticity that controls hippocampal learning. *Proc Natl Acad Sci U S A*. 2019;116(14):7113-22.
272. Bastock R, Strutt D. The planar polarity pathway promotes coordinated cell migration during *Drosophila* oogenesis. *Development*. 2007;134(17):3055-64.
273. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 2000;6(4):389-95.
274. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407(6801):249-57.
275. Szabo DR, Baghy K, Szabo PM, Zsippai A, Marczell I, Nagy Z, et al. Antitumoral effects of 9-cis retinoic acid in adrenocortical cancer. *Cell Mol Life Sci*. 2014;71(5):917-32.
276. Bird IM, Mason JI, Rainey WE. Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. *Endocrinology*. 1994;134(6):2468-74.
277. Richards AM, Nicholls MG, Espiner EA, Ikram H, Cullens M, Hinton D. Diurnal patterns of blood pressure, heart rate and vasoactive hormones in normal man. *Clin Exp Hypertens A*. 1986;8(2):153-66.
278. Funder JW, Feldman D, Edelman IS. The roles of plasma binding and receptor specificity in the mineralocorticoid action of aldosterone. *Endocrinology*. 1973;92:994-1004.
279. Lombes M, Kenouch S, Souque A, Farman N, Rafestin-Oblin M-E. The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 β -hydroxysteroid dehydrogenase. *Endocrinology*. 1994;135(3):834-40.
280. Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M. The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. *Nucl Recept Signal*. 2007;5:e012.
281. Zennaro MC, Fernandes-Rosa F. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Mineralocorticoid receptor mutations. *J Endocrinol*. 2017;234(1):T93-T106.
282. Berger S, Bleich M, Schmid W, Cole TJ, Peters J, Watanabe H, et al. Mineralocorticoid receptor knockout mice: pathophysiology of Na⁺ metabolism. *Proc Natl Acad Sci U S A*. 1998;95(16):9424-9.
283. Bleich M, Warth R, Schmidt-Hieber M, Schulz-Baldes A, Hasselblatt P, Fisch D, et al. Rescue of the mineralocorticoid receptor knock-out mouse. *Pflugers Arch*. 1999;438(3):245-54.
284. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. *Cell*. 1995;83(6):835-9.
285. Funder JW. Mineralocorticoids, glucocorticoids, receptors and response elements. *Science*. 1993;259(5098):1132-3.
286. Liu W, Wang J, Yu G, Pearce D. Steroid receptor transcriptional synergy is potentiated by disruption of the DNA-binding domain dimer interface. *Mol Endocrinol*. 1996;10(11):1399-406.

287. Binart N, Lombes M, Baulieu EE. Distinct functions of the 90 kDa heat-shock protein (hsp90) in oestrogen and mineralocorticosteroid receptor activity: effects of hsp90 deletion mutants. *Biochem J.* 1995;311(Part 3):797-804.
288. Pratt WB. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. *Annu Rev Pharmacol Toxicol.* 1997;37:297-326.
289. Bruner KL, Derfoul A, Robertson NM, Guerriero G, Fernandes-Alnemri T, Alnemri ES, et al. The unliganded mineralocorticoid receptor is associated with heat shock proteins 70 and 90 and the immunophilin FKBP-52. *Recept Signal Transduct.* 1997;7(2):85-98.
290. Galigniana MD, Erlejman AG, Monte M, Gomez-Sanchez C, Piwien-Pilipuk G. The hsp90-FKBP52 complex links the mineralocorticoid receptor to motor proteins and persists bound to the receptor in early nuclear events. *Mol Cell Biol.* 2010;30(5):1285-98.
291. Le Billan F, Khan JA, Lamribet K, Viengchareun S, Bouligand J, Fagart J, et al. Cistrome of the aldosterone-activated mineralocorticoid receptor in human renal cells. *FASEB J.* 2015;29(9):3977-89.
292. Le Billan F, Amazit L, Bleakley K, Xue QY, Pussard E, Lhadj C, et al. Corticosteroid receptors adopt distinct cyclical transcriptional signatures. *FASEB J.* 2018;32(10):5626-39.
293. Jaffe IZ, Mendelsohn ME. Angiotensin II and aldosterone regulate gene transcription via functional mineralocorticoid receptors in human coronary artery smooth muscle cells. *Circ Res.* 2005;96(6):643-50.
294. Geerling JC, Kawata M, Loewy AD. Aldosterone-sensitive neurons in the rat central nervous system. *J Comp Neurol.* 2006;494(3):515-27.
295. Fitzsimons JT. Angiotensin, thirst, and sodium appetite. *Physiol Rev.* 1998;78(3):583-686.
296. de Kloet ER, Van Acker SA, Sibug RM, Oitzl MS, Meijer OC, Rahmouni K, et al. Brain mineralocorticoid receptors and centrally regulated functions. *Kidney Int.* 2000;57(4):1329-36.
297. Gomez-Sanchez EP. Intracerebroventricular infusion of aldosterone induces hypertension in rats. *Endocrinology.* 1986;118(2):819-23.
298. Sakai RR, McEwen BS, Fluharty SJ, Ma LY. The amygdala: site of genomic and nongenomic arousal of aldosterone-induced sodium intake. *Kidney Int.* 2000;57(4):1337-45.
299. van Weert L, Buurstede JC, Sips HCM, Vettorazzi S, Mol IM, Hartmann J, et al. Identification of mineralocorticoid receptor target genes in the mouse hippocampus. *J Neuroendocrinol.* 2019;31(8):e12735.
300. Zennaro MC, Hubert EL, Fernandes-Rosa FL. Aldosterone resistance: structural and functional considerations and new perspectives. *Mol Cell Endocrinol.* 2012;350(2):206-15.
301. Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, et al. Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A.* 1999;96:2514-9.
302. Muller OG, Parnova RG, Centeno G, Rossier BC, Firsov D, Horisberger JD. Mineralocorticoid effects in the kidney: correlation between alphaENaC, GILZ, and Sgk-1 mRNA expression and urinary excretion of Na⁺ and K⁺. *J Am Soc Nephrol.* 2003;14(5):1107-15.
303. Liang X, Peters KW, Butterworth MB, Frizzell RA. 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. *J Biol Chem.* 2006;281(24):16323-32.
304. Staub O, Rotin D. Role of ubiquitylation in cellular membrane transport. *Physiol Rev.* 2006;86(2):669-707.

305. Fakitsas P, Adam G, Daidie D, van Bemmelen MX, Fouladkou F, Patrignani A, et al. Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation. *J Am Soc Nephrol*. 2007;18(4):1084-92.
306. Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, et al. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na⁽⁺⁾ channel cell surface expression. *EMBO J*. 2001;20(24):7052-9.
307. Soundararajan R, Pearce D, Hughey RP, Kleyman TR. Role of epithelial sodium channels and their regulators in hypertension. *J Biol Chem*. 2010;285(40):30363-9.
308. Rossi GP, Sacchetto A, Pavan E, Palatini P, Graniero GR, Canali C, et al. Remodeling of the left ventricle in primary aldosteronism due to Conn's adenoma. *Circulation*. 1997;95(6):1471-8.
309. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *J Am Coll Cardiol*. 2005;45(8):1243-8.
310. Farquharson CA, Struthers AD. Aldosterone induces acute endothelial dysfunction in vivo in humans: evidence for an aldosterone-induced vasculopathy. *Clin Sci (Lond)*. 2002;103(4):425-31.
311. Caprio M, Newfell BG, la Sala A, Baur W, Fabbri A, Rosano G, et al. Functional mineralocorticoid receptors in human vascular endothelial cells regulate intercellular adhesion molecule-1 expression and promote leukocyte adhesion. *Circ Res*. 2008;102(11):1359-67.
312. Selye H. Production of Nephrosclerosis by Overdosage with Desoxycorticosterone Acetate. *Can Med Assoc J*. 1942;47(6):515-9.
313. Selye H. Protection by a steroid-spirolactone against certain types of cardiac necroses. *Proc Soc Exp Biol Med*. 1960;104:212-3.
314. Pitt B, Zannad F, Remme W, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. *N Engl J Med*. 1999;341(10):709-17.
315. Pitt B, White H, Nicolau J, Martinez F, Gheorghide M, Aschermann M, et al. Eplerenone reduces mortality 30 days after randomization following acute myocardial infarction in patients with left ventricular systolic dysfunction and heart failure. *J Am Coll Cardiol*. 2005;46(3):425-31.
316. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *N Engl J Med*. 2011;364(1):11-21.
317. Swedberg K, Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Shi H, et al. Eplerenone and atrial fibrillation in mild systolic heart failure: results from the EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization And Survival Study in Heart Failure) study. *J Am Coll Cardiol*. 2012;59(18):1598-603.
318. Leopold JA, Dam A, Maron BA, Scribner AW, Liao R, Handy DE, et al. Aldosterone impairs vascular reactivity by decreasing glucose-6-phosphate dehydrogenase activity. *Nat Med*. 2007;13(2):189-97.
319. Farquharson CA, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and suppresses vascular angiotensin I/angiotensin II conversion in patients with chronic heart failure. *Circulation*. 2000;101(6):594-7.
320. Shibata S, Nagase M, Yoshida S, Kawarazaki W, Kurihara H, Tanaka H, et al. Modification of mineralocorticoid receptor function by Rac1 GTPase: implication in proteinuric kidney disease. *Nat Med*. 2008;14(12):1370-6.
321. Chrysostomou A, Becker G. Spironolactone in addition to ACE inhibition to reduce proteinuria in patients with chronic renal disease. *N Engl J Med*. 2001;345(12):925-6.

322. Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, et al. Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-gamma, and proinflammatory adipokines. *Circulation*. 2008;117(17):2253-61.
323. McCurley A, Jaffe IZ. Mineralocorticoid receptors in vascular function and disease. *Mol Cell Endocrinol*. 2012;350(2):256-65.
324. Brilla CG, Pick R, Tan LB, Janicli JS, Weber KT. Remodeling of the rat right and left ventricle in experimental hypertension. *Circ Res*. 1990;67:1355-64.
325. Rocha R, Rudolph AE, Friedrich GE, Nachowiak DA, Kekec BK, Blomme EA, et al. Aldosterone induces a vascular inflammatory phenotype in the rat heart. *Am J Physiol Heart Circ Physiol*. 2002;283(5):H1802-10.
326. Rickard AJ, Young MJ. Corticosteroid receptors, macrophages and cardiovascular disease. *J Mol Endocrinol*. 2009;42(6):449-59.
327. Keidar S, Kaplan M, Pavlotzky E, Coleman R, Hayek T, Hamoud S, et al. Aldosterone administration to mice stimulates macrophage NADPH oxidase and increases atherosclerosis development: a possible role for angiotensin-converting enzyme and the receptors for angiotensin II and aldosterone. *Circulation*. 2004;109(18):2213-20.
328. Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ. Deletion of mineralocorticoid receptors from macrophages protects against deoxycorticosterone/salt-induced cardiac fibrosis and increased blood pressure. *Hypertension*. 2009;54(3):537-43.
329. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schutz G, et al. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. *J Clin Invest*. 2010;120(9):3350-64.
330. Fraccarollo D, Berger S, Galuppo P, Kneitz S, Hein L, Schutz G, et al. Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. *Circulation*. 2011;123(4):400-8.
331. Rickard AJ, Morgan J, Chrissobolis S, Miller AA, Sobey CG, Young MJ. Endothelial cell mineralocorticoid receptors regulate deoxycorticosterone/salt-mediated cardiac remodeling and vascular reactivity but not blood pressure. *Hypertension*. 2014;63(5):1033-40.
332. Gueret A, Harouki N, Favre J, Galmiche G, Nicol L, Henry JP, et al. Vascular Smooth Muscle Mineralocorticoid Receptor Contributes to Coronary and Left Ventricular Dysfunction After Myocardial Infarction. *Hypertension*. 2016;67(4):717-23.
333. Lavall D, Selzer C, Schuster P, Lenski M, Adam O, Schafers HJ, et al. The mineralocorticoid receptor promotes fibrotic remodeling in atrial fibrillation. *J Biol Chem*. 2014;289(10):6656-68.
334. Zennaro MC, Caprio M, Fève B. Mineralocorticoid receptors in the metabolic syndrome. *Trends Endocrinol Metab*. 2009;20(9):444-51.
335. Burton TJ, Cope G, Wang J, Sim JC, Azizan EA, O'Shaughnessy KM, et al. Expression of the epithelial Na(+) channel and other components of an aldosterone response pathway in human adrenocortical cells. *Eur J Pharmacol*. 2009;613(1-3):176-81.
336. Romero DG, Yanes LL, de Rodriguez AF, Plonczynski MW, Welsh BL, Reckelhoff JF, et al. Disabled-2 is expressed in adrenal zona glomerulosa and is involved in aldosterone secretion. *Endocrinology*. 2007;148(6):2644-52.
337. Czirjak G, Fischer T, Spat A, Lesage F, Enyedi P. TASK (TWIK-related acid-sensitive K⁺ channel) is expressed in glomerulosa cells of rat adrenal cortex and inhibited by angiotensin II. *Mol Endocrinol*. 2000;14(6):863-74.

10 Appendix

10.1 A gain-of-function mutation in the *CLCN2* chloride channel gene causes primary aldosteronism

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A gain-of-function mutation in the *CLCN2* chloride channel gene causes primary aldosteronism

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Primary aldosteronism is the most common and curable form of secondary arterial hypertension. We performed whole-exome sequencing in patients with early-onset primary aldosteronism and identified a de novo heterozygous c.71G>A/p.Gly24Asp mutation in the *CLCN2* gene, encoding the voltage-gated CIC-2 chloride channel¹, in a patient diagnosed at 9 years of age. Patch-clamp analysis of glomerulosa cells of mouse adrenal gland slices showed hyperpolarization-activated Cl⁻ currents that were abolished in *Clcn2*^{-/-} mice. The p.Gly24Asp variant, located in a well-conserved 'inactivation domain'^{2,3}, abolished the voltage- and time-dependent gating of CIC-2 and strongly increased Cl⁻ conductance at resting potentials. Expression of CIC-2^{Asp24} in adrenocortical cells increased expression of aldosterone synthase and aldosterone production. Our data indicate that *CLCN2* mutations cause primary aldosteronism. They highlight the important role of chloride in aldosterone biosynthesis and identify CIC-2 as the foremost chloride conductor of resting glomerulosa cells.

Arterial hypertension is a major cardiovascular risk factor⁴. Primary aldosteronism is the most common and curable form of secondary arterial hypertension, with an estimated prevalence of ~10% in referred patients and 4% in primary care⁵, and a prevalence of up to 20% in patients with resistant hypertension⁶. Primary aldosteronism results from autonomous aldosterone production in the adrenal cortex⁷, caused in most cases by a unilateral aldosterone-producing adenoma or bilateral adrenal hyperplasia (BAH). It is diagnosed on the basis of hypertension associated with an increased aldosterone-to-renin ratio and often hypokalemia⁸. In comparison to essential hypertension, increased aldosterone levels in primary aldosteronism are associated with increased cardiovascular risk, in particular for coronary artery disease, heart failure, renal damage, and stroke^{9,10}.

Gain-of-function mutations in different genes, encoding cation channels (*KCNJ5*¹¹, *CACNA1D*^{12,13}, and *CACNA1H*^{14,15}) and ATPases (*ATP1A1* and *ATP2B3*^{12,16}), regulating intracellular ion homeostasis and plasma membrane potential, have been described in aldosterone-producing adenoma and familial forms of primary aldosteronism, but the pathophysiology of many cases is still unknown.

We performed whole-exome sequencing on germline DNA from 12 patients with young-onset hypertension and hyperaldosteronism diagnosed before 25 years of age. Two index cases were investigated together with their parents and unaffected sibling to search for de novo variants. A de novo germline *CLCN2* variant, c.71G>A (NM_004366; p.Gly24Asp), was identified in subject K1011-1, but not in her asymptomatic parents (K1011-3 and K1011-4) and sibling (K1011-2; Fig. 1a,b and Table 1). The variant was absent from more than 120,000 alleles in the Exome Aggregation Consortium (ExAC) database and in our in-house database. We did not find additional *CLCN2* variants among the other 11 investigated individuals. *CLCN2* encodes the chloride channel CIC-2. The variant p.Gly24Asp is located in its N-terminal cytoplasmic domain (Fig. 1c,d). Gly24 is highly conserved in CIC-2 from species as distant as zebrafish and *Xenopus tropicalis* (Fig. 1c).

The patient carrying the *CLCN2* variant encoding p.Gly24Asp is a 9-year-old girl who presented with severe headache and vomiting lasting for 1 year (Table 1). The child was developmentally normal, first born to a non-consanguineous couple. There was a history of mild hypertension in the maternal grandmother and granduncle. Blood pressure was 172/100 mm Hg, and heart rate was 120 beats/min. The rest of the examination was normal. Her work-up showed persistent hypokalemia (serum K⁺ ranging from 1.8 to 2.4 meq/L), elevated serum aldosterone (868.1 pg/ml, reference range 12–340 pg/ml), and suppressed plasma renin activity (0.11 ng/ml/h, reference range 1.9 to 6.0 ng/ml/h in an upright posture), suggestive of primary aldosteronism. Abdominal computed tomography (CT) scans showed no adrenal abnormalities. Other parameters including 24-h urinary vanillylmandelic acid and serum cortisol were normal. Hypertension was initially managed with amlodipin, enalapril, and atenolol. Once the diagnosis of primary aldosteronism was made, spironolactone was added, enalapril was stopped, and the doses of amlodipin and atenolol were reduced. Serum K⁺ normalized. A positive glucocorticoid suppression test (with aldosterone at 949.3 pg/ml at baseline and 56.9 pg/ml after oral administration of 0.5 mg of dexamethasone every 6 h for 48 h) suggested the possibility of glucocorticoid-remediable aldosteronism (GRA), a rare familial

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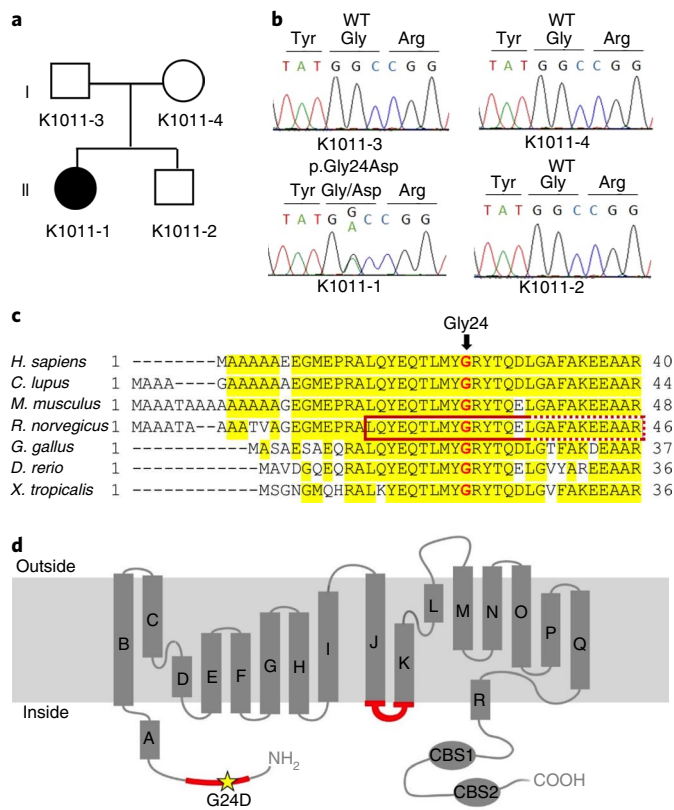


Fig. 1 | A *CLCN2* variant identified in a patient with early-onset primary aldosteronism. a, Pedigree of kindred K1011. The subject with primary aldosteronism is shown with a filled symbol, and non-affected subjects are shown with unfilled symbols. **b**, Sanger sequencing chromatograms showing the wild-type *CLCN2* sequence of the unaffected parents (K1011-3 and K1011-4) and brother (K1011-2) and the *CLCN2* variant c.71G>A (p.Gly24Asp) identified in the patient with early-onset primary aldosteronism (K1011-1). **c**, Alignment and conservation of residues encoded by *CLCN2* orthologs. The red box indicates the N-terminal inactivation domain of CIC-2. Several deletions and mutations mapping to this region of rat CIC-2 lead to constitutively open CIC-2 channels (solid line) or partially open channels (dashed line)³. Residues that are conserved among more than three sequences are highlighted in yellow. **d**, Position of the disease-causing p.Gly24Asp variant in the CIC-2 protein (schematic transmembrane topology drawing modified from ref. ³⁶). Inactivation domains previously identified by structure-function analysis in the N terminus³ and an intracellular loop² of CIC-2 are shown in red. Several point mutations and deletions affecting these domains open the CIC-2 channel^{2,3} similarly to the p.Gly24Asp substitution described here. CBS1 and CBS2 are cystathionine- β -synthase domains that can affect gating of CIC channels²³.

form of hyperaldosteronism¹⁷. However, genetic analysis for a chimeric *CYP11B1-CYP11B2* gene was negative. The child's hypertension has been well controlled over 18 months with prednisolone at 5 mg/m²/d, spironolactone, and amlodipin. On treatment, her serum aldosterone and plasma renin levels are 421 pg/ml (reference range 25 to 392 pg/ml) and 8.22 μ U/ml (4.4 to 46.1 μ U/ml), respectively. After exclusion of GRA by genetic testing, prednisolone treatment was stopped.

Cl⁻ conductance can regulate the excitability of neuronal, muscle, and endocrine cells^{18–21}. In zona glomerulosa cells, adrenocorticotrophic hormone (ACTH)-activated Cl⁻ currents have been described²², but their outward rectification sets them apart from hyperpolarization-activated CIC-2 currents. CIC-2 is expressed in almost all tissues¹ and may have roles in ion homeostasis and

Table 1 | Clinical and biological characteristics of individuals carrying *CLCN2* variants

	K1011-1	K963-1	K1044-1
Sex	F	F	F
Age at HTN dg (years)	9	19	29
Age at primary aldosteronism dg (years)	9	27	48
SBP at primary aldosteronism dg (mm Hg)	172	139	173
DBP at primary aldosteronism dg (mm Hg)	100	90	114
Lowest plasma K ⁺ (mmol/L)	1.8	2.9	2.5
Urinary aldosterone (nmol/24 h)	ND	60	ND
Plasma aldosterone (pmol/L)	2,406	927	1,061
Plasma renin (mU/L)	0.9	1.9	<1
ARR (pmol/mU)	481.2	185.4	212.2
Adrenal abnormalities on imaging	No	No	No
Lateralization at AVS	ND	No	No

Hormonal data were obtained at diagnosis of primary aldosteronism. For comparison within this table, plasma aldosterone levels for patient K1011-1 were converted to pmol/L and plasma renin activity was converted to plasma renin concentration. For ARR calculation, renin values <5 were transformed to 5. The conversion factor used for plasma aldosterone was 1 ng/L = 2.77 pmol/L, and the conversion factor used for plasma renin was 1 ng/ml/h = 8.2 mU/L. dg, diagnosis; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; ARR, aldosterone-to-renin ratio; ND, not determined; AVS, adrenal venous sampling

transepithelial transport²³. *Cln2*^{-/-} mice display early postnatal retinal and testicular degeneration²⁴ as well as leukodystrophy^{25,26}; in humans, *CLCN2* loss-of-function mutations result in leukodystrophy²⁷ that may be associated with azoospermia²⁸. These phenotypes have been ascribed to a role for CIC-2 in extracellular ion homeostasis^{24,25}.

Data retrieved from a transcriptome analysis including 11 human adrenal glands²⁹ showed high expression of *CLCN2* in human adrenal cortex (Supplementary Table 1). In mice, western blots showed similar expression of CIC-2 in whole adrenal gland as in brain (Fig. 2a), which expresses substantial, physiologically important amounts of CIC-2²⁵. Patch-clamp analysis of glomerulosa cells in situ showed typical hyperpolarization-activated currents in wild-type mice, but not in *Cln2*^{-/-} mice (Fig. 2b,c). The magnitude of these currents was similar to those observed in Bergmann glia, which prominently express CIC-2²⁶. The almost complete absence of Cl⁻ currents in *Cln2*^{-/-} cells demonstrates that under resting conditions CIC-2 mediates the bulk of glomerulosa cell Cl⁻ currents.

The CIC-2 p.Gly24Asp variant is located in a highly conserved inactivation domain^{2,3} of the channel. Deletions and point mutations mapping to this region and to an intracellular loop² (highlighted in Fig. 1c,d) lead to 'open' CIC-2 channels that have lost their sensitivity to voltage, cell swelling, or external pH^{2,3}. Likewise, insertion of the p.Gly24Asp variant drastically changed voltage-dependent gating of CIC-2 (Fig. 2d,e,h) and dramatically increased current amplitudes when the human protein was expressed in *Xenopus laevis* oocytes (Fig. 2d–g). When measured at -80 mV, the approximate resting potential of glomerulosa cells³⁰, current amplitudes from the mutant channel were much larger than those for the wild-type channel (Fig. 2d–f). Linear, ohmic currents like those of the mutant channel might be due to unspecific electrical leaks; however, the currents of both wild-type and mutant CIC-2 were markedly reduced when extracellular chloride was replaced by iodide (Fig. 2d–f), in agreement with the Cl⁻>I⁻ selectivity of CIC channels in general²³ and CIC-2 in particular¹. The activation of CIC-2 by an acidic

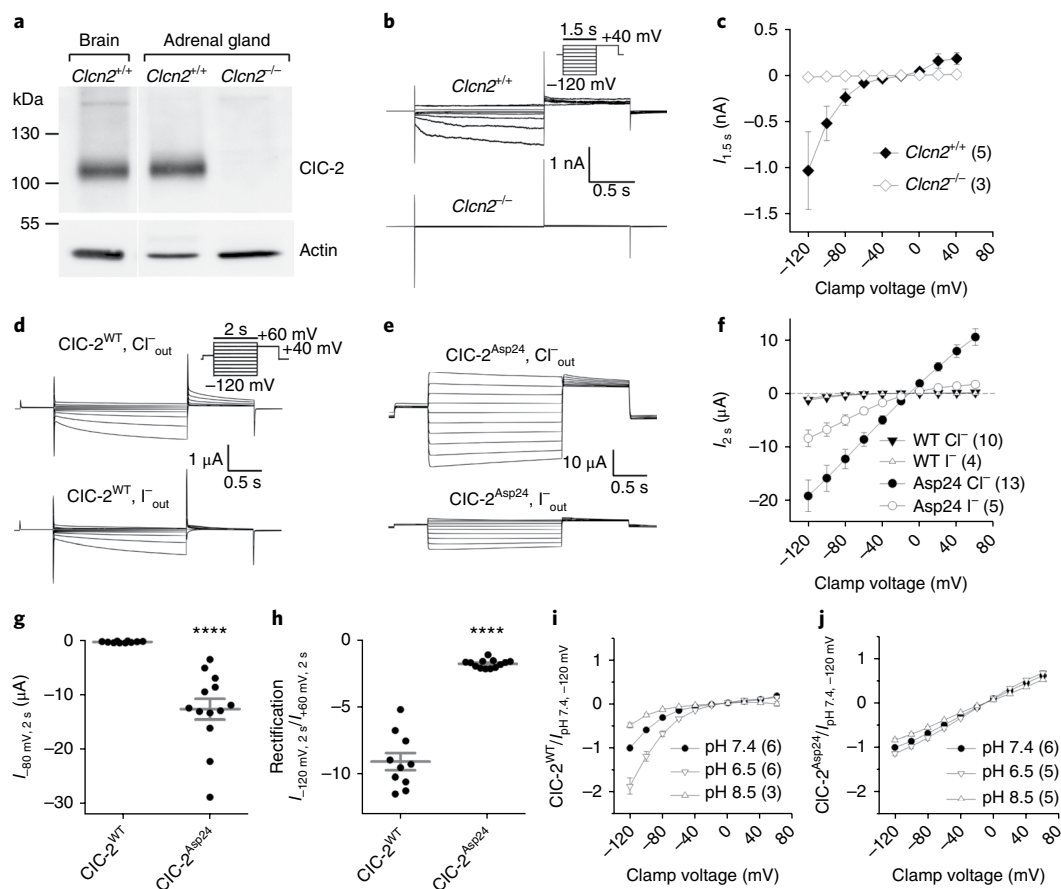


Fig. 2 | CIC-2 expression in mouse adrenal glands and electrophysiological analyses of wild-type and mutant channels. **a**, CIC-2 immunoblot of brain and adrenal glands from *Clcn2*^{+/+} and *Clcn2*^{-/-} mice. All lanes are from the same blot, which was cut where indicated. Similar amounts of protein were loaded with actin serving as a loading control. This blot is representative of three independent experiments. **b**, Representative whole-cell chloride current traces of mouse zona glomerulosa cells from *Clcn2*^{+/+} (top) and *Clcn2*^{-/-} (bottom) adrenal slices using voltage steps as indicated above. **c**, Mean \pm s.e.m. currents measured after 1.5 s from the experiments in **b** plotted as a function of clamp voltage. The number of cells analyzed is given in parentheses. **d,e**, Representative chloride current traces measured by two-electrode voltage-clamp from *Xenopus* oocytes injected with either human CIC-2^{WT} (**d**) or CIC-2^{Asp24} (**e**) cRNA, using the protocol shown in **d**. For some measurements (below), extracellular chloride was replaced with iodide. **f**, Mean \pm s.e.m. currents measured in **d** and **e** plotted as a function of voltage. The number of cells analyzed is given in parentheses. **g,h**, Summary of Cl⁻ currents at -80 mV ($I_{-80\text{ mV}, 2\text{ s}}$) (**g**) and current ratios ($I_{-120\text{ mV}, 2\text{ s}}/I_{+60\text{ mV}, 2\text{ s}}$) as a measure of rectification (**h**) (always measured at 2 s) for panels **d-f**. **** $P < 0.0001$, Mann-Whitney two-tailed test. **i,j**, Effect of external pH on currents mediated by CIC-2^{WT} (**i**) and CIC-2^{Asp24} (**j**) in *Xenopus* oocytes. Currents were normalized to mean currents from the respective construct measured after 2 s at -120 mV and at pH 7.4. The number of oocytes analyzed is given in parentheses; error bars, s.e.m.

extracellular pH can be almost abolished by mutations mapping to the inactivation domain². Likewise, the CIC-2^{Asp24} mutant had largely reduced pH sensitivity (Fig. 2i,j and Supplementary Fig. 1). In conclusion, the p.Gly24Asp variant results in a strong gain of function, in line with the dominant disease phenotype of the mutation that is present in the heterozygous state. It also suggests a pathophysiological mechanism in which a strong increase in Cl⁻ currents may depolarize glomerulosa cells, thereby opening voltage-gated Ca²⁺ channels and activating transcriptional programs via an increase in cytosolic Ca²⁺.

Expression of the mutant CIC-2^{Asp24} channel in human adrenocortical H295R-S2 cells and, conversely, knockdown of CIC-2 by shRNA significantly affected aldosterone production and expression of steroidogenic enzymes. Despite similar expression of CIC-2 in H295R-S2 cells stably transfected to express CIC-2^{Asp24} and wild-type CIC-2 (CIC-2^{WT}) (Fig. 3a,b), aldosterone synthase expression (Fig. 3a,c) and aldosterone production (Fig. 3d,e) were significantly increased in CIC-2^{Asp24}-expressing cells. Stimulation with angiotensin II (Ang II; 10 nM) or K⁺ (12 mM) increased aldosterone production in cells expressing CIC-2^{WT} (Fig. 3e). A further increase was

observed in cells expressing CIC-2^{Asp24} after Ang II stimulation, but not after K⁺ stimulation (Fig. 3e). Nevertheless, also after stimulation, aldosterone production in cells expressing CIC-2^{Asp24} was significantly higher than in cells expressing CIC-2^{WT} (Fig. 3e). Infection of H295R-S2 cells with shRNA constructs targeting CIC-2 reduced *CLCN2* expression by ~50% (Supplementary Fig. 2a) as compared with a scrambled shRNA and significantly reduced aldosterone production, both at baseline and after stimulation (Supplementary Fig. 2b), suggesting that even CIC-2^{WT} currents, although much smaller than currents from the CIC-2^{Asp24} mutant, increase the excitability of H295R-S2 adrenocortical cells. These changes were paralleled in both models by concomitant modifications of the expression of steroidogenic genes. An increase in the mRNA expression of *CYP11B2* (encoding aldosterone synthase; Fig. 3f), *STAR* (encoding the steroidogenic acute regulatory protein; Fig. 3g), and *CYP21A2* (encoding steroid 21-hydroxylase; Fig. 3h) was observed in CIC-2^{Asp24} as compared with CIC-2^{WT}-overexpressing cells under basal conditions. Ang II increased expression of *STAR* and *CYP11B2*, while K⁺ stimulation increased mRNA expression of *CYP11B2*. Conversely, knockdown of CIC-2 led to a decrease in

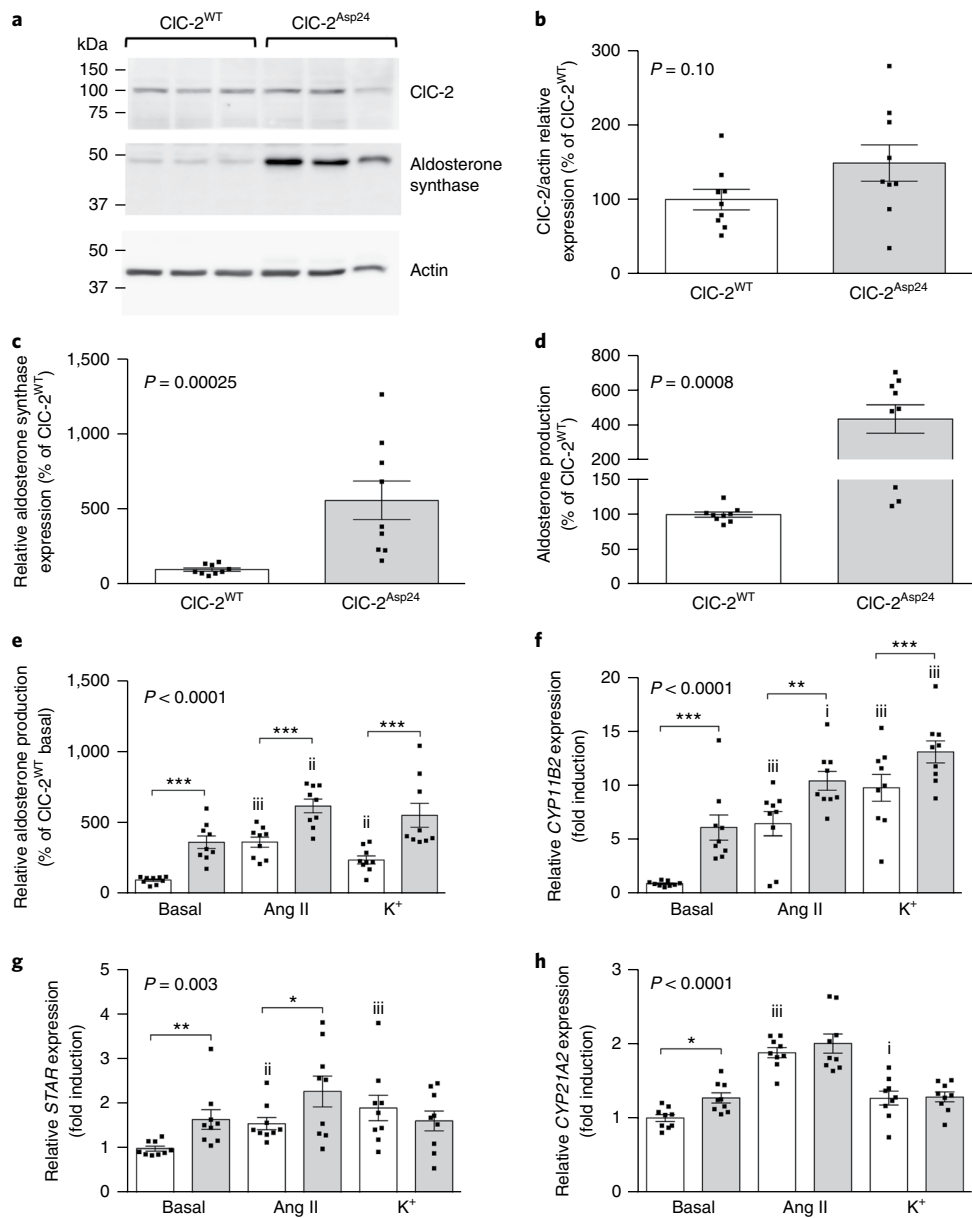


Fig. 3 | Effect of CIC-2^{WT} and mutant CIC-2^{Asp24} channels on aldosterone production and expression of genes and proteins involved in aldosterone biosynthesis. **a**, Western blots for CIC-2 and aldosterone synthase of H295R-S2 cells stably transfected to express CIC-2^{WT} or mutant CIC-2^{Asp24}. These blots are representative of three independent experiments, with actin serving as a loading control. **b**, Quantification of CIC-2 protein levels in CIC-2^{WT} and CIC-2^{Asp24} H295R-S2 cells (t test, $P = 0.10$, $F = 3.19$). **c**, Quantification of aldosterone synthase expression in CIC-2^{WT} and CIC-2^{Asp24} H295R-S2 cells (t test, $P = 0.0025$, $F = 136$). **d**, Basal aldosterone production by H295R-S2 cells transfected to express CIC-2^{WT} or mutant CIC-2^{Asp24} (t test, $P = 0.0008$, $F = 142$). **e**, Basal and stimulated aldosterone production by H295R-S2 cells transfected to express CIC-2^{WT} (open bars) or mutant CIC-2^{Asp24} (filled bars) (one-way ANOVA, $P < 0.0001$, $F = 23.46$). **f–h**, Basal and stimulated mRNA expression of *CYP11B2* (one-way ANOVA, $P < 0.001$, $F = 18.39$) (**f**), *STAR* (Kruskal-Wallis, $P = 0.0033$) (**g**), and *CYP21A2* (one-way ANOVA, $P < 0.0001$, $F = 23.27$) (**h**) in H295R-S2 cells transfected to express CIC-2^{WT} (open bars) or mutant CIC-2^{Asp24} (filled bars). Quantifications of protein expression (using actin as a loading control) and aldosterone production are presented as the percentage of the value for CIC-2^{WT}-expressing cells under basal conditions, and the results of mRNA expression are presented as fold induction relative to CIC-2^{WT}-expressing cells under basal conditions. Values of all experiments are presented as the means \pm s.e.m. of three independent experiments performed in experimental triplicate ($n = 9$) for each condition. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; i, $P < 0.05$ for the stimulated versus basal condition; ii, $P < 0.01$ for the stimulated versus basal condition; iii, $P < 0.001$ for the stimulated versus basal condition.

CYP11B2 expression under all conditions (Supplementary Fig. 2c). These data further support the notion that a gain-of-function *CLCN2* mutation may depolarize the cell, activate the steroidogenic pathway, and increase aldosterone production. While knockdown of CIC-2 influences aldosterone production in H295R-S2 cells, which have a resting potential of about -65 mV (Fig. 4a,b), this may not be the case in native glomerulosa cells. Because their membrane

voltage (V_m) is close to the K⁺ equilibrium potential³⁰, they are unlikely to markedly hyperpolarize upon loss of CIC-2. No changes in blood pressure have been reported for mice or patients lacking CIC-2^{24,25,27}, but this issue has not been investigated in detail.

We next explored the effect of the CIC-2 p.Gly24Asp variant on membrane potential and calcium influx through voltage-gated calcium channels. These studies were performed with the perforated

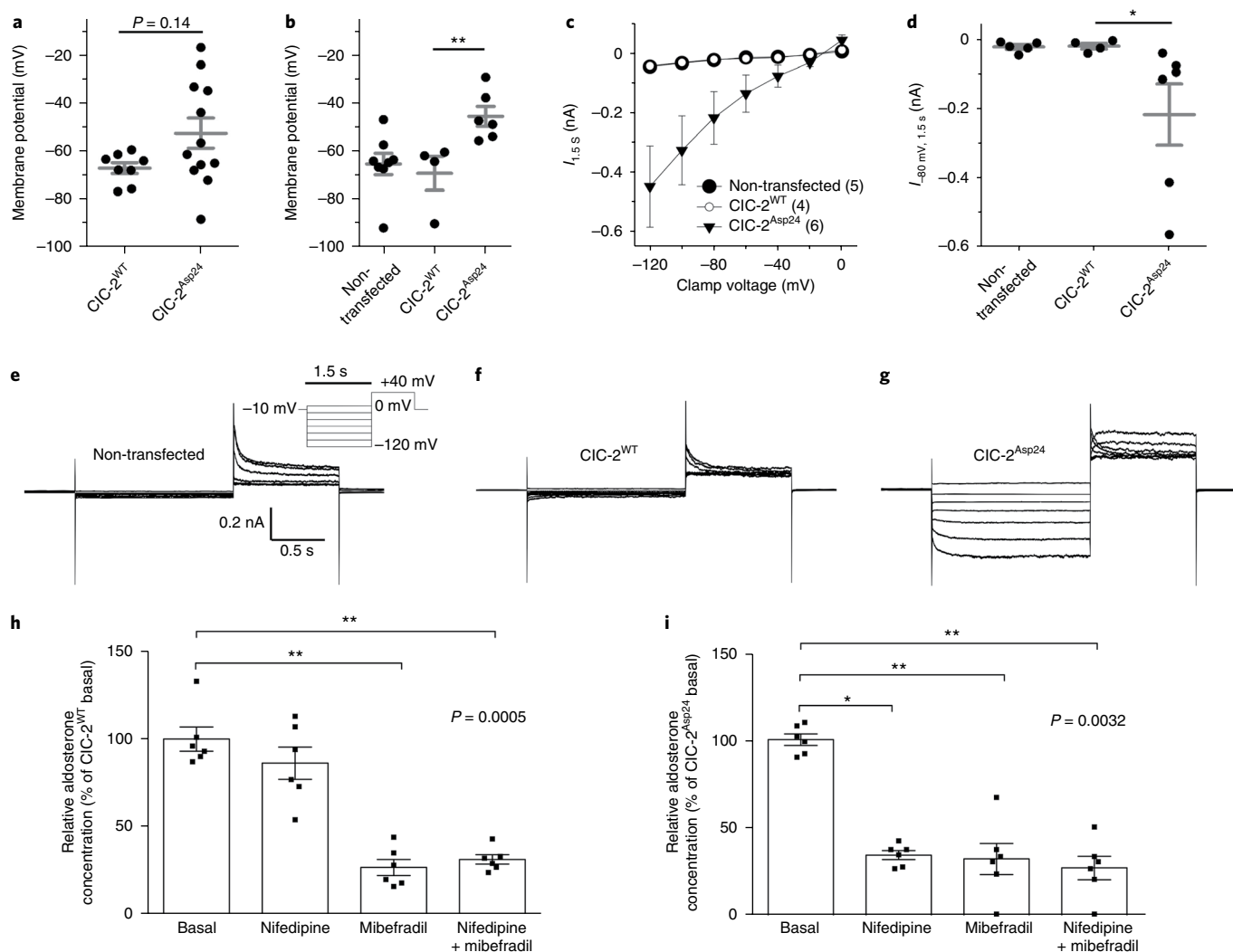


Fig. 4 | Functional impact of the CIC-2^{Asp24} variant. a–g, Effect on membrane potential (V_m) and plasma membrane anion currents in H295R-S2 cells. **a**, Resting membrane potential of the CIC-2^{WT} and CIC-2^{Asp24} channels in the stably transfected H295R-S2 cells that were used to determine aldosterone secretion. Note the strong mean depolarization (CIC-2^{WT}, -67 ± 2 mV ($n=8$); CIC-2^{Asp24}, -52 ± 6 mV ($n=12$)), which, however, was not significant (two-tailed Mann-Whitney, $P=0.14$) owing to large variability in V_m for CIC-2^{Asp24}-expressing cells that were not clonally selected. **b**, Similarly determined values of V_m for non-transfected and transiently transfected H295R-S2 cells (non-transfected, -65 ± 4 mV ($n=8$); CIC-2^{WT}, -69 ± 7 mV ($n=4$); CIC-2^{Asp24}, -46 ± 4 mV ($n=6$); ** $P=0.0095$, two-tailed Mann-Whitney test). **c,d**, Corresponding plasma membrane currents measured after 1.5 s under conditions eliminating cation inward currents plotted as a function of voltage (**c**) or as individual values at the physiological V_m of glomerulosa cells (**d**). The number of cells analyzed is given in parentheses. * $P=0.019$, two-tailed Mann-Whitney test. **e–g**, Corresponding averaged current traces with 20-mV voltage steps between 0 and -120 mV for non-transfected cells (**e**) and cells expressing CIC-2^{WT} (**f**) and CIC-2^{Asp24} (**g**). **h,i**, Effect of calcium channel blockers on aldosterone production in H295R-S2 cells expressing CIC-2^{WT} (Kruskal-Wallis, $P=0.0005$) (**h**) and CIC-2^{Asp24} (Kruskal-Wallis, $P=0.0032$) (**i**). Values represent the means \pm s.e.m. of two independent experiments performed in experimental triplicate ($n=6$) for each condition. Dunn's post test for the treated versus basal condition: * $P < 0.05$, ** $P < 0.01$.

patch-clamp technique, which does not disturb the intracellular chloride concentration and is required to see the full effect of inactivation domain^{2,3} mutations^{31,32}. In the stably transfected H295R-S2 cells used to investigate steroidogenesis (Fig. 3), there was a trend toward V_m being depolarized in CIC-2^{Asp24}- as compared to CIC-2^{WT}-overexpressing cells (mean values of roughly -52 and -67 mV, respectively) (Fig. 4a). However, because these cell lines were not clonal, the variability was large and the difference was not statistically significant.

We therefore resorted to transient transfection of H295R-S2 cells, which allowed us to select CIC-2-expressing cells by fluorescence of cotransfected GFP (Fig. 4b–g). Although these cells express CIC-2 less efficiently than *Xenopus* oocytes (compare Figs. 4f and 2d) and HEK cells^{31,32}, CIC-2^{Asp24}-expressing cells displayed robust

Cl⁻ currents that lacked strong voltage dependence (Fig. 4c–g). The observed increase in currents may reflect increases in both the currents for individual channels and the number of channels; both must be considered when analyzing the pathogenic effects of ion channel mutants. This increase in currents correlated with a strong depolarization from $V_m = -69 \pm 7$ mV in CIC-2^{WT}-expressing cells to -46 ± 4 mV in CIC-2^{Asp24}-expressing cells (Fig. 4b), indicating that chloride concentration in H295R-S2 cells is higher than predicted from the electrochemical equilibrium. This depolarization may open voltage-dependent calcium channels. Indeed, nifedipine (an L-type calcium channel blocker) and/or mibefradil (a T-type calcium channel blocker) strongly reduced aldosterone production in cells expressing the CIC-2^{Asp24} mutant (Fig. 4i). The involvement of L-type calcium channels appeared to be larger in CIC-2^{Asp24}-expressing

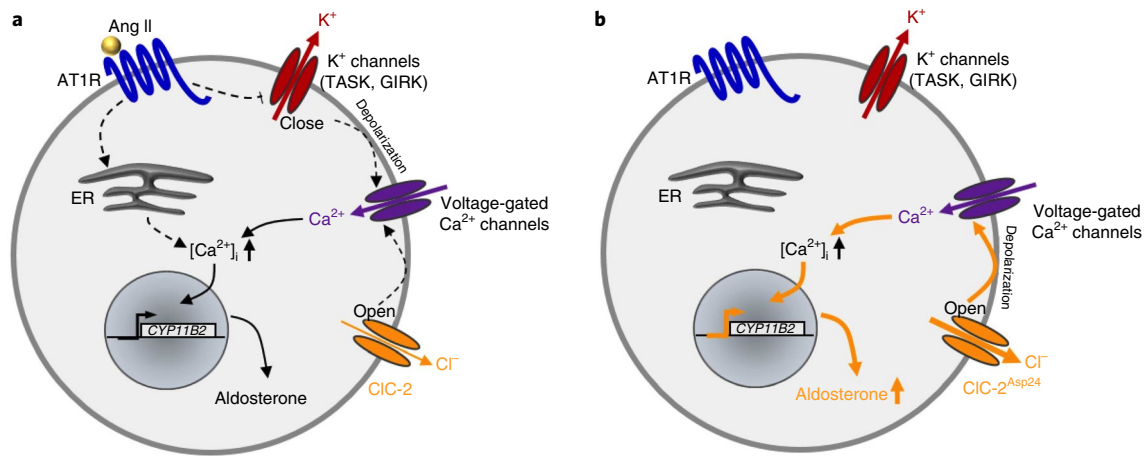


Fig. 5 | Proposed model for autonomous aldosterone secretion in adrenal zona glomerulosa cells with the CIC-2^{Asp24} mutant. **a**, In unstimulated conditions, the zona glomerulosa cell membrane potential closely follows the potassium resting potential at approximately -80 mV. Increasing extracellular K^+ concentration, or inhibition of K^+ channels by Ang II through its receptor (AT1R), leads to cell membrane depolarization, opening of voltage-gated Ca^{2+} channels, and increased intracellular calcium concentrations, the major trigger for aldosterone biosynthesis. Binding of Ang II to AT1R also leads to G_{α_q} -mediated signaling and IP_3 -mediated release of Ca^{2+} from the endoplasmic reticulum (ER). **b**, The CIC-2 p.Gly24Asp variant abolishes the voltage-dependent gating of CIC-2. The resulting pronounced increase in Cl^- currents at resting potentials is proposed to result in cell depolarization, opening of voltage-gated Ca^{2+} channels, stimulation of Ca^{2+} signaling, and, ultimately, increased expression of steroidogenic genes and aldosterone production. $[Ca^{2+}]_i$, intracellular calcium concentration.

cells (Fig. 4h), possibly because of the depolarized plasma membrane potential of these cells, which is required to open L-type calcium channels³³. However, we cannot exclude the possibility that nifedipine acted partially through T-type calcium channels, which are also blocked by this compound at depolarized voltages³⁴.

To investigate whether the *CLCN2* mutation encoding p.Gly24Asp could be involved in other forms of primary aldosteronism, we sequenced exon 2 of *CLCN2* in 100 patients with BAH. While the *CLCN2* mutation encoding p.Gly24Asp was not identified among these subjects, we found two rare *CLCN2* variants, c.197G>A (p.Arg66Gln, rs755883734) and c.143C>G (p.Pro48Arg, rs115661422) in two subjects (Supplementary Fig. 3). The minor allele frequencies of these variants were very low in the ExAC database (*CLCN2* p.Arg66Gln, 0.00003; *CLCN2* p.Pro48Arg, 0.0017). Both variants failed to significantly change CIC-2 currents in *Xenopus* oocytes in which the mutant proteins were heterologously expressed (Supplementary Fig. 4), in spite of a previously described³⁵ moderate reduction in CIC-2^{Arg48} current amplitudes. Nonetheless, it is noteworthy that the two patients were diagnosed with hypertension at a young age, at 29 and 19 years, respectively (Table 1), and in both cases during pregnancy. Finally, sequencing the *CLCN2* exons encoding the N-terminal domain (exons 1 and 2) and the loop between helices J and K (exon 10), corresponding to the CIC-2 inactivation domains^{2,3}, in 20 additional patients with hypertension before 20 years of age did not identify additional mutations. Among these patients, nine had a history of hypertension before the age of 15 years (one before 10 years), indicating that *CLCN2* mutations might underlie forms of primary aldosteronism with very young onset.

In conclusion, we show that a gain-of-function mutation affecting the CIC-2 chloride channel underlies a genetic form of secondary arterial hypertension and identify CIC-2 as the foremost chloride conductor of resting glomerulosa cells. We suggest that increased Cl^- currents induced by the CIC-2 p.Gly24Asp variant could depolarize the zona glomerulosa cell membrane, thereby opening voltage-gated calcium channels that trigger autonomous aldosterone production by increasing intracellular Ca^{2+} concentrations (Fig. 5b, orange arrows). We hypothesize that the increased Cl^- currents may overcome the hyperpolarizing currents of K^+ channels that normally determine the glomerulosa cell resting potential.

The inhibition of these potassium channels, for example upon Ang II stimulation, or the depolarizing currents mediated by these channels upon increases in extracellular K^+ are the main mechanisms triggering aldosterone production under physiological conditions (Fig. 5a, dashed black arrows).

Not only mutations mapping to the N-terminal CIC-2 inactivation domain^{2,3}, like the p.Gly24Asp variant found here, but also those mapping to the cytoplasmic linker between transmembrane helices J and K may cause primary aldosteronism (Fig. 1d). Several point mutations affecting this linker result in constitutively open CIC-2 channels². We propose both regions as potential hotspots for mutations causing primary aldosteronism. The discovery that a chloride channel is involved in primary aldosteronism opens new and unexpected perspectives for the pathogenesis and treatment of arterial hypertension.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at <https://doi.org/10.1038/s41588-018-0053-8>.

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References

- Thiemann, A., Gründer, S., Pusch, M. & Jentsch, T. J. A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* **356**, 57–60 (1992).
- Jordt, S. E. & Jentsch, T. J. Molecular dissection of gating in the CIC-2 chloride channel. *EMBO J.* **16**, 1582–1592 (1997).
- Gründer, S., Thiemann, A., Pusch, M. & Jentsch, T. J. Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. *Nature* **360**, 759–762 (1992).
- NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *Lancet* **389**, 37–55 (2017).
- Hannemann, A. & Wallaschofski, H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies—a review of the current literature. *Horm. Metab. Res.* **44**, 157–162 (2012).
- Calhoun, D. A. Hyperaldosteronism as a common cause of resistant hypertension. *Annu. Rev. Med.* **64**, 233–247 (2013).

7. Zennaro, M. C., Boulkroun, S. & Fernandes-Rosa, F. Genetic causes of functional adrenocortical adenomas. *Endocr. Rev.* **38**, 516–537 (2017).
8. Funder, J. W. et al. The management of primary aldosteronism: case detection, diagnosis, and treatment: an Endocrine Society Clinical Practice Guideline. *J. Clin. Endocrinol. Metab.* **101**, 1889–1916 (2016).
9. Savard, S., Amar, L., Plouin, P. F. & Steichen, O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension* **62**, 331–336 (2013).
10. Rossi, G. P. et al. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J. Am. Coll. Cardiol.* **48**, 2293–2300 (2006).
11. Choi, M. et al. K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* **331**, 768–772 (2011).
12. Azizan, E. A. et al. Somatic mutations in *ATP1A1* and *CACNA1D* underlie a common subtype of adrenal hypertension. *Nat. Genet.* **45**, 1055–1060 (2013).
13. Scholl, U. I. et al. Somatic and germline *CACNA1D* calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat. Genet.* **45**, 1050–1054 (2013).
14. Scholl, U. I. et al. Recurrent gain of function mutation in calcium channel *CACNA1H* causes early-onset hypertension with primary aldosteronism. *eLife* **4**, e06315 (2015).
15. Daniil, G. et al. *CACNA1H* mutations are associated with different forms of primary aldosteronism. *EBioMedicine* **13**, 225–236 (2016).
16. Beuschlein, F. et al. Somatic mutations in *ATP1A1* and *ATP2B3* lead to aldosterone-producing adenomas and secondary hypertension. *Nat. Genet.* **45**, 440–444 (2013).
17. Lifton, R. P. et al. A chimaeric 11 β -hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* **355**, 262–265 (1992).
18. Moss, S. J. & Smart, T. G. Constructing inhibitory synapses. *Nat. Rev. Neurosci.* **2**, 240–250 (2001).
19. Rinke, I., Artmann, J. & Stein, V. ClC-2 voltage-gated channels constitute part of the background conductance and assist chloride extrusion. *J. Neurosci.* **30**, 4776–4786 (2010).
20. Koch, M. C. et al. The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science* **257**, 797–800 (1992).
21. Guo, J. H. et al. Glucose-induced electrical activities and insulin secretion in pancreatic islet β -cells are modulated by CFTR. *Nat. Commun.* **5**, 4420 (2014).
22. Chorvátová, A., Gendron, L., Bilodeau, L., Gallo-Payet, N. & Payet, M. D. A Ras-dependent chloride current activated by adrenocorticotropin in rat adrenal zona glomerulosa cells. *Endocrinology* **141**, 684–692 (2000).
23. Jentsch, T. J. Discovery of ClC transport proteins: cloning, structure, function and pathophysiology. *J. Physiol.* **593**, 4091–4109 (2015).
24. Bösl, M. R. et al. Male germ cells and photoreceptors, both dependent on close cell–cell interactions, degenerate upon ClC-2 Cl⁻ channel disruption. *EMBO J.* **20**, 1289–1299 (2001).
25. Blanz, J. et al. Leukoencephalopathy upon disruption of the chloride channel ClC-2. *J. Neurosci.* **27**, 6581–6589 (2007).
26. Hoegg-Beiler, M. B. et al. Disrupting MLC1 and GlialCAM and ClC-2 interactions in leukodystrophy entails glial chloride channel dysfunction. *Nat. Commun.* **5**, 3475 (2014).
27. Depienne, C. et al. Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study. *Lancet Neurol.* **12**, 659–668 (2013).
28. Di Bella, D. et al. Subclinical leukodystrophy and infertility in a man with a novel homozygous *CLCN2* mutation. *Neurology* **83**, 1217–1218 (2014).
29. Boulkroun, S. et al. Prevalence, clinical, and molecular correlates of *KCNJ5* mutations in primary aldosteronism. *Hypertension* **59**, 592–598 (2012).
30. Spät, A. & Hunyady, L. Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol. Rev.* **84**, 489–539 (2004).
31. Varela, D., Niemeyer, M. I., Cid, L. P. & Sepúlveda, F. V. Effect of an N-terminus deletion on voltage-dependent gating of the ClC-2 chloride channel. *J. Physiol.* **544**, 363–372 (2002).
32. Pusch, M., Jordt, S. E., Stein, V. & Jentsch, T. J. Chloride dependence of hyperpolarization-activated chloride channel gates. *J. Physiol.* **515**, 341–353 (1999).
33. Barrett, P. Q. et al. Role of voltage-gated calcium channels in the regulation of aldosterone production from zona glomerulosa cells of the adrenal cortex. *J. Physiol.* **594**, 5851–5860 (2016).
34. Perez-Reyes, E., Van Deusen, A. L. & Vitko, I. Molecular pharmacology of human Ca_v3.2 T-type Ca²⁺ channels: block by antihypertensives, antiarrhythmics, and their analogs. *J. Pharmacol. Exp. Ther.* **328**, 621–627 (2009).
35. Paul, J. et al. Alterations in the cytoplasmic domain of CLCN2 result in altered gating kinetics. *Cell. Physiol. Biochem.* **20**, 441–454 (2007).
36. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T. & MacKinnon, R. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* **415**, 287–294 (2002).

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Author contributions

M.-C.Z., F.L.F.-R., G.D., I.J.O., and T.J.J. designed experiments and wrote the manuscript. T.M.S., M.-C.Z., T.S., and F.L.F.-R. performed and analyzed whole-exome sequencing. M.-C.Z., F.L.F.-R., G.D., R.E.Z., and S.B. performed and analyzed the results of in vitro studies on H295R-S2 cells. I.J.O. performed electrophysiological studies for which the data were analyzed by I.J.O. and T.J.J. C.G. characterized adrenal glands from wild-type and *Clcn2*^{-/-} mice and performed western blots. V.J., X.J., L.A., and H.L. were responsible for patient recruitment, medical care, and clinical data acquisition. All authors revised the manuscript draft. C.G. and R.E.Z. contributed equally to this work.

Competing interests

The authors declare no competing financial interests.

Additional information

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Methods

Patients. Patients with primary aldosteronism were recruited within the COMETE (CORTico- et MEDullo-surrénale, les Tumeurs Endocrines) network (COMETE-HEGP protocol, authorization CPP 2012-A00508-35) or in the context of genetic screening for familial hyperaldosteronism at the Genetics Department of the HEGP. Methods for screening and subtype identification of primary aldosteronism were according to the Endocrine Society guidelines⁸. In patients diagnosed with primary aldosteronism, a thin-slice CT scan or magnetic resonance imaging (MRI) of the adrenal and/or an adrenal venous sampling (AVS) was performed to differentiate between unilateral and bilateral aldosterone hypersecretion. All patients gave written informed consent for genetic and clinical investigation. Procedures were in accordance with institutional guidelines (Assistance Publique–Hôpitaux de Paris).

DNA isolation. DNA from peripheral blood leukocytes was extracted using the QIAamp DNA midi kit (Qiagen) or salt extraction.

Whole-exome sequencing and variant detection. Exomes were enriched in solution and indexed with SureSelect XT Human All Exon 50 Mb kits (Agilent). Sequencing was performed as 100-bp paired-end runs on HiSeq 2000 systems (Illumina). Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and base calling were performed using Illumina Real Time Analysis. CASAVA 1.8 was used for demultiplexing. BWA (v 0.5.9) with standard parameters was used for read alignment against human genome assembly hg19 (GRCh37). We performed single-nucleotide variant and small insertion and deletion (indel) calling specifically for the regions targeted by the exome enrichment kit, using SAMtools (v 0.1.18). Subsequently, variant quality was determined using the SAMtools varFilter script. We used default parameters, with the exception of the maximum read depth (-D) and the minimum *P* value for base quality bias (-2), which we set to 9,999 and 1×10^{-400} , respectively. Additionally, we applied a custom script to mark all variants with adjacent bases of low median base quality. All variants were then annotated using custom Perl scripts. Software is available on request (see URLs). Annotation included information about known transcripts (UCSC Known Genes and RefSeq genes), known variants (dbSNP v 135), type of mutation, and—if applicable—amino acid change in the corresponding protein. The annotated variants were then inserted into our in-house database. To reduce false positives, we filtered out variants that were already present in our database, had variant quality less than 40, or failed one of the filters from the filter scripts. We then manually investigated the raw read data of the remaining variants using the Integrative Genomics Viewer (IGV).

Sanger sequencing. *CLCN2* DNA was amplified using the intron-spanning primers described in Supplementary Table 2. PCR was performed on 100 ng of DNA in a final volume of 25 μ l containing 0.75 mM MgCl₂, 400 nM of each primer, 200 μ M deoxynucleotide triphosphate, and 1.25 U Taq DNA Polymerase (Sigma). Cycling conditions for *CLCN2* were as previously described³⁷ with an annealing temperature of 60 °C. Direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

Site-directed mutagenesis. The CIC-2^{Asp24} construct was generated by site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Agilent). The mutation was introduced into the human CIC-2 cDNA fragment inserted into the pFROG expression vector³⁸, and the presence of the mutation was confirmed by Sanger sequencing.

Western blotting. The membrane fractions of tissue homogenate from brain and adrenal glands of adult *Clcn2*^{+/+} and *Clcn2*^{-/-} mice were isolated and lysed in 50 mM Tris pH 6.8, 140 mM NaCl, 0.5 mM EDTA, and 2% SDS with protease inhibitors (4 mM Pefabloc and Complete EDTA-free protease inhibitor cocktail, Roche). Equal amounts of protein were separated by SDS-PAGE (10% polyacrylamide) and blotted onto nitrocellulose. Rabbit polyclonal antibodies against a modified C-terminal CIC-2 peptide have been described previously²⁶. Blots were reprobbed with mouse antibody to β -actin (clone AC-74, Sigma, A2228, 1:1,000) as a loading control.

H295R-S2 cells were lysed using RIPA buffer (Bio Basic Canada) with protease and phosphatase inhibitor mini tablets, EDTA free (Thermo Scientific). Proteins were solubilized for 30 min at 4 °C, under end-over-end rotation, and then centrifuged at 13,000 r.p.m. for 15 min at 4 °C. Equal amounts of protein were subjected to 10% SDS-PAGE and transferred onto nitrocellulose. Membranes were blotted with rabbit antibody to CIC-2 (1:500), mouse antibody to aldosterone synthase (1:500, clone CYP11B2-41-13, kindly provided by C. Gomez-Sanchez, University of Mississippi³⁹), and mouse antibody to β -actin (A2228, 1:10,000, Sigma).

Electrophysiological recordings. Patch-clamp analysis was performed in adrenal sections from wild-type and *Clcn2*^{-/-} mice²⁴, similarly to previously described experiments⁴⁰. Bicarbonate-buffered buffers were used, which were continuously bubbled with 95% O₂ and 5% CO₂. Briefly, adrenal glands were removed and

placed into cold low-Ca²⁺ solution composed of (in mM) 140 NaCl, 2 KCl, 26 NaHCO₃, 10 glucose, 5 MgCl₂, and 0.1 CaCl₂, pH 7.4. The adrenal glands, after removal of surrounding fat tissue, were embedded in 3% low-melting agarose, sectioned at 70 μ m (Leica, VT1200S), and incubated at room temperature in solution containing (in mM) 140 NaCl, 2 KCl, 26 NaHCO₃, 10 glucose, 2 MgCl₂, and 2 CaCl₂, and adjusted to pH 7.4. After at least 1 h, slices were transferred to a recording chamber and imaged with a 60 \times objective and DIC optics (Olympus, BX51WI). Cells of the zona glomerulosa were identified by their rosette organization. Electrical signals were acquired at room temperature using a microelectrode amplifier (Multiclamp, 700B) and software (Clampex 10.3, Molecular Devices). As expected, cells when patched with a K⁺-based solution displayed spontaneous spiking that could be stimulated with Ang II. For measuring Cl⁻ currents, patch pipettes were filled with solution containing (in mM) 117.5 CsMeSO₃, 17.5 CsCl, 4 NaCl, 10 HEPES, 1 EGTA, and 1 MgCl₂, adjusted to pH 7.3, while the bath solution contained (in mM) 117 NMDG-Cl, 23 NMDG-HCO₃, 5 CsCl, 1.3 MgCl₂, 9 glucose, and 2 CaCl₂, adjusted to pH 7.3. Voltage steps from +40 to -120 mV from a holding potential of -10 mV were used, with a final 1-s step at +40 mV. Signals were digitized at 10 kHz, filtered at 2 kHz, and stored offline for analysis with Clampfit software 10.4.

For two-electrode voltage-clamp in *Xenopus* oocytes, human wild-type and Gly24Asp CIC-2 cRNAs were prepared from pFROG vectors (Ambion mMMESSAGE mMACHINE T7 kit) and injected into *Xenopus* oocytes, at 13.8 ng and 9.2 ng per cell, respectively. Following 2 d of expression at 17 °C, two-electrode voltage-clamp was performed at room temperature using a TurboTEC amplifier (npi electronic) and pClamp software (Molecular Devices) to elicit CIC-2 currents (2-s steps from +60 mV to -120 mV with a final 1-s step at +40 mV) in ND109 solution containing (in mM) 109 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 2 HEPES and adjusted to pH 7.4. To test for the typical Cl⁻ > I⁻ selectivity of CIC channels, currents were sequentially measured in solutions containing 109 mM Cl⁻ or 109 mM I⁻. To determine the pH sensitivity of currents, ND109 was buffered with 5 mM MES for pH 6.5 and with 5 mM Tris for pH 8.5. Offline analysis was performed with Clampfit software 10.4. Statistical significance was assessed using the Mann–Whitney test (Prism, GraphPad).

For patch-clamp analysis of transiently transfected H295R-S2 cells, cells were seeded at 30% confluence onto coverslips coated with poly-L-lysine (Sigma). Once adhered, they were transfected (X-fect) with bicistronic plasmids encoding emGFP (for identification of transfected cells) and, after an IRES sequence, CIC-2^{WT} or CIC-2^{Asp24}. Cells were measured 12–24 h later. Both transiently and stably transfected cells were measured using a Multiclamp 700B amplifier (Axon Instruments), and gramicidin-perforated patch-clamp was performed so that the intracellular chloride concentration was not disturbed. The tips of patch pipettes were first filled with gramicidin-free internal pipette solution, containing (in mM) 100 KMeSO₃, 30 KCl, 4 NaCl, 10 HEPES, 1 MgCl₂, 1 EGTA, and 3 MgATP (pH 7.3; 280 mOsm/L), and then backfilled with the same solution containing 25 μ g/ml gramicidin. GFP-expressing cells were selected for analysis. Approximately 20 min following tight giga-seal formation, stable membrane potential measurements (*I* = 0 configuration) could be acquired with access resistances of <100 M Ω in bath solution containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 1.8 MgCl₂, and 1.8 CaCl₂ (pH 7.4; 300 mOsm/L). When adequate access resistance was attained (<35 M Ω), a Na⁺- and K⁺-free bath solution containing (in mM) 140 CsCl, 10 HEPES, 1.8 MgCl₂, 1.8 CaCl₂, and 20 sucrose (pH 7.3; 300 mOsm/L) was perfused to measure anion membrane currents in the voltage-clamp configuration (1-s steps from 0 mV to -120 mV from a holding clamp of -10 mV). Measurements were performed at room temperature (22–24 °C). Data are presented as means \pm s.e.m.

Functional studies in H295R-S2 cells. The human adrenocortical carcinoma cell line H295R strain 2 (H295R-S2), kindly provided by W. E. Rainey (University of Michigan)⁴¹, was cultured in DMEM/Eagle's F12 medium (Gibco, Life Technologies) supplemented with 2% Ultrosor G (PALL Life Sciences), 1% insulin/transferrin/selenium premix (Gibco, Life Technologies), 10 mM HEPES (Gibco, Life Technologies), and 1% penicillin and streptomycin (Gibco, Life Technologies) and maintained in a 37 °C humidified atmosphere (5% CO₂).

For overexpression experiments, H295R-S2 cells were seeded into tissue culture dishes 100 in groups of 5,000,000 cells per dish and maintained in the conditions described. After 24 h, cells were resuspended in 100 μ l of Nucleofector R solution (AMAXA kit, Lonza) and transfected with 3 μ g of the CIC-2^{WT} or CIC-2^{Asp24} pFROG construct or a GFP construct, using the electroporation program P-20. To select only stably transfected cells, 48 h after transfection, cells were changed to medium containing 500 μ g/ml G418-geneticin (Gibco) and used after all GFP-transfected cells were dead. G418 selection was maintained during all functional studies. For aldosterone measurements and RNA extraction, cells were serum deprived in DMEM/F12 medium containing 0.1% Ultrosor G for 24 h and then incubated for another 24 h with fresh medium containing 0.1% Ultrosor G with no secretagogue or vehicle (basal), with the secretagogues Ang II (10 nM) or K⁺ (12 mM), or with calcium channel blockers nifedipine (L-type calcium channel blocker, 10 μ M, Sigma) or mibefradil (T-type calcium channel blocker, 10 μ M, Sigma). At the end of the incubation time, supernatant and cells from each well were harvested for aldosterone measurement and RNA extraction. Three

experiments using aldosterone secretagogues ($n=9$) and two experiments using calcium channel blockers ($n=6$) were independently conducted in triplicate.

Human CIC-2-targeting (TRCN0000427876) and non-mammalian control (SHC002V) MISSION shRNA lentiviral transduction particles were obtained from Sigma-Aldrich. The shRNA sequences were inserted into the TRC2 pLKO-puro plasmid backbone. For lentiviral infections, the manufacturer's protocol was followed with slight modifications. 1×10^4 H295R-S2 cells were seeded in a 96-well plate. After 24 h, medium was changed and supplemented with 2 $\mu\text{g}/\text{ml}$ polybrene (Sigma). Lentiviral particles were then added at a multiplicity of infection of 10 and the medium was changed after overnight incubation. For selection, 2 $\mu\text{g}/\text{ml}$ puromycin (Gibco) was added to the medium. After two passages, cells were characterized in terms of mRNA expression and aldosterone production after incubation with or without secretagogue as mentioned above.

RNA extraction and RT-qPCR. Total RNA was extracted in TRIzol reagent (Ambion, Life Technologies) according to the manufacturer's recommendations. After DNase I treatment (Life Technologies), 500 ng of total RNA was reverse transcribed (iScript reverse transcriptase, Bio-Rad). Primers used for qPCR are described in Supplementary Table 3. qPCR was performed using SYBR Green (Sso Advanced Universal SYBR Green Supermix, Bio-Rad) on a C1000 touch thermal cycler from Bio-Rad (CFX96 Real-Time System), according to the manufacturer's instructions. Controls without template were included to verify that fluorescence was not overestimated from primer-dimer formation or PCR contaminations. RT-qPCR products were analyzed in a postamplification fusion curve to ensure that a single amplicon was obtained. Normalization for RNA quantity and reverse transcriptase efficiency was performed against three reference genes (geometric mean of the expression of 18 S rRNA, *HPRT1*, and *GAPDH*), in accordance with the MIQE guidelines⁴². Quantification was performed using the standard curve method. Standard curves were generated using serial dilutions from a cDNA pool of all samples of each experiment, yielding a correlation coefficient of at least 0.98 in all experiments.

Aldosterone and protein assays. Aldosterone levels were measured in cell culture supernatants by ELISA. Antibody to aldosterone and aldosterone-3-CMO-biotin were kindly provided by C. Gomez-Sanchez⁴³. Aldosterone concentrations were normalized to cell protein concentrations (determined using Bradford protein assays).

Statistical analyses. Quantitative variables are reported as means \pm s.e.m. when a Gaussian distribution was present or as medians and interquartile ranges when no Gaussian distribution was present. Pairwise comparisons were done with unpaired *t* tests and Mann-Whitney tests, respectively; multiple comparisons were done with the ANOVA test followed by a test for pairwise comparison of

subgroups according to Bonferroni when a Gaussian distribution was present or Kruskal-Wallis followed by Dunn's test when no Gaussian distribution was present. Comparisons between two groups were performed with two-tailed *t* tests or two-tailed Mann-Whitney tests. A *P* value of <0.05 was considered significant. For functional experiments, all results are expressed as means \pm s.e.m. of three separate experiments performed in triplicate for CIC-2 overexpression studies with secretagogues, two separate experiments performed in triplicate for CIC-2 expression studies with calcium channel blockers, and two to four separate experiments performed in triplicate for CIC-2 knockdown studies. Analyses were performed using Prism5 (GraphPad).

URLs. Software used to analyze the whole-exome sequencing data, <https://ihg4.helmholtz-muenchen.de/cgi-bin/mysql/snv-vcf/login.pl>.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.





Data availability. The data that support the findings of this study are available from the authors upon reasonable request; see author contributions for specific datasets. Disease-causing variants have been submitted to ClinVar. Exome data are available upon request within a scientific cooperation.

References

- Hubert, E. L. et al. Mineralocorticoid receptor mutations and a severe recessive pseudohypoaldosteronism type 1. *J. Am. Soc. Nephrol.* **22**, 1997–2003 (2011).
- Günther, W., Lüchow, A., Cluzeaud, F., Vandewalle, A. & Jentsch, T. J. CIC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl. Acad. Sci. USA* **95**, 8075–8080 (1998).
- Gomez-Sanchez, C. E. et al. Development of monoclonal antibodies against human CYP11B1 and CYP11B2. *Mol. Cell. Endocrinol.* **383**, 111–117 (2014).
- Hu, C., Rusin, C. G., Tan, Z., Guagliardo, N. A. & Barrett, P. Q. Zona glomerulosa cells of the mouse adrenal cortex are intrinsic electrical oscillators. *J. Clin. Invest.* **122**, 2046–2053 (2012).
- Wang, T. et al. Comparison of aldosterone production among human adrenocortical cell lines. *Horm. Metab. Res.* **44**, 245–250 (2012).
- Bustin, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622 (2009).
- Gomez-Sanchez, C. E. et al. The production of monoclonal antibodies against aldosterone. *Steroids* **49**, 581–587 (1987).

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A gain-of-function mutation in the *CLCN2* chloride channel gene causes primary aldosteronism

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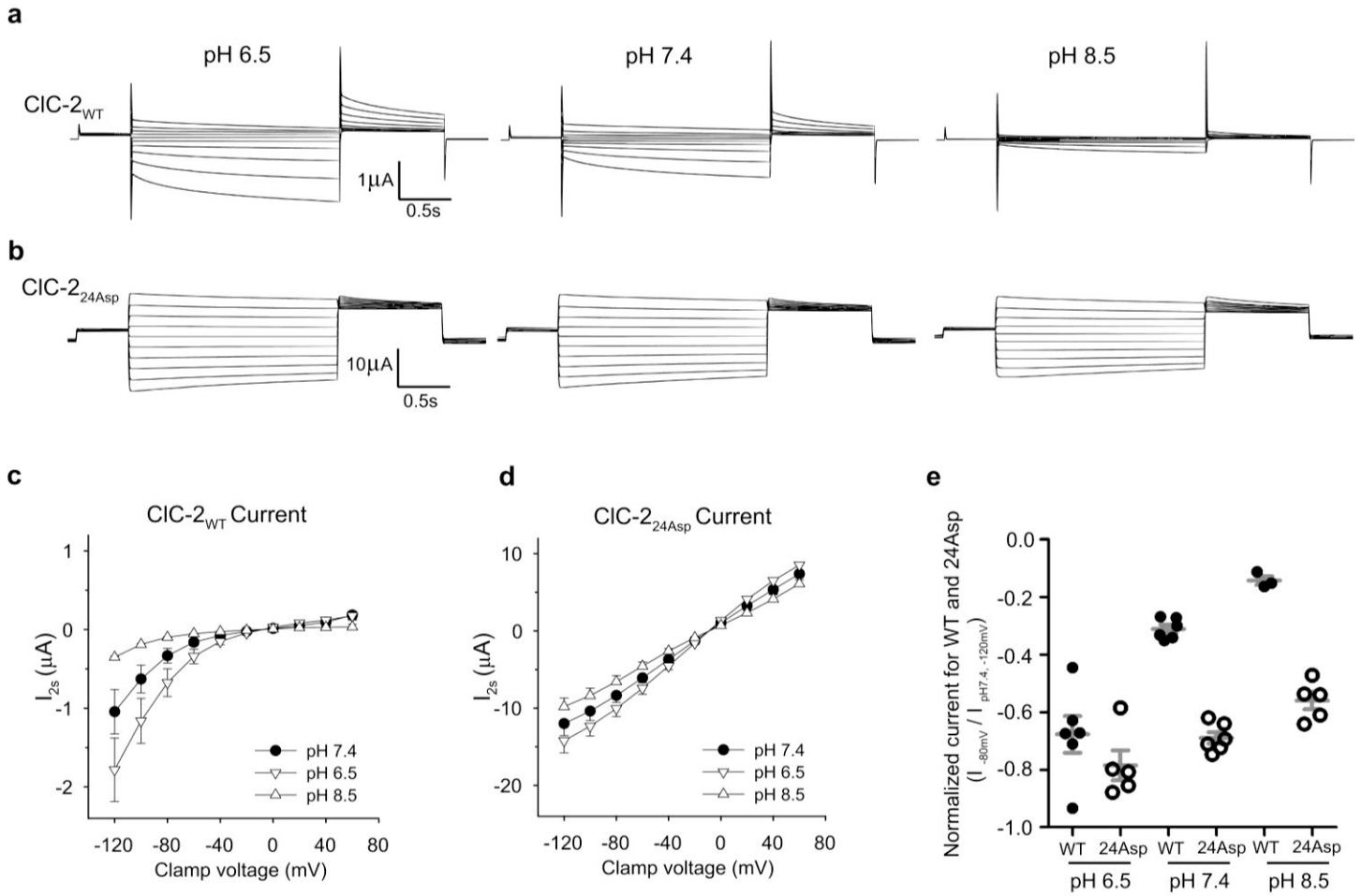
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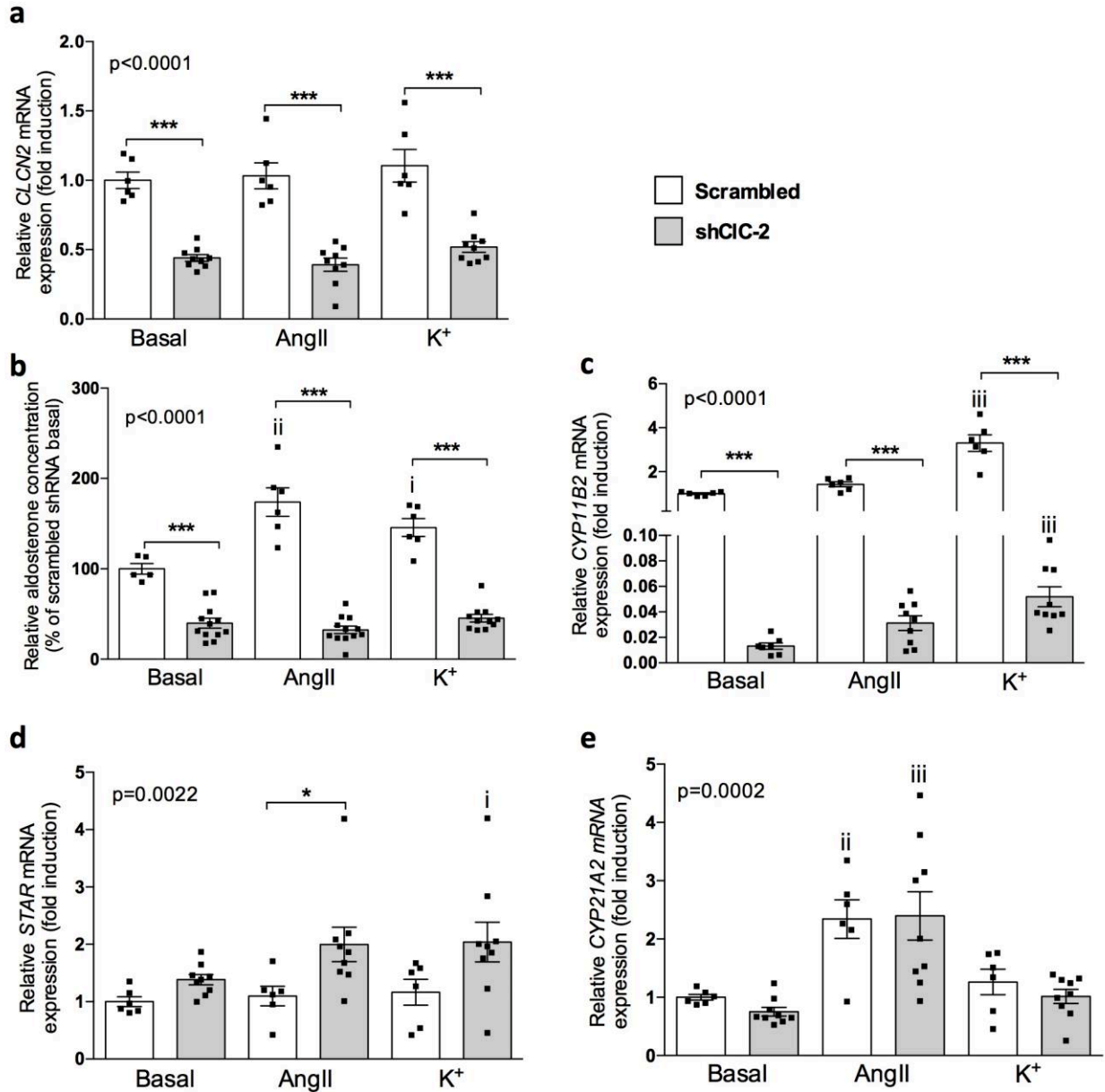
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Supplementary Figure 1

Dependence of CIC-2^{WT} and CIC-2^{Asp24} currents on external pH

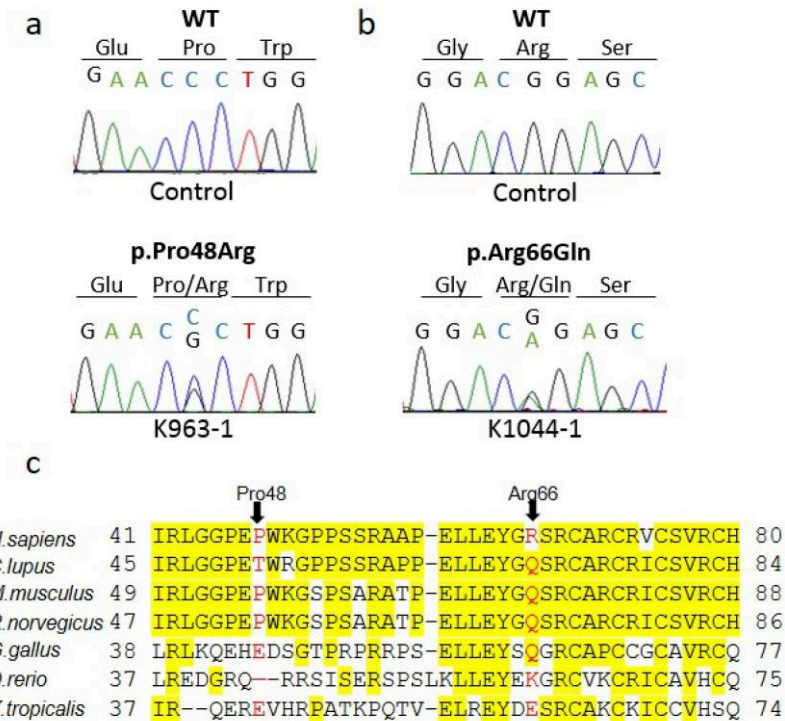
WT and mutant channels were expressed in *Xenopus* oocytes and measured by two-electrode voltage-clamp using a pulse protocol that clamped the oocytes in 2-s-long 20-mV steps from +60 to -120 mV. **a,b**, Representative current traces obtained from WT (**a**) and G24D mutant (**b**) CIC-2 at indicated pH values. **c,d**, Mean CIC-2^{WT} (**c**) and CIC-2^{Asp24} (**d**) currents measured after 2 s as a function of voltage and pH. $n = 3-6$ oocytes; error bars, s.e.m. (**e**) Currents at -80 mV (approximately the resting voltage of glomerulosa cells) from CIC-2^{WT} (filled circles) and CIC-2^{Asp24} (open circles) normalized to respective currents at -120 mV at pH 7.4. Note the large pH dependence of WT currents, which is strongly reduced but not abolished by the Gly24Asp mutation.



Supplementary Figure 2

Effect of CIC-2 downregulation on aldosterone production and expression of genes involved in aldosterone biosynthesis

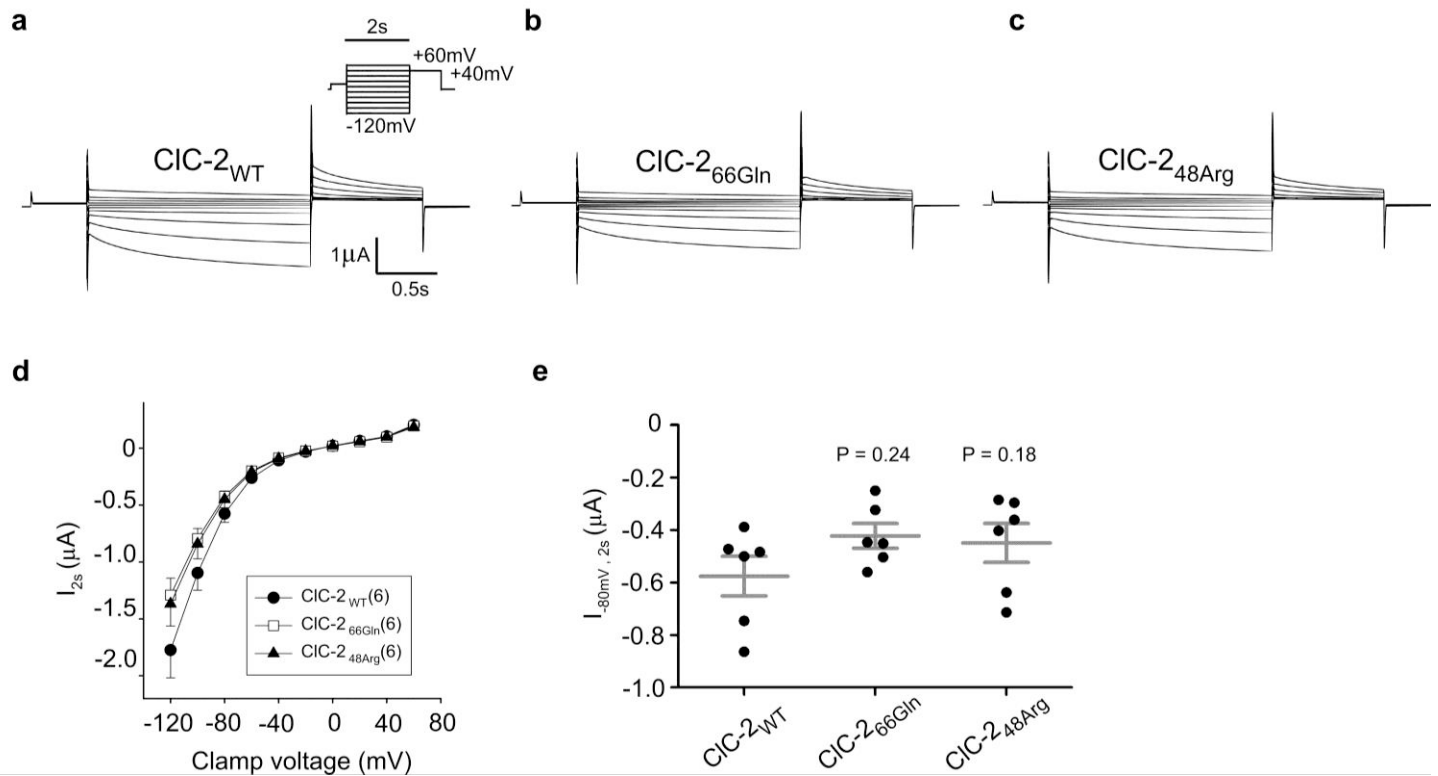
a, Basal and stimulated (Ang II or K⁺) mRNA expression of *CLCN2* in H295R-S2 cells infected with scrambled (open bars) or CIC-2 (filled bars) shRNA (one-way ANOVA, $P < 0.0001$, $F = 28.11$). **b**, Basal and stimulated aldosterone production by H295R-S2 cells infected with scrambled or CIC-2 shRNA. **c–e**, Basal and stimulated mRNA expression of *CYP11B2* (one-way ANOVA, $P < 0.0001$, $F = 84$) (**c**), *STAR* (Kruskal–Wallis, $P = 0.0022$) (**d**), and *CYP21A2* (Kruskal–Wallis, $P = 0.0002$) (**e**) in H295R-S2 cells transfected with scrambled or CIC-2 shRNA. Results of mRNA expression are represented as fold induction of cells infected with scrambled shRNA in basal conditions. Values of all experiments are represented as means \pm s.e.m. of two independent experiments performed in experimental triplicate for each condition ($n = 6$ for scrambled shRNA, $n = 12$ for CIC-2 shRNA). * $P < 0.05$; *** $P < 0.001$; (i) $P < 0.05$ stimulated versus basal condition; (ii) $P < 0.01$ stimulated versus basal condition; (iii) $P < 0.001$ stimulated versus basal condition.



Supplementary Figure 3

CLCN2 variants identified in subjects with bilateral adrenal hyperplasia

a, Sanger sequencing chromatograms showing the *CLCN2* wild-type sequence and the *CLCN2* variant c.143C>G (p.Pro48Arg) identified in subject K963-1 with bilateral adrenal hyperplasia. **b**, Sanger sequencing chromatograms showing the *CLCN2* wild-type sequence and the *CLCN2* variant c.197G>A (p.Arg66Gln) identified in subject K1044-1 with bilateral adrenal hyperplasia. **c**, Alignment and conservation of residues encoded by CIC-2 orthologs. Residues that are conserved among more than three sequences are highlighted in yellow.



Supplementary Figure 4

Electrophysiological analyses of CIC-2^{Gln66} and CIC-2^{Arg48} channels

a–c, Representative chloride current traces measured by two-electrode voltage-clamp from *Xenopus* oocytes injected with 9.2 ng of human CIC-2^{WT} (a), CIC-2^{Gln66} (b), or CIC-2^{Arg48} (c) cRNA. d, Mean ± s.e.m. currents measured after 2 s from experiments in a–c plotted as a function of clamp voltage. The number of cells, obtained from two different batches of oocytes is indicated in parentheses. e, Summary of Cl⁻ currents at -80 mV and after 2 s for a–c. Statistical analyses for CIC-2^{Gln66} and CIC-2^{Arg48} were performed by comparison with CIC-2^{WT}, Mann–Whitney test.

Supplementary material

A gain-of-function mutation in the *CLCN2* chloride channel gene causes primary aldosteronism

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Supplementary Table 1: Expression of plasma membrane chloride channels in the human adrenal cortex.

Gene name	protein	mRNA expression*
<i>CLCN2</i>	CIC-2	1.65±0.23/1.16±0.18**
<i>CLCN1</i>	CIC-1	0.51±0.07
<i>CFTR</i>	CFTR	0.46±0.05
<i>LRRC8A</i> ^{\$}	LRRC8A	1.09±0.22
<i>LRRC8B</i> [#]	LRRC8B	0.62±0.14/0.42±0.041**
<i>LRRC8C</i> [#]	LRRC8C	2.11±0.22
<i>LRRC8D</i> [#]	LRRC8D	9.95±0.84
<i>LRRC8E</i> [#]	LRRC8E	0.79±0.11
<i>TMEM16A</i>	Anoctamin-1	0.44±0.065

*mRNA expression was retrieved from a transcriptome study including 123 APA and 11 CA ¹. Values represent median centred, log2-transformed and model-adjusted expression levels respresented as mean±SEM. **Values represent expression levels detected by two different probes. ^{\$}Essential and [#]non-essential components of the volume-regulated anion channel (VRAC) ².

Supplementary Table 2. Primers used for *CLCN2* sequence

<i>Exon</i>	Forward primer	Reverse primer
1	CAGGACAGAGCCGGAACC	GGACAGGATTAGGGTAGGCC
2	CATAAGCATGGTCCACTCCC	AGCAGCTCTAATGGCCTCTG
10	AGGCTCCTTTTCACTCAGGT	CCTGTTTTGACTGGGCCATT

Supplementary Table 3. Primers used for real-time RT-qPCR

Gene Symbol	Forward primer	Reverse primer
<i>18S</i>	CCCTGCCTTTGTACACACC	CGATCCGAGGGCCTCACTA
<i>HPRT</i>	CTCAACTTTAACTGGAAAGAATGTC	TCCTTTTCACCAGCAAGCT
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
<i>CLCN2</i>	TTGATCCTGCTCCCTTCCAG	CATAAGCATGGTCCACTCCC
<i>StAR</i>	ATGAGTAAAGTGGTCCCAGATG	ACCTTGATCTCCTTGACATTGG
<i>CYP21A2</i>	GAGTAGTCTCCCAAGGACAGGT	GTGGTGCTGAACTCCAAGAGGA
<i>CYP11B2</i>	GTGTGGAAGGAGCACTTTGAGG	GATGCCTGTGTAGTGTTGAGGC

References

1. Boulkroun, S. *et al.* Prevalence, Clinical, and Molecular Correlates of KCNJ5 Mutations in Primary Aldosteronism. *Hypertension* **59**, 592-8 (2012).
2. Voss, F.K. *et al.* Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. *Science* **344**, 634-8 (2014).

10.2 Molecular genetics of Conn adenomas in the era of exome analysis

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Molecular genetics of Conn adenomas in the era of exome analysis

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Molecular genetics of Conn adenomas in the era of exome analysis.

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■ Summary

Aldosterone-producing adenomas (APA) are a major cause of primary aldosteronism (PA), the most common form of secondary hypertension. Exome analysis of APA has allowed the identification of recurrent somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* in more than 50 % of sporadic cases. These gain of function mutations in ion channels and pumps lead to increased and autonomous aldosterone production. In addition, somatic *CTNNB1* mutations have also been identified in APA. The *CTNNB1* mutations were also identified in cortisol-producing adenomas and adrenal cancer, but their role in APA development and the mechanisms specifying the hormonal production or the malignant phenotype remain unknown. The role of the somatic mutations in the regulation of aldosterone production is well understood, while the impact of these mutations on cell proliferation remains to be established. Furthermore, the sequence of events leading to APA formation is currently the focus of many studies. There is evidence for a two-hit model where the somatic mutations are second hits occurring in a previously remodeled adrenal cortex. On the other hand, the APA-driver mutations were also identified in aldosterone-producing cell clusters (APCC) in normal adrenals, suggesting that these structures may represent precursors for APA development. As PA due to APA can be cured by surgical removal of the affected adrenal gland, the identification of the underlying genetic abnormalities by novel biomarkers could improve diagnostic and therapeutic approaches of the disease. In this context, recent data on steroid profiling in peripheral venous samples of APA patients and on new drugs capable of inhibiting mutated potassium channels provide promising preliminary data with potential for translation into clinical care.

Introduction

Arterial hypertension (HT) is a worldwide health problem which affects ~25 % of the global population [1], resulting in an estimated 9.4 million deaths or approximately 12.8 % of all deaths (Global Health Observatory data, WHO). A vast and diverse array of drugs exists for the treatment of HT, such as diuretics, antagonists of the renin-angiotensin-aldosterone system, notably angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers, calcium channel blockers, vasodilators, β adrenergic blocking agents. Optimal blood pressure control, however, is still far from being achieved in up to two thirds of the hypertensive population. In a certain proportion of cases, HT can arise from a specific disease; endocrine hypertension, a frequent form of secondary HT, emerges following a dysregulation of one or more hormones that are involved in blood pressure regulation. Primary aldosteronism (PA), also known as Conn's syndrome, is the most frequent form of secondary hypertension with estimates of up to 10 % of cases in referred patients, 4 % in primary care [2] and 20 % in patients with resistant hypertension [3,4]. PA is mainly due to aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasias (BAH, or idiopathic hyperaldosteronism [IHA]). The clinical picture of patients with PA consists of HT, a high aldosterone to renin ratio, which has become one of the major diagnostic tools for PA alongside different confirmation tests and adrenal venous sampling for subtype diagnosis, and variable hypokalemia and metabolic alkalosis [5]. PA is associated to an increased risk of cardiovascular complications, which occur beyond the effect of hypertension, such as coronary artery disease, heart failure, myocardial infarction, atrial fibrillation and renal damage. Different studies insist on the importance of screening most hypertensive patients for PA to either confirm or exclude the diagnosis [6,7]. Indeed, early PA diagnosis can improve prognosis and prevent the development of target organ damage.

Although the management of PA in hypertensive patients has come a long way and the treatment is much better established, the prognosis of unilateral PA depends on different criteria such as age, sex, BMI, age upon diagnosis and the duration of hypertension [7,8]. Indeed, younger patients and female patients show a better clinical outcome after adrenalectomy in comparison to older or male patients [7].

Aldosterone biosynthesis in the adrenal cortex

The human adrenal cortex is composed of three distinct zones that are characterized by their respective functions. Steroid hormones are synthesized following the sequential enzymatic breakdown of cholesterol by different cytochrome P450 enzymes as well as hydroxysteroid dehydrogenases, the particularity of each zone lies in the expression of specific steroidogenic enzymes and the ability for each to have its own

regulators. The most outer zone of the adrenal cortex, the zona glomerulosa (ZG), expresses aldosterone synthase (encoded by *CYP11B2*), which catalyzes the hydroxylation at the C11 position of the 11-deoxycorticosterone into corticosterone, furthermore, its hydroxylation at C18 into 18(OH)corticosterone followed by an oxidation of C18's hydroxyl group giving as an end result aldosterone. The main trigger for aldosterone biosynthesis is the activation of intracellular calcium signaling in the zona glomerulosa which is induced by either angiotensin II (Ang II) from the renin-angiotensin system or by extracellular potassium levels. The zona fasciculata (ZF) of the adrenal cortex mainly produces cortisol. In this process, the conversion of progesterone into 17-hydroxyprogesterone is catalyzed by the activity of 17 α -hydroxylase which is not expressed in ZG cells. 17-hydroxyprogesterone undergoes a hydroxylation at C21 by the 21-hydroxylase enzyme, and finally a hydroxylation at position C11 by 11 β -hydroxylase (encoded by *CYP11B1*) forming cortisol. The main regulator of cortisol production is the hypothalamus-pituitary-adrenal (HPA) axis primarily through the adrenocorticotrophic hormone (ACTH).

Ang II is one of the major regulators of aldosterone secretion by ZG cells. The binding of AngII to its receptor (AT1) will lead to the activation of the G α q-phospholipase C-mediated pathway, increasing inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol concentrations. Ultimately, IP3 is responsible for increased intracellular calcium concentration due to calcium release from intracellular stores. AngII also inhibits the background TWIK-related acid-sensitive potassium channel (TASK), as well as GIRK4 and the Na⁺, K⁺-ATPase, leading to a cell membrane depolarization [9]. This will lead to opening of voltage-gated Ca²⁺ channels, also increasing intracellular calcium concentrations (figure 1A and B).

The other main stimulator for aldosterone biosynthesis is the increase in extracellular potassium levels. In the normal ZG cell at a resting state, the cell membrane potential is hyperpolarized, the reason is that the membrane potential follows closely the equilibrium potential of potassium in these cells which largely express potassium channels. Small increases in extracellular potassium levels cause ZG cell membrane depolarization. The depolarization of the ZG cell membrane leads to the opening of voltage-gated Ca²⁺ channels and an increase in intracellular calcium levels resulting in the activation of calcium signaling. Calcium signaling acts by increasing the release of deesterified cholesterol from cytoplasmic stores, as well as cholesterol delivery to the outer mitochondrial membrane and then to the inner mitochondrial membrane by increasing the expression of the steroid acute regulatory protein (StAR). Calcium signaling also increases the expression of cofactors required for p450 cytochrome enzymes. Calcium/Calmodulin binding in the cytosol of the ZG cell induces the activation of protein kinases that regulate phosphorylation of transcription factors involved in *CYP11B2* transcriptional induction, mainly nuclear

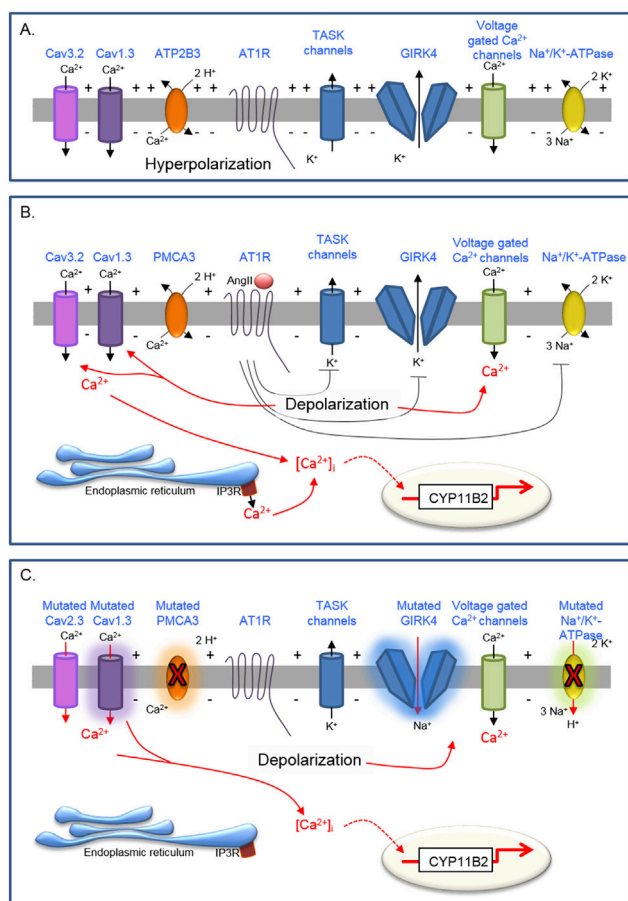


FIGURE 1

Regulation of aldosterone biosynthesis in zona glomerulosa cells

A. In basal conditions, zona glomerulosa cells are in a hyperpolarized state due to the activity of potassium channels at the cell membrane. B. The binding of AngII to its receptor AT1R or the increase of extracellular K⁺ concentration lead to inhibition of K⁺ currents through TASK and GIRK4 channels, followed by cell membrane depolarization; AngII also inhibits the activity of the Na⁺/K⁺-ATPase pump (*ATP1A1*) activity. This depolarization leads to the opening of voltage-gated calcium channels on the cell membrane increasing Ca²⁺ concentrations in the cytosol. AngII also induces, through inositol triphosphate (IP3), the release of Ca²⁺ from the sarco/endoplasmic reticulum. The increased intracellular Ca²⁺ concentration leads to the activation of the calcium signaling pathway, the major trigger for aldosterone biosynthesis. C. In pathological conditions, mutations affecting specific ion channels (*CACNA1D*, *CACNA1H*, *KCNJ5*) and ATPases (*ATP1A1*, *ATP2B3*) lead to constitutively depolarized ZG cell membrane or directly to increased intracellular Ca²⁺ concentrations, constitutively activating Ca²⁺ signaling. The net result is an increased expression of *CYP11B2* and an autonomous aldosterone biosynthesis.

receptor subfamily 4 group A 1 and 2 (*NR4A1* and *NR4A2* coding for NUR77/NGF1B and NURR1 respectively) and the cyclic AMP-responsive element-binding protein (CREB).

Genetic abnormalities in aldosterone-producing adenomas (APA)

PA is due to inappropriate aldosterone production by the adrenal cortex in spite of the suppression of the renin-angiotensin system. In the last years, whole exome sequencing (WES) performed on DNA from APA led to the identification of recurrent somatic mutations in genes coding for ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*). These genes are essential for regulating intracellular ion homeostasis and cell membrane potential. All these mutations promote an

increased intracellular calcium signaling through cell membrane depolarization and opening of voltage-dependent calcium channels, or impaired intracellular calcium recycling, therefore leading to high aldosterone levels by constitutive expression of *CYP11B2* (figure 1C).

In a large multicenter study from the European Network for the Study of Adrenal Tumors (ENS@T) that analyzed somatic mutations in APA from 474 patients [10], hot spot regions for mutations in *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* were sequenced. Somatic mutations were identified in 54.2 % of APA, with *KCNJ5* being the most prevalent at 38 %, *CACNA1D* at 9.3 %, *ATP1A1* at 5.3 % and *ATP2B3* 1.7 % of these mutations. However, *KCNJ5* mutations are more prevalent in Asian populations, with up to 76 % of prevalence [11–15]. These observations were

corroborated by a meta-analysis of clinical and genetic data from 1636 patients with APA showing an overall prevalence of *KCNJ5* mutations of 43 %, with higher prevalence in patients from Asia [16]. Some APAs also carry somatic mutations in the gene that codes for β -catenin (*CTNNB1*), less common mutations have also been identified in *PRKACA* (encoding Protein Kinase cAMP-Activated Catalytic Subunit α) [17-19].

KCNJ5 codes for an inwardly rectifying K^+ channel, which is the G-protein-activated inward rectifier potassium channel GIRK4 (also known as Kir3.4). It is mainly expressed in the ZG of the adrenal cortex. *KCNJ5* mutations were found to be more frequent in female and younger patients, and the expression of GIRK4 in APA was found to be correlated to the mutation status [20]. GIRK4 is composed of 2 membrane spanning helices with one pore-forming region in between and N- and C- termini that contribute to the pore structure [21]. Choi et al. identified two somatic *KCNJ5* mutations mapping to the selectivity filter of GIRK4 (p.Gly151Arg and p.Leu168Arg). In addition to these two mutations (the most prevalent mutations in APA), the majority of the *KCNJ5* mutations described are located within or near the selectivity filter, rendering the channel permeable to sodium, which leads to chronic cell membrane depolarization [22]. Transient transfection of *KCNJ5* mutants in HAC-15 resulted in a calcium-dependent increase in *CYP11B2* expression and aldosterone biosynthesis in the cells; the mutant GIRK4, however, did not induce any increase in proliferation but rather a reduced cell viability or sodium-induced cell death [23,24]. This leaves the question of the role of *KCNJ5* mutations on the cell proliferation and APA formation unanswered in tumors where these mutations occur. *KCNJ5* mRNA expression is not affected by *KCNJ5* mutations, but APA harboring *KCNJ5* mutations show decreased GIRK4 protein expression when compared with adjacent ZG, allowing the differentiation from APA harboring other mutations or without mutations identified [20,25].

More than 20 mutations have been identified in *CACNA1D* (encoding the voltage-dependent L-type calcium channel subunit alpha-1D, Cav1.3) [26]. The Cav1.3 calcium channel consists of 4 repeat domains, each one consisting of six transmembrane segments, with a membrane-associated loop between S5 and S6 [27-29]. Mutations occurring in *CACNA1D* are gain of function mutations that lead to a decrease in the threshold of the voltage-dependent activation or impaired channel inactivation, which is followed by increased intracellular calcium concentrations and thereby an induction of aldosterone biosynthesis [28,29].

ATP1A1 and *ATP2B3* are members of the P-type family of ATPases and are composed of 10 transmembrane domains (M1-M10) with intracellular N- and C- termini. *ATP1A1* codes for the Na^+ -ATPase alpha-1 subunit. Mutations in this pump lead to a loss of its activity and affinity to K^+ and to an inward proton or sodium leak, which has been proposed to induce aldosterone production through cell membrane depolarization and increased calcium influx [28,30]. Nevertheless, transient transfection of -two of

the described *ATP1A1* mutations in the adrenocortical cell line H295R did not result in modifications of basal cytosolic calcium levels, and barely increased potassium-stimulated calcium concentrations, in spite of depolarizing the cells and stimulating aldosterone biosynthesis. In these cells, Stindl et al. found that there was an increased intracellular acidification, which was suggested to regulate *CYP11B2* biosynthesis [31].

ATP2B3 codes for the plasma membrane calcium-transporting ATPase 3 (PMCA3). Mutations of PMCA3 are found in the transmembrane domain M4 and result in the deletion of different amino acids in the region between Leu422 and Leu433. One mutation in particular, p.Leu425_Val426del, leads to reduced calcium export which is due to the loss of the physiological pump functions, and an increased intracellular calcium signaling due to the depolarization-activated Ca^{2+} channels [32]. Recently, a second mechanism explaining aldosterone production due to *ATP2B3* mutations was suggested. *ATP2B3* mutations induce an increase in calcium influx by the opening of depolarization-activated calcium channels and by a possible calcium leak through the mutated PMCA3 [32].

CACNA1H encoding the pore-forming $\alpha 1$ subunit of the T-type voltage-dependent calcium channel Cav3.2 has been recently shown to be involved in familial forms of PA in some cases associated with developmental disorders [33,34]. In addition, it was also described as germline mutation in a patient with APA [34]. This channel consists of a single polypeptide chain of four homologous domains (I-IV), each one containing six transmembrane spans (S1-S6) and cytoplasmic C- and N- Termini. Mutant Cav3.2 channels show significant changes in their electrophysiological properties, specifically a shift in activation towards more negative voltages and modifications of their inactivation properties. Consequently, the channels are activated at less depolarized voltages leading to activation of calcium signaling and autonomous aldosterone production [33,34]. A germline *CACNA1H* variant was identified in one patient with APA without somatic mutations and improvement after adrenalectomy [33,34]. This case suggest that *CACNA1H* might be a susceptibility gene for different types of PA, including APA.

The Wnt/ β -Catenin signaling pathway has been shown to play an important role in the development of the adrenal cortex and in aldosterone biosynthesis [35]. This signaling pathway is constitutively active in ~ 70 % of APA [36]. In unstimulated conditions, β -catenin is located in the cytosol, and is part of the axin complex along with adenomatous polyposis coli protein (APC), axin, Glycogen Synthase Kinase-3 β (GSK-3 β) and Casein Kinase-1 β . Eventually, β -catenin in this complex will be phosphorylated resulting in its degradation by the proteasome, and preventing its translocation to the nucleus and the activation of different Wnt target-genes. The activation of the pathway occurs through binding of Wnt ligand to its receptor Frizzled resulting in the inhibition of the phosphorylation of β -catenin, which dissociates from the axin complex and translocates to the nucleus

where it induces the expression of Wnt target-genes, most notably the transcription factors T-cell factor (*TCF*) and lymphocyte enhancer factor (*LEF*), through its actions as a transcriptional coactivator [35]. Mutations in the *CTNNB1* gene, encoding β -Catenin, have been described in 2–5 % of APA [17,19]. The description of somatic *CTNNB1* mutations associated with higher expression of luteinizing hormone–chorionic gonadotropin receptor (LHCGR) and gonadotropin-releasing hormone receptor (GNRHR) in APA diagnosed during pregnancy or menopause suggested that pregnancy may reveal an underlying PA [37]. Other studies, however, showed high expression of GNRHR and LHCGR in more than 40 % of APA [38,39], and the presence of *CTNNB1* mutations both in females and males [17,19]. Further studies are necessary to establish the mechanism of *CTNNB1* mutations in the development of APA.

To a much lesser extent, somatic *PRKACA* (encoding the catalytic α subunit of Protein Kinase A) mutations have been described in APA [18]. Rhayem et al. identified somatic mutations of the *PRKACA* gene in two patients with APA by whole exome sequencing. The mutation p.Leu206Arg, previously identified in cortisol-producing adenoma (CPA) [40–42], was found in one patient with PA and Cushing syndrome. The second mutation (p.His88Asp) was identified in a patient without cortisol hypersecretion [18]. This particular mutation was not associated with a gain of function, the mechanism underlying increased PKA signaling and tumorigenesis in CPA. The role of these mutations on aldosterone secretion and their frequency in APA remains to be established.

CTNNB1 mutations and *PRKACA* mutations are also identified in CPA. Other evidence for an overlap of genetic determinants of aldosterone and cortisol excess have been described, including the cortisol co-secretion observed in a subset of APA, notably those harboring *KCNJ5* mutations [43]. The complex mechanisms that would explain how the same mutations could end up in two different hormonal phenotypes remain to be discovered.

Clinical correlates of somatic mutations

The discovery of clinical or biochemical surrogate markers of somatic mutations in APA could be of benefit for the management of the disease. Different studies described the higher prevalence of somatic *KCNJ5* mutations in women and in young patients with APA [10,16,44]. *KCNJ5* mutations were also associated to higher levels of plasma aldosterone and larger tumors [16], and with higher left ventricular mass index [45]. *CACNA1D* mutations were associated with smaller APA [10]. More recently, *KCNJ5* mutations were described as a predictor of better outcome in young patients with APA [46]. Promising data for the identification of the underlying APA genotype came from a study that analyzed steroid profiles in adrenal and peripheral venous plasma samples from APA patients by liquid chromatography–tandem mass spectrometry [47]. The authors identified a 7-steroid fingerprint in peripheral venous samples

allowing to correctly classify 92 % of the APA according to the genotype. Additionally, specific steroid profiles were associated with *KCNJ5* mutations, in particular the presence of significantly higher hybrid steroids 18-hydroxycortisol and 18-oxocortisol. This approach may be translated into clinical care, allowing to identify the APA genotype from peripheral venous plasma samples before surgery. This could be useful for the selection of patients for adrenal vein sampling.

Heterogeneity of aldosterone-producing adenomas (APA)

In spite of the fact that the relation between aldosterone production and the presence of somatic mutations is well established, the impact of these mutations on nodule/APA formation and cell proliferation is still far from fully understood. APA present a highly pronounced molecular heterogeneity not only on a mutational status, but also in terms of aldosterone synthase expression within the same APA. Recent studies showed the presence of different somatic mutations in different aldosterone-producing nodules from the same adrenal [48,49], suggesting that somatic mutations are independent events occurring in a previously remodeled adrenal cortex. In the same context, Nanba et al. described one case of a patient that was diagnosed with PA and Cushing syndrome with double adrenocortical adenomas, one harboring a *KCNJ5* somatic mutation and the other a *PRKACA* somatic mutation [50]. Furthermore, in APA exhibiting heterogeneity of aldosterone synthase expression, APA-driver mutations were identified only in positive aldosterone synthase regions [51]. Interestingly, two different mutations were identified in the same APA, lying in two distinct positive aldosterone synthase regions [51]. These findings suggest that somatic mutations are second hits in APA development that emerge from specific mechanisms that remain to be elucidated. Supporting this hypothesis, our group described the occurrence of a germline *APC* mutation and a somatic *KCNJ5* mutation leading to the development of an APA in a young patient with severe unilateral PA, bilateral macronodular adrenal hyperplasia and Gardner syndrome [52], suggesting a two-hit model for APA development with the *APC* mutation driving nodule formation and the *KCNJ5* mutation being responsible for aldosterone hypersecretion.

Another theory was suggested by Nishimoto et al., in a study that describes aldosterone-producing cell clusters (APCC) in being the origin behind APA development [53]. APCCs are structures of outer morphological ZG cells in contact with the capsule and inner ZF-like cells, expressing CYP11B2 but not CYP11B1 [53,54]. They are found in normal adrenal tissue and in adrenals with APA. APCC express important amounts of aldosterone synthase in normal and pathological conditions. In a later study, Nishimoto et al. sequenced DNA from APCCs that were collected from normal adrenal glands and identified mutations in APA-driver genes in up to 35 % of the collected samples;

specifically, mutations in *CACNA1D*, *ATP1A1* and *ATP2B3*. Interestingly, no mutations in *KCNJ5* were reported, which is the most frequently mutated gene in APA [55]. The authors suggest that APCCs could represent cellular precursors that could lead to APA with their specific mutations through unknown mechanisms. On the other hand, they propose that APCCs with *KCNJ5* could be rarer, or that APCCs that develop *KCNJ5* mutations tend to become APAs quite quickly and are hard to be witnessed before the APA development [55]. It was suggested that the sequence of events leading to APA development from an APCC occurs through the development of structures called possible APCC-to-APA translational lesions (pAATL) [56]. pAATL are composed by an outer APCC-like portion and inner micro-APA (mAPA)-like portion. The genetic characterization of pAATL is complex. In one adrenal, a *KCNJ5* mutation was identified only in the mAPA-like portion of a pAATL, not observed in the APCC-like portion and was different from the mutation identified in an APA within the same adrenal. This suggests that the APA and the pAATL do not share the same origin and that the *KCNJ5* mutation leads to differentiation of the mAPA portion from the APCC. In a second adrenal, both portions of the pAATL carried an *ATP1A1* mutation indicating its clonal origin. Although the model whereby APA arise from APCC through pAATL and mAPA is intriguing, further studies are required to better clarify the suite of genomic events involved in this transition.

Conclusion

The role of each mutation in the regulation of aldosterone production is well studied, while the impact of these mutations

on cell proliferation remains to be established. In the future, it would be of relevance to distinguish additional biomarkers or the development of techniques that are able to identify somatic mutations in APA. This could be of interest since PA is the most frequent form of secondary hypertension and is curable by the surgical removal of the APA carrying adrenal if recognized early enough. An additional benefit is the possibility of developing new diagnostic and therapeutic approaches. This is particularly the case for the use of macrolides in the detection and treatment of APA with *KCNJ5* mutations [57]. A recent work has shown that macrolide antibiotics, including roxithromycin, are potent inhibitors of *KCNJ5* channels carrying the most frequent mutations p.Gly151Arg and p.Leu168Arg. Use of clarithromycin in primary cultures from APA showed a significant inhibition of *CYP11B2* gene expression and aldosterone production [58]. These compounds could therefore be used to identify patients carrier of APA with *KCNJ5* mutations and as targeted treatments in patients who are not candidates for surgery.

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References

- [1] Collaboration, N.C.D.R.F. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *Lancet* 2017;389:37-55.
- [2] Hannemann A, Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies—a review of the current literature. *Horm Metab Res* 2012;44:157-62.
- [3] Calhoun DA, Nishizaka MK, Zaman MA, Thakkar RB, Weissmann P. Hyperaldosteronism among black and white subjects with resistant hypertension. *Hypertension* 2002;40:892-6.
- [4] Douma S, Petidis K, Doulmas M, Papaefthimiou P, Triantafyllou A, Kartali N, et al. Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet* 2008;371:1921-6.
- [5] Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The management of primary aldosteronism: case detection, diagnosis, and treatment: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2016;101:1889-916.
- [6] Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, et al. Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 2008;93:3266-81.
- [7] Williams TA, Lenders JWM, Mulatero P, Burrello J, Rottenkolber M, Adolf C, et al. Outcomes after adrenalectomy for unilateral primary aldosteronism: an international consensus on outcome measures and analysis of remission rates in an international cohort. *Lancet Diabetes Endocrinol* 2017;5:689-99.
- [8] Mulatero P, Monticone S, Bertello C, Viola A, Tizzani D, Iannaccone A, et al. Long-term cardio- and cerebrovascular events in patients with primary aldosteronism. *J Clin Endocrinol Metab* 2013;98:4826-33.
- [9] Spat A, Hunyady L, Szanda G. Signaling interactions in the adrenal cortex. *Front Endocrinol* 2016;7:17.
- [10] Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, Boulkroun S, et al. Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* 2014;64:354-61.
- [11] Hong AR, Kim JH, Song YS, Lee KE, Seo SH, Seong MW, et al. Genetics of aldosterone-producing adenoma in Korean patients. *Plos One* 2016;11:e0147590.
- [12] Taguchi R, Yamada M, Nakajima Y, Satoh T, Hashimoto K, Shibusawa N, et al. Expression and mutations of *KCNJ5* mRNA in Japanese

- patients with aldosterone-producing adenomas. *J Clin Endocrinol Metab* 2012;97:1311–9.
- [13] Wang B, Li X, Zhang X, Ma X, Chen L, Zhang Y, et al. Prevalence and characterization of somatic mutations in Chinese aldosterone-producing adenoma patients. *Medicine (Baltimore)* 2015;94:e708.
- [14] Wu VC, Huang KH, Peng KY, Tsai YC, Wu CH, Wang SM, et al. Prevalence and clinical correlates of somatic mutation in aldosterone producing adenoma-Taiwanese population. *Sci Rep* 2015;5:11396.
- [15] Zheng FF, Zhu LM, Nie AF, Li XY, Lin JR, Zhang K, et al. Clinical characteristics of somatic mutations in Chinese patients with aldosterone-producing adenoma. *Hypertension* 2015;65:622–8.
- [16] Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW, Rossi GP. A meta-analysis of somatic KCNJ5 K(+) channel mutations in 1636 patients with an aldosterone-producing adenoma. *J Clin Endocrinol Metab* 2015;100:E1089–E1095.
- [17] Akerstrom T, Maharjan R, Sven Willenberg H, Cupisti K, Ip J, Moser A, et al. Activating mutations in CTNNB1 in aldosterone producing adenomas. *Sci Rep* 2016;6:19546.
- [18] Rhayem Y, Perez-Rivas LG, Dietz A, Bathon K, Gebhard C, Riester A, et al. PRKACA somatic mutations are rare findings in aldosterone-producing adenomas. *J Clin Endocrinol Metab* 2016;101:3010–7.
- [19] Scholl UI, Healy JM, Thiel A, Fonseca AL, Brown TC, Kunstman JW, et al. Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype. *Clin Endocrinol (Oxf)* 2015;83:779–89.
- [20] Boulkroun S, Golib Dzib JF, Samson-Couterie B, Rosa FL, Rickard AJ, Meatchi T, et al. KCNJ5 mutations in aldosterone producing adenoma and relationship with adrenal cortex remodeling. *Mol Cell Endocrinol* 2013;371:221–7.
- [21] Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE. The G-protein-gated atrial K⁺ channel IKACH is a heteromultimer of two inwardly rectifying K⁺ channel proteins. *Nature* 1995;374:135–41.
- [22] Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, et al. K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* 2011;331:768–72.
- [23] Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP, Gomez-Sanchez CE. Potassium channel mutant KCNJ5 T158A expression in HAC-15 cells increases aldosterone synthesis. *Endocrinology* 2012;153:1774–82.
- [24] Scholl UI, Nelson-Williams C, Yue P, Grekin R, Wyatt RJ, Dillon MJ, et al. Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. *Proc Natl Acad Sci U S A* 2012;109:2533–8.
- [25] Fernandes-Rosa FL, Amar L, Tissier F, Bertherat J, Meatchi T, Zennaro MC, et al. Functional histopathological markers of aldosterone producing adenoma and somatic KCNJ5 mutations. *Mol Cell Endocrinol* 2015;408:220–6.
- [26] Zennaro MC, Boulkroun S, Fernandes-Rosa F. Genetic causes of functional adrenocortical adenomas. *Endocr Rev* 2017;38:516–37.
- [27] Catterall WA. Signaling complexes of voltage-gated sodium and calcium channels. *Neurosci Lett* 2010;486:107–16.
- [28] Azizan EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, et al. Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nat Genet* 2013;45:1055–60.
- [29] Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, et al. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet* 2013;45:1050–4.
- [30] Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet* 2013;45:440–4, 444e1–2.
- [31] Stindl J, Tauber P, Sterner C, Tegmeier I, Warth R, Bandulik S. Pathogenesis of adrenal aldosterone-producing adenomas carrying mutations of the Na⁺/K⁺-ATPase. *Endocrinology* 2015;156:4582–91.
- [32] Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, et al. Cellular pathophysiology of an adrenal adenoma-associated mutant of the plasma membrane Ca²⁺-ATPase ATP2B3. *Endocrinology* 2016;157:2489–99.
- [33] Scholl UI, Stolting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, et al. Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. *Elife* 2015;4:e06315.
- [34] Daniil G, Fernandes-Rosa FL, Chemin J, Blesneac I, Beltrand J, Polak M, et al. CACNA1H mutations are associated with different forms of primary aldosteronism. *EBio-Medicine* 2016;13:225–36.
- [35] El Wakil A, Lalli E. The Wnt/beta-catenin pathway in adrenocortical development and cancer. *Mol Cell Endocrinol* 2011;332:32–7.
- [36] Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, et al. WNT/beta-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Hum Mol Genet* 2014;23:889–905.
- [37] Teo AE, Garg S, Shaikh LH, Zhou J, Karet Frankl FE, Gurnell M, et al. Pregnancy, primary aldosteronism, and adrenal CTNNB1 mutations. *N Engl J Med* 2015;373:1429–36.
- [38] Nakamura Y, Hattangady NG, Ye P, Satoh F, Morimoto R, Ito-Saito T, et al. Aberrant gonadotropin-releasing hormone receptor (GnRHr) expression and its regulation of CYP11B2 expression and aldosterone production in adrenal aldosterone-producing adenoma (APA). *Mol Cell Endocrinol* 2014;384:102–8.
- [39] Nicolini G, Balzan S, Morelli L, Iacconi P, Sabatino L, Ripoli A, et al. LH, progesterone, and TSH can stimulate aldosterone in vitro: a study on normal adrenal cortex and aldosterone producing adenoma. *Horm Metab Res* 2014;46:318–21.
- [40] Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, et al. Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nat Genet* 2014;46:613–7.
- [41] Cao Y, He M, Gao Z, Peng Y, Li Y, Li L, et al. Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science* 2014;344:913–7.
- [42] Beuschlein F, Fassnacht M, Assie G, Calebiro D, Stratakis CA, Osswald A, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. *N Engl J Med* 2014;370:1019–28.
- [43] Arlt W, Lang K, Stith AJ, Dietz AS, Rhayem Y, Bancos I, et al. Steroid metabolome analysis reveals prevalent glucocorticoid excess in primary aldosteronism. *JCI Insight* 2017;2.
- [44] Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, et al. Prevalence, clinical, and molecular correlates of *knj5* mutations in primary aldosteronism. *Hypertension* 2012;59:592–8.
- [45] Rossi GP, Cesari M, Letizia C, Seccia TM, Cicala MV, Zinamosca L, et al. KCNJ5 gene somatic mutations affect cardiac remodelling but do not preclude cure of high blood pressure and regression of left ventricular hypertrophy in primary aldosteronism. *J Hypertens* 2014;32:1514–21 [discussion 1522].
- [46] Kitamoto T, Omura M, Suematsu S, Saito J, Nishikawa T. KCNJ5 mutation as a predictor for resolution of hypertension after surgical treatment of aldosterone-producing adenoma. *J Hypertens* 2018;36:619–27.
- [47] Williams TA, Peitzsch M, Dietz AS, Dekkers T, Bidlingmaier M, Riester A, et al. Genotype-specific steroid profiles associated with aldosterone-producing adenomas. *Hypertension* 2016;67:139–45.
- [48] Dekkers T, ter Meer M, Lenders JW, Hermus AR, Schultze Kool L, Langenhuijsen JF, et al. Adrenal nodularity and somatic mutations in primary aldosteronism: one node is the culprit? *J Clin Endocrinol Metab* 2014;99:E1341–51.
- [49] Fernandes-Rosa FL, Giscos-Douriez I, Amar L, Gomez-Sanchez CE, Meatchi T, Boulkroun S, et al. Different somatic mutations in multinodular adrenals with aldosterone-producing adenoma. *Hypertension* 2015;66:1014–22.
- [50] Nanba K, Omata K, Tomlins SA, Giordano TJ, Hammer GD, Rainey WE, et al. Double adrenocortical adenomas harboring independent

- KCNJ5 and PRKACA somatic mutations. *Eur J Endocrinol* 2016;175:K1-6.
- [51] Nanba K, Chen AX, Omata K, Vinco M, Giordano TJ, Else T, et al. Molecular heterogeneity in aldosterone-producing adenomas. *J Clin Endocrinol Metab* 2016;101:999-1007.
- [52] Vouillarmet J, Fernandes-Rosa F, Graeppl-Dulac J, Lantelme P, Decaussin-Petrucci M, Thivolet C, et al. Aldosterone-producing adenoma with a somatic KCNJ5 mutation revealing APC-dependent familial adenomatous polyposis. *J Clin Endocrinol Metab* 2016;101:3874-8.
- [53] Nishimoto K, Nakagawa K, Li D, Kosaka T, Oya M, Mikami S, et al. Adrenocortical zonation in humans under normal and pathological conditions. *J Clin Endocrinol Metab* 2010;95:2296-305.
- [54] Boulkroun S, Samson-Couterie B, Dzib JF, Lefebvre H, Louiset E, Amar L, et al. Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. *Hypertension* 2010;56:885-92.
- [55] Nishimoto K, Tomlins SA, Kuick R, Cani AK, Giordano TJ, Hovelson DH, et al. Aldosterone-stimulating somatic gene mutations are common in normal adrenal glands. *Proc Natl Acad Sci U S A* 2015;112:E4591-99.
- [56] Nishimoto K, Seki T, Kurihara I, Yokota K, Omura M, Nishikawa T, et al. Case report: nodule development from subcapsular aldosterone-producing cell clusters causes hyperaldosteronism. *J Clin Endocrinol Metab* 2016;101:6-9.
- [57] Scholl UI, Abriola L, Zhang C, Reimer EN, Plummer M, Kazmierczak BI, et al. Macrolides selectively inhibit mutant KCNJ5 potassium channels that cause aldosterone-producing adenoma. *J Clin Invest* 2017;127:2739-50.
- [58] Caroccia B, Prisco S, Seccia TM, Piazza M, Maiolino G, Rossi GP. Macrolides blunt aldosterone biosynthesis: a proof-of-concept study in KCNJ5 mutated adenoma cells ex vivo. *Hypertension* 2017;70:1238-42.

10.3 Molecular mechanisms in primary aldosteronism

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REVIEW

Molecular mechanisms in primary aldosteronism

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Abstract

Primary aldosteronism (PA) is the most common form and an under-diagnosed cause of secondary arterial hypertension, accounting for up to 10% of hypertensive cases and associated to increased cardiovascular risk. PA is caused by autonomous overproduction of aldosterone by the adrenal cortex. It is mainly caused by a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia. Excess aldosterone leads to arterial hypertension with suppressed renin, frequently associated to hypokalemia. Mutations in genes coding for ion channels and ATPases have been identified in APA, explaining the pathophysiology of increased aldosterone production. Different inherited genetic abnormalities led to the distinction of four forms of familial hyperaldosteronism (type I to IV) and other genetic defects very likely remain to be identified. Somatic mutations are identified in APA, but also in aldosterone-producing cell clusters (APCCs) in normal adrenals, in image-negative unilateral hyperplasia, in transitional lesions and in APCC from adrenals with bilateral adrenal hyperplasia (BAH). Whether these structures are precursors of APA or whether somatic mutations occur in a proliferative adrenal cortex, is still a matter of debate. This review will summarize our knowledge on the molecular mechanisms responsible for PA and the recent discovery of new genes related to early-onset and familial forms of the disease. We will also address new issues concerning genomic and proteomic changes in adrenals with APA and discuss adrenal pathophysiology in relation to aldosterone-producing structures in the adrenal cortex.

Key Words

- ▶ hypertension
- ▶ adrenal
- ▶ primary aldosteronism
- ▶ ion channels
- ▶ calcium

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Introduction

Primary aldosteronism (PA) is due to excessive and autonomous aldosterone production by the adrenal cortex. It is the most frequent form of secondary hypertension with an estimated prevalence up to 10% in referred patients, 4% in primary care (Hannemann & Wallaschofski 2012, Monticone *et al.* 2017) and 20% in patients with resistant hypertension (Calhoun *et al.* 2002, Douma *et al.* 2008). PA is characterized by an increased aldosterone-to-renin ratio, which is the principal feature used for screening, often hypokalemia and metabolic alkalosis and an

increased risk of cardiovascular complications. The prevalence of left ventricular hypertrophy is higher in patients with PA even after adjustment for hypertension duration (Rossi *et al.* 1996). Moreover, PA patients have a significantly higher prevalence of coronary artery disease, nonfatal myocardial infarction, heart failure and atrial fibrillation (Savard *et al.* 2013). The two major causes of PA are APA and BAH, also called idiopathic hyperaldosteronism (IHA). Although guidelines for the management of PA have been published and are

widely used in reference centers (Funder *et al.* 2016), improvements for better diagnosis, subtype identification and treatment for non-operable patients are urgently required to allow for improved care and prevention of cardiovascular complications.

Recently, whole exome sequencing had allowed identifying several somatic genetic abnormalities in APA, providing a pathogenic model for PA development. Recurrent somatic mutations were identified in genes coding for ion channels (*KCNJ5* Choi *et al.* 2011 and *CACNA1D* Azizan *et al.* 2013, Scholl *et al.* 2013) and ATPases (*ATP1A1* and *ATP2B3*, Azizan *et al.* 2013, Beuschlein *et al.* 2013) regulating intracellular ionic homeostasis and cell membrane potential. Germline mutations were identified in familial forms of PA, allowing to distinguish four different forms of familial hyperaldosteronism (FH), based on the underlying genetic defect (Fernandes-Rosa *et al.* 2018, Scholl *et al.* 2018, Zennaro 2019). These mutations increase intracellular calcium concentrations, leading to the activation of the calcium signaling pathway and to increased aldosterone production by increasing the expression of *CYP11B2*, coding for aldosterone synthase. In addition, somatic mutations were also identified in *CTNNB1* coding for b-catenin in a small proportion of APA (Teo *et al.* 2015, Scholl *et al.* 2015a, Akerstrom *et al.* 2016) and mutations in *PRKACA* (encoding protein kinase cAMP-activated catalytic subunit α) have been described in two cases (Rhayem *et al.* 2016).

Even though the consequences of these mutations on aldosterone production are well demonstrated, their impact on cell proliferation and APA formation is not well understood. Different pathogenic mechanisms have been proposed, including different hits being responsible for adrenocortical cell proliferation and hormonal secretion or the development of APA from aldosterone-producing cell clusters (APCC) of the adrenal cortex.

Steroid biosynthesis in the adrenal cortex

The adrenal cortex is composed of three separate layers, the zona glomerulosa, the zona fasciculata and the zona reticularis. This zonation is functional, each zone producing specific steroid hormones due to the zone-specific expression of two enzymes, the aldosterone synthase and the 11β -hydroxylase. Cholesterol is the common precursor in steroid hormone biosynthesis through a process that implicates multiple enzymatic steps, the major effectors being enzymes belonging to the cytochrome P450 family. In zona glomerulosa, where aldosterone is produced, cholesterol is converted

into 11-deoxycorticosterone through the successive actions of the cytochrome P450 side-chain cleavage (*CYP11A1*), the 3β -hydroxysteroid dehydrogenase type II (*HSD3B2*) and the 21α -hydroxylase (*CYP21A2*). Aldosterone synthase (encoded by the *CYP11B2* gene) catalyzes the three final steps in aldosterone biosynthesis. The first step consists in the hydroxylation at the C11 position of the intermediate steroid deoxycorticosterone into corticosterone, followed by a hydroxylation at the C18 position and the formation of 18-hydroxy-corticosterone. The last step is the oxidation of the hydroxyl group on C18, finally resulting in the production of aldosterone (Connell & Davies 2005).

Cortisol biosynthesis occurs in the zona fasciculata, where 17α -hydroxylase is expressed. This enzyme is responsible for the conversion of pregnenolone and progesterone to 17-OH-pregnenolone and 17-OH-progesterone, precursors of cortisol, whereas 21 -hydroxylase induces a hydroxylation in the C11 position of the 17OH-progesterone producing 11-deoxycortisol. The final step of cortisol biosynthesis is the hydroxylation in the C21 position of 11-deoxycortisol by 11β -hydroxylase, encoded by *CYP11B1* and specifically expressed in the zona fasciculata and reticularis (Connell & Davies 2005).

Aldosterone production is mainly regulated by the renin angiotensin system and potassium concentrations and, to a lesser extent, by the adrenocorticotrophic hormone (ACTH) (Connell & Davies 2005). The renin angiotensin system plays a major role in sodium homeostasis. In response to a decrease in blood volume, dehydration or hyponatremia, renin is secreted by the juxta-glomerulus apparatus of the kidney and cleaves the angiotensinogen produced in the liver to produce angiotensin I. In the lungs, angiotensin I is converted by the angiotensin-converting enzyme (ACE) to angiotensin II (Ang II). Ang II acts via its AT1 receptor expressed in zona glomerulosa cells, activating a $G\alpha_q$ -phospholipase C-mediated pathway, inducing the production of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol. IP3 binding to its receptor leads to calcium release from the endoplasmic reticulum, thus increasing intracellular calcium concentrations. In parallel, Ang II also inhibits the potassium channels TASK (TWIK-related acid-sensitive potassium channel) and GIRK4 (G-protein-activated inward rectifier potassium channel) and the Na^+/K^+ ATPase, therefore inducing cell membrane depolarization, and the opening of voltage-dependent membrane calcium channels, leading to calcium influx into the cell and activation of calcium signaling (Spat & Hunyady 2004, Oki *et al.* 2012a) (Fig. 1A and B).

Activation of calcium signaling increases the availability of cholesterol in the cell, by stimulating the activity of the enzyme cholesterol ester hydrolase, but also by increasing the expression of StAR (steroid acute regulatory protein), which is responsible for cholesterol transport into the mitochondria, and of other steroidogenic enzymes (Spat & Hunyady 2004). Calcium/calmodulin-dependent protein kinases also activate transcription factors such as nuclear receptor subfamily 4 group A members 1 and 2 (NUR77/NGF1B and NURR1 encoded by NR4A1 and NR4A2 respectively), which increase transcription of the *CYP11B2* gene (Bassett *et al.* 2004b).

Cortisol production is regulated by the hypothalamic–pituitary–adrenal axis via ACTH. ACTH binds to the

melanocortin receptor 2 (MC2R), inducing an increase in intracellular cAMP levels via the activation of a $G\alpha s$ protein. cAMP activates PKA signaling following its binding to the regulatory subunits of PKA (Ruggiero & Lalli 2016). The duration of ACTH stimulation affects its role in the biosynthesis of steroids. Short-term stimulation increases the availability of intracellular cholesterol and its transfer to the internal membrane of the mitochondria, primarily through the phosphorylation of StAR at the Ser195 position; it also induces the expression of steroidogenic enzymes. However, long-term stimulation by ACTH induces transcriptional regulation of StAR and steroidogenic enzymes. Particularly, PKA signaling activates transcription factors such as steroidogenic

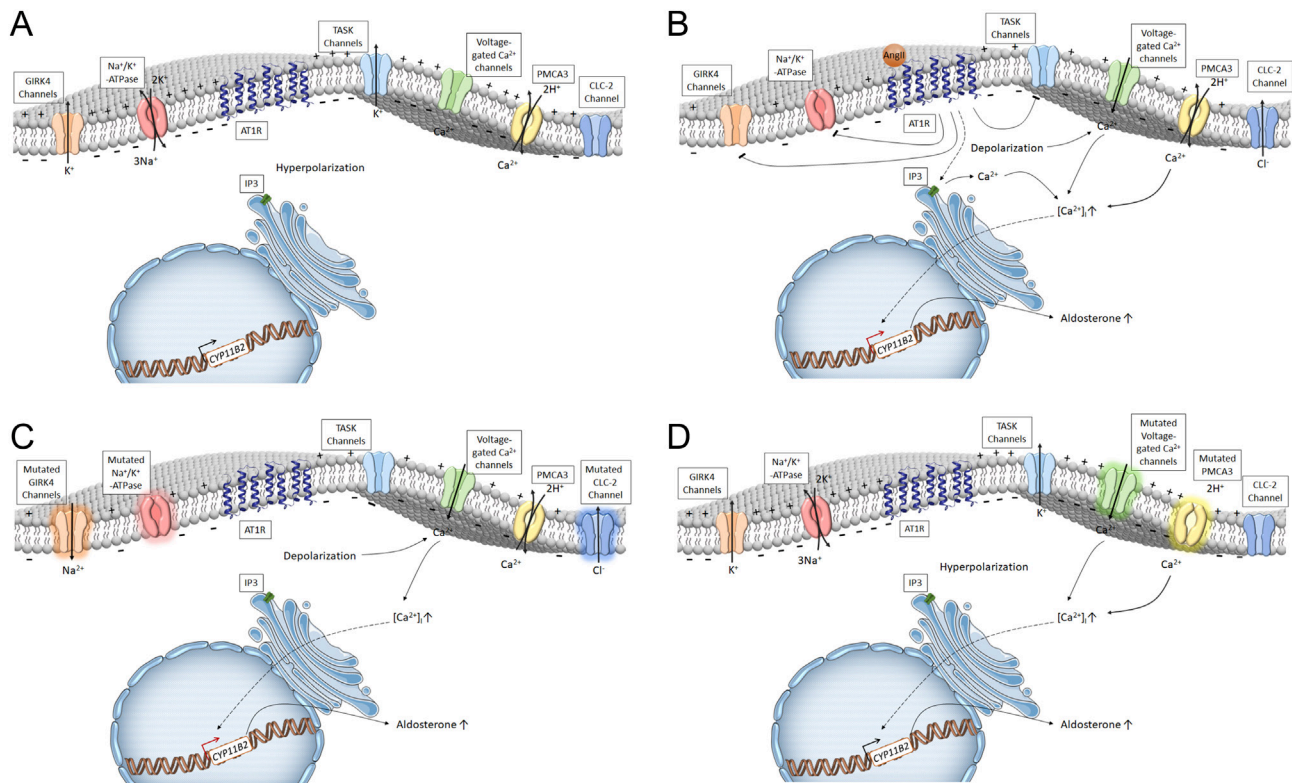


Figure 1

Regulation of aldosterone biosynthesis in normal conditions and in PA. (A) In unstimulated conditions, zona glomerulosa cells are hyperpolarized. This state is maintained by the activity of potassium channels such as TASK and GIRK4 and the Na⁺/K⁺ ATPase pump. (B) Increases in extracellular potassium levels inhibit K⁺ currents of TASK channels and lead to cell membrane depolarization. Activation of AngII signaling through its receptor (AT1R) on the surface of ZG cells inhibits K⁺ channels and the Na⁺/K⁺ ATPase, which leads to cell membrane depolarization, opening of voltage-gated Ca²⁺ channels and increased intracellular calcium concentrations. Intracellular calcium activates signaling pathways leading to *CYP11B2* expression and thus increasing aldosterone biosynthesis. Activation of AT1R also activates a Gαq-phospholipase C-mediated pathway, inducing an increase in the concentration of inositol 1,4,5-triphosphate (IP3), which binds to its receptor expressed on the endoplasmic reticulum, inducing the release of calcium from intracellular stores. (C) Mutations in *KCNJ5* encoding the potassium channel GIRK4 are responsible for a loss of selectivity of the channel and lead to sodium influx into the cell; mutations in *ATP1A1* encoding the α1 subunit of the Na⁺/K⁺ ATPase increase intracellular Na⁺ and H⁺ concentrations; mutations in *CLCN2*, encoding the chloride channel CLC-2, increase chloride conductance at resting potentials; all these abnormalities lead to membrane depolarization of glomerulosa cells. Cell membrane depolarization induces opening of voltage-gated calcium channels, increased intracellular calcium concentrations and activation of calcium signaling, triggering increased expression of *CYP11B2* and aldosterone production. (D) Mutations in *CACNA1H* and *CACNA1D* encoding the voltage-gated calcium channels Cav3.2 and Cav1.3 as well as mutations in *ATP2B3*, encoding the Ca²⁺ pump PMCA3, directly increase intracellular calcium concentrations and lead to increased aldosterone biosynthesis through activation of calcium signaling.

factor 1 (SF-1), cAMP-responsive element-binding protein (CREB), cAMP-responsive element modulator and CCAAT/enhancer-binding protein and activator protein 1, which regulate the expression of steroidogenic genes (Ruggiero & Lalli 2016).

ACTH stimulation also induces aldosterone production by similar mechanisms. Moreover, ACTH stimulates calcium flow into zona glomerulosa cells by activating L-type calcium channels. However, in the case of a chronic ACTH stimulation, *CYP11B2* expression is inhibited, which causes a reduction in aldosterone production, as opposed to the expression of *CYP11B1* which is responsible of cortisol production (Vinson 2016).

There exist other factors that regulate aldosterone production in a paracrine fashion. Among them, serotonin (5-HT) produced by mastocytes stimulates steroid production (El Ghorayeb *et al.* 2016) through mechanisms involving the 5-HT receptor type 4 (HTR4) (Lefebvre *et al.* 1993, Lefebvre *et al.* 2001) which is expressed in adrenocortical cells. Activation of HTR4 activates adenylyl cyclase and induces calcium influx into the cell through T-type channels, thus regulating cortisol and aldosterone production (Louiset *et al.* 2017). Remarkably, activation of 5-HT4 by agonists such as cisapride or zacopride induces aldosterone production in healthy volunteers (Lefebvre *et al.* 2000).

Genetic alterations in PA

The mechanisms that regulate aldosterone production are severely altered in PA. PA is the most common form of secondary arterial hypertension, with an estimated prevalence of 10% in referred patients in specialized centers and up to 5% in the general hypertensive population (Rossi *et al.* 2006, Hannemann & Wallaschofski 2012, Monticone *et al.* 2017). Although the majority of cases are sporadic, PA may be transmitted as a Mendelian trait in familial forms of the disease. Familial forms of PA account for 1–5% of cases and are transmitted as autosomal dominant traits. Four different forms have been described, based on the underlying genetic defect. Similarly, recurrent somatic mutations in different genes have been identified in up to 88% of APA (Nanba *et al.* 2018).

Germline mutations in FH

FH type I

FH-I or glucocorticoid-remediable aldosteronism (GRA) is an autosomal dominant disease that accounts for 0.5–1% of PA in adult patients, but up to 3% in pediatric cohorts

(Medeau *et al.* 2005, Aglony *et al.* 2011, Mulatero *et al.* 2011, Pallauf *et al.* 2012). It is characterized by early and severe hypertension, often associated to hypokalemia. Peculiar features of FH-I are the presence of hybrid steroids 18-hydroxycortisol and 18-oxocortisol in the urines and the suppression of aldosterone by dexamethasone (Sutherland *et al.* 1966). These features are a consequence of the underlying genetic defect, which consists in the formation of a chimeric gene between the regulatory regions of *CYP11B1* and the coding regions of *CYP11B2* due to unequal crossing over during meiosis. This results in ectopic expression of *CYP11B2* in the zona fasciculata of the adrenal cortex, and ACTH-regulated expression of aldosterone synthase (Lifton *et al.* 1992, Stowasser *et al.* 2000). Those patients can be efficiently treated with low-dose glucocorticoids (Stowasser *et al.* 2000); however, cardiovascular consequences of high aldosterone excess may manifest even in young normotensive patients (Stowasser *et al.* 2005) and patients with FH-I are at increased cardiovascular risk, with increased number of cerebrovascular events at a young age (Litchfield *et al.* 1998).

FH type II

FH-II is a non-glucocorticoid-remediable form of familial hyperaldosteronism, transmitted in an autosomal dominant fashion, found in 1.2–6% of adult patients with PA. Patients display a familial history of PA, and different subtypes of the disease (APA or BAH) may be present within the same family (Stowasser & Gordon 2001, Medeau *et al.* 2005, Mulatero *et al.* 2011, Pallauf *et al.* 2012). FH-II is not distinguishable from other sporadic forms of PA, the diagnosis is based on the identification of two or more affected family members. While a genetic locus was associated with FH-II on 7p22 (Lafferty *et al.* 2000), no pathogenic mutations were identified in several candidate genes located in this chromosomal region. It is only very recently that use of whole exome sequencing allowed the discovery of a new gene associated to FH-II. Two groups have indeed identified several gain-of-function mutations in the *CLCN2* gene coding for the CLC-2 chloride channel in patients with FH-II and early-onset PA and described for the first time the implication of a chloride channel in the regulation of aldosterone production (Fig. 1C).

In one study, a *de novo* heterozygous p.Gly24Asp mutation in the *CLCN2* gene was identified in a patient diagnosed at 9 years of age with hypertension due to PA, associated to profound hypokalemia (Fernandes-Rosa *et al.* 2018). This gain-of-function mutation affects a highly

conserved region in the CIC-2 chloride channel, in which mutations lead to constitutive activation of the channel (Grunder *et al.* 1992). The channel is highly expressed in the human and mouse adrenal zona glomerulosa and patch clamp analyses of zona glomerulosa cells from mouse adrenal gland slices show *bona fide* chloride currents, which are abolished in *Clcn2* knockout mice, indicating that CIC-2 is the foremost chloride channel in the adrenal zona glomerulosa (Fernandes-Rosa *et al.* 2018). Expression of the mutated CIC-2 in adrenocortical cells leads to increased chloride currents and depolarization of the zona glomerulosa cell membrane, opening voltage-gated calcium channels and triggering autonomous aldosterone production (Fig. 1C). This work indicates for the first time the implication of an anion channel in the regulation of aldosterone biosynthesis, PA and hypertension (Fernandes-Rosa *et al.* 2018).

In the other study, whole exome sequencing of three members of a large Australian family with FH-II previously described by Stowasser *et al.* (Stowasser *et al.* 1992, Lafferty *et al.* 2000) allowed the identification of a germline mutation in the *CLCN2* gene, p.Arg172Gln, in affected family members. The same mutation was identified in three additional cases with early-onset PA, one within a familial context, another occurring *de novo*. Furthermore, the authors identified four additional germline *CIC-2* variants, p.Met22Lys, p.Tyr26Asn, p.Lys362del and p.Ser865Arg in four unrelated patients with early-onset PA (Scholl *et al.* 2018). Immunohistochemistry confirmed the expression of CIC-2 in human adrenal zona glomerulosa. Mutant channels showed gain of function, with higher open probabilities at the glomerulosa resting potential.

Altogether, these results indicate that *CLCN2* should be included in the panel of genes that are screened in patients who develop early-onset PA and in families with a diagnosis of FH-II. Mutations may be located in different domains of CIC-2 and therefore affect channel function at different degrees, which explains phenotypic heterogeneity and the presence of relatively mild cases.

FH type III and germline *KCNJ5* mutations

FH-III is a non-glucocorticoid-remediable form of FH, showing early-onset severe hypertension, hyperaldosteronism, profound hypokalemia and high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol (Geller *et al.* 2008). Patients require bilateral adrenalectomy to control blood pressure, which shows massive bilateral hyperplasia of the adrenal cortex, although mild forms have also been described.

Recurrent germline mutations in the *KCNJ5* gene (coding for the potassium channel GIRK4) have been identified in FH-III (Choi *et al.* 2011). *KCNJ5* codes for the G protein-activated inward rectifier potassium channel 4 (GIRK4), which is composed of two membrane-spanning domains (M1 and M2), one pore-forming region (H5) and cytoplasmic N- and C-termini that participate in the formation of pore structure. The mutations are located in a highly conserved domain within or near the channel selectivity filter. They induce a gain of function, with a loss of potassium selectivity in favor of an increased unspecific sodium influx into the cytoplasm. This results in cell membrane depolarization, increased intracellular calcium concentrations, activation of Ca²⁺ signaling and autonomous aldosterone biosynthesis (Choi *et al.* 2011, Oki *et al.* 2012b) (Fig. 1C). Different *KCNJ5* mutations have been described in the literature, which not all are associated with severe PA (Mulatero *et al.* 2013). Indeed, *KCNJ5* mutations were also identified in patients diagnosed with mild PA in a context of FH-II, with no BAH at imaging and blood pressure controlled by conventional medication. The genetic diagnosis allowed reclassifying those patients into mild forms of FH-III. Remarkably, mutations associated with milder phenotypes have more pronounced functional effects *in vitro*. In particular, the channel's selectivity was less affected by the p.Thr158Ala mutation compared to the channels carrying the other mutations (p.Leu168Arg and p.Gly151Arg) (Choi *et al.* 2011). The p.Gly151Glu mutation, identified in mild cases diagnosed as FH-II, generates significantly larger Na⁺ currents than the p.Gly151Arg mutation and leads to sodium-dependent cell death (Scholl *et al.* 2012), with strong depolarization of adrenocortical cells expressing the mutated channel (Mulatero *et al.* 2012).

FH type IV and germline *CACNA1H* mutations

FH-IV is a non-glucocorticoid-remediable form of FH, which has been attributed to germline *CACNA1H* mutations. Familial analysis showed that despite autosomal dominant transmission of the mutation, differences could be observed within family members on a clinical level, indicating incomplete penetrance of the disease (Scholl *et al.* 2015b).

CACNA1H codes for the $\alpha 1$ subunit of the voltage-gated T-type calcium channel Cav3.2. The structure of this subunit consists of four homologous domains (named from I to IV) where each domain contains six transmembrane helices (S1–S6). A recurrent p.Met1549Val mutation in *CACNA1H* was first identified in five patients

with hypertension due to PA by age 10 years and their relatives. The mutation affects the electrophysiological properties of the channel, inducing specifically a shift in the voltage sensitivity of the channel to a more negative current and a modification of its inactivating properties, which triggers an opening of the channel at more negative membrane potentials, allowing activation of calcium signaling in the absence of stimulation (Scholl *et al.* 2015b) (Fig. 1D). Scholl *et al.* demonstrated in their study that cells transfected with the Cav3.2 1549Val had more aldosterone biosynthesis as well as higher *CYP11B2* expression when compared to cells transfected with the WT Cav3.2 channel (Reimer *et al.* 2016).

In a similar study, Daniil *et al.* identified four germline mutations in the *CACNA1H* gene in PA patients with different phenotypic presentations. Two of the variants (p.Val1951Glu and p.Pro2083Leu) in this study are located in the cytoplasmic C terminus of the channel. A p.Ser196Leu variant was located in the S4 segment of domain I of the channel, and one mutation, p.Met1549Ile, is located in the S6 segment of domain III. The p.Met1549Ile mutation, affecting the same amino acid described by Scholl *et al.* in patients with FH-IV, was identified in a patient with early-onset PA and multiplex developmental disorder. Cav3.2 p.Ser196Leu and p.Pro2083Leu were found in two patients with FH-II, and p.Val1951Glu was identified in one patient with APA. Electrophysiological analysis revealed significant changes in the Ca²⁺ current properties for all mutants, suggesting a gain-of-function phenotype. Moreover, transfection of the mutant Cav3.2 in the adrenocortical cell line H295R-S2 led to increased aldosterone production and increases the expression of steroidogenic enzymes following potassium stimulation (Daniil *et al.* 2016).

Germline *CACNA1D* mutations in PA

CACNA1D mutations have been described in two children with PASNA (primary aldosteronism, seizures and neurological abnormalities) (Scholl *et al.* 2013). *CACNA1D* codes for the $\alpha 1$ subunit of the voltage-gated L-type calcium channel Cav1.3. Again, these mutations induce a gain of function, with the channel opening at lower voltages. This leads to excessive aldosterone production by increasing Ca²⁺ entry that causes an increased intracellular Ca²⁺-mediated signaling with induction of aldosterone biosynthesis (Azizan *et al.* 2013, Scholl *et al.* 2013).

The first case was identified in a 3-year-old female who suffered severe hypertension, biventricular hypertrophy, neurological abnormalities and aldosteronism as a

newborn, associated with high aldosterone levels, a high aldosterone-to-renin ratio and hypokalemia. Genetic analyses identified a germline mutation in *CACNA1D* (p.Gly403Asp), a variant that had also been identified in APA (Scholl *et al.* 2013, 2015a). The second subject exhibited cerebral palsy and complex seizures just after birth, with HT and hyperaldosteronism with hyperkalemia at age 5 years. Genetic analysis leads to the identification of a *CACNA1D* mutation (p.Ile770Met) also found as recurrent mutation in APA (Scholl *et al.* 2013, Korah & Scholl 2015) (see below).

Somatic mutations in APA

Numerous recurrent somatic mutations have been identified in APA. These affect genes coding for ion channels (*KCNJ5* Choi *et al.* 2011 and *CACNA1D* Azizan *et al.* 2013, Scholl *et al.* 2013) also involved in inherited forms. In addition, recurrent mutations have been identified in genes coding for ATPases (*ATP1A1* which codes for the $\alpha 1$ subunit of the Na⁺/K⁺-ATPase Azizan *et al.* 2013, Beuschlein *et al.* 2013 and *ATP2B3* encoding the plasma membrane calcium-transporting ATPase 3 (PMCA3) Beuschlein *et al.* 2013) that regulate the intracellular ionic homeostasis and membrane potential. Na⁺/K⁺-ATPase and PMCA3 are members of the P-type family of ATPases and are composed of ten transmembrane domains (M1–M10) with intracellular N- and C-tails. *ATP1A1* mutations affect amino acids in the transmembrane helices M1, M4, and M9 of the $\alpha 1$ subunit. They lead to a loss of pump activity and a reduction in its affinity for potassium as well as an inward leakage of sodium, inducing membrane depolarization (Azizan *et al.* 2013, Beuschlein *et al.* 2013). In addition, mutant pumps may induce a proton leak (Azizan *et al.* 2013), leading to intracellular acidification which contributes to aldosterone production. Indeed, expression of mutant $\alpha 1$ subunits of the Na⁺/K⁺ ATPase carrying the L104R, V332G and G99R mutations in adrenocortical NCI-H295R cells failed to increase intracellular Ca²⁺ concentrations, but showed intracellular acidification, which caused increased aldosterone production (Stindl *et al.* 2015). Under physiological conditions, the PMCA3 extrudes one cytosolic Ca²⁺ in exchange for two H⁺. PMCA3 mutations occur in the transmembrane domain M4 and induce the deletion of a stretch of amino acids between Leu425 and Leu433 (Beuschlein *et al.* 2013, Fernandes-Rosa *et al.* 2014). Cells expressing the mutant PMCA3 have a reduced capacity to export Ca²⁺, suggesting a loss

of the physiological pump function. In addition, they induce an increased Ca^{2+} influx possibly due to opening of depolarization-activated Ca^{2+} channels or to Ca^{2+} leak through the mutated pump (Tauber *et al.* 2016). All these mutations induce an increase in intracellular calcium levels, followed by an activation of calcium signaling and therefore increase the biosynthesis of aldosterone.

A study on 474 subjects presenting an APA, performed within the European Network for the Study of Adrenal Tumors (ENS@T), found mutations in 54.2% of APA, where 38% of these were found in the *KCNJ5* gene, 9.3% in *CACNA1D*, 5.3% in *ATP1A1* and 1.7% in *ATP2B3* (Fernandes-Rosa *et al.* 2014). Patients presenting mutations in *KCNJ5* were mostly women and younger patients, while APA with *CACNA1D* mutations were smaller than the other mutational groups (Fernandes-Rosa *et al.* 2014). A recent meta-analysis on 1636 patients from 13 studies showed a prevalence of *KCNJ5* mutations of 43%, with much higher frequencies (up to 77%) in populations from East Asia in comparison to studies performed on European populations. APA harboring *KCNJ5* mutations were more frequent in women and associated with larger tumors and a higher plasma aldosterone concentration when compared with mutation-negative APA. APA where *KCNJ5* mutations are present are also associated with APA composed mainly of ZF-like cells. Other mutations were associated mainly with male gender and smaller tumors (Lenzini *et al.* 2015).

In a recent study, Nanba *et al.* performed CYP11B2 immunohistochemistry on 75 adrenal samples from white American patients, in order to identify APA and other aldosterone-producing regions prior to genetic analysis. DNA was then extracted directly from formalin-fixed paraffin-embedded (FFPE) tissue, positive for CYP11B2 expression. Complete next-generation sequencing of all genes involved in PA identified somatic mutations in 88% of APA (Nanba *et al.* 2018). Again, *KCNJ5* mutations were the most frequent mutational events (43%), followed by *CACNA1D* (21%), *ATP1A1* (17%), *ATP2B3* (4%) and *CTNNB1* (3%) mutations. Also, *KCNJ5* mutations were more frequent in women (70 vs 24% in men). This study shows that CYP11B2 immunohistochemistry-guided next-generation sequencing allows the identification of a larger portion of somatic mutations in APA. Similar results were obtained by analyzing adrenocortical adenomas from 79 black patients with PA. Somatic mutations in aldosterone driver genes were found in 89% of APAs. Interestingly, *CACNA1D* mutations were the most frequent (42%) ones in APA from patients with African ancestry, followed by

KCNJ5 (34%), *ATP1A1* (8%) and *ATP2B3* mutations (4%) (Nanba *et al.* 2019).

Other less frequent mutations that play an important role in APA were identified in *CTNNB1* and *PRKACA*. *CTNNB1* codes for β -catenin. This protein is a central element of the Wnt/ β -catenin signaling pathway, which has an essential role in the development of the adrenal cortex, in the differentiation of the zona glomerulosa and steroid hormone biosynthesis (El Wakil & Lalli 2011). In unstimulated conditions, β -catenin is present in the cytoplasm and is associated to the degradation complex with adaptor proteins such as axin, APC (adenomatous polyposis coli), two serine/threonine kinases, casein kinase 1 ($\text{CK1}\alpha$) and glycogen synthase kinase 3 β (GSK3 β). This complex phosphorylates β -catenin, which is then recognized by the ubiquitin ligase E3 that results in its destruction by the proteasome, thus preventing its translocation into the nucleus and the activation of different Wnt target genes. Activation of the Wnt/ β -catenin signaling pathway by binding of Wnt to its receptor frizzled inhibits the phosphorylation of β -catenin which dissociates from the axin complex and translocates to the nucleus where it induces the expression of Wnt target genes, by binding to the transcription factors TCF (T-cell factor) and LEF (lymphocyte enhancer factor) as a transcriptional co-activator (MacDonald *et al.* 2009). It has been shown that β -catenin indirectly regulates *CYP11B2* expression via transcriptional induction of the nuclear receptors NURR1 and NUR77 (Berthon *et al.* 2014). The Wnt/ β -catenin signaling pathway has been shown to be constitutively active in 70% of APA (Berthon *et al.* 2014). Mutations in *CTNNB1* have been found in 2–5% of APA and are more frequent in women (Teo *et al.* 2015, Scholl *et al.* 2015a, Akerstrom *et al.* 2016). Mutations specifically occur in exon 3, which codes for the serine/threonine residue which when phosphorylated results in the degradation of β -catenin (Berthon *et al.* 2010). To a much lesser extent, somatic mutations in *PRKACA*, which codes for the cAMP-dependent protein kinase catalytic subunit α , have been described in APA (Rhayem *et al.* 2016). A study performed on a cohort of 12 patients showed a prevalence of 1.6% of this mutation. One of the described mutations in this study was new (p.His88Asp) (Rhayem *et al.* 2016), whereas the other mutation, p.Leu206Arg, had been previously described as a recurrent somatic mutation in patients with cortisol-producing adenoma and Cushing's syndrome (Goh *et al.* 2014). Mutations in both *PRKACA* and *CTNNB1* have been mainly associated with cortisol-producing adenomas and/or adrenocortical

cancer. Therefore, the mechanisms explaining how the same mutations result in two different hormonal phenotypes remain to be established.

Genomic and proteomic alterations in APA

Despite the major advances on the genetics of PA, many questions remain open concerning the pathophysiology of the disease, in particular, the mechanisms and dynamics of adenoma formation. Several studies have explored genomic and proteomic alterations in APA, to better understand the mechanisms involved in its development. A recent extremely original work performed deep quantitative proteomic and phosphoproteomic profiling on APA and adjacent non-tumoral adrenal cortex from six patients with PA. The authors identified, out of 5555 proteins common to all samples, 18 which were significantly downregulated and 11 significantly upregulated in all APA. Proteome data confirmed that upregulation of the steroidogenic enzymes HSD3B2, CYP21A2, CYP11B2 and of proteins involved in cholesterol uptake (lipolysis-stimulated lipoprotein receptor (LSR)) is involved in the increase of steroidogenesis in APA (Swierczynska *et al.* 2019). This study also showed that steroidogenic enzymes are regulated by phosphorylation; in particular, HSD3B2 is phosphorylated at Ser95 or 96 and CYP21A2 has a novel phosphorylation site at Ser489. An altered composition of the extracellular matrix (ECM) was also revealed in APA, with reduction in proteins related to collagen and collagen fibril assembly, enzymes involved in extracellular matrix turnover and structural components of the extracellular matrix. The deregulation of kinases affecting cytoskeleton remodeling and axonal guidance-related pathways suggests that APA development may be accompanied or be related to changes in cytoskeleton rearrangements and possibly innervation. Remarkably, the GTPase RHOC controlling actin organization, which was upregulated in APA, significantly increased the expression of CYP11B2 at the transcriptional level in transfected adrenocortical H295R cells.

APA also showed higher levels of proteins involved in N-glycosylation and enzymes involved in GABA degradation. Remarkably, the activity of the main regulators of steroidogenesis, MC2R (receptor for ACTH) and AT1R (receptor for Ang II) is affected by N-glycosylation and the level and the activity of N-glycoproteins like lipoprotein receptors (LDLR and SR-B1) and many ion channels (TASK1, TASK3, TREK1, Kv1.4, CACNA1D) are affected by N-glycosylation. The inhibition of TASK1, TASK3 and KV1.4 glycosylation decreases the number of

cell-surface channels, leading to a reduced current flow, protein instability and intracellular channel retention (Watanabe *et al.* 2004, Mant *et al.* 2013, Swierczynska *et al.* 2019). Reduced GABAergic signaling in APA may also modulate aldosterone production. Indeed, in physiological conditions, GABAergic signaling mediates a decrease of steroidogenesis *in vivo* in the rat adrenal cortex (Mishunina & Kononenko 2002). Finally, one of the most dysregulated pathways in APAs appeared to be the mTORC1 signaling pathway. Its activity was increased in APA; this pathway is involved in cell proliferation and steroidogenesis in APA cells and immortalized adrenocortical cells. Thus, the increase of its activation may lead to an increased steroidogenic output and adenoma development. This opens new perspectives on the benefits of inhibiting mTORC1 in patients with APA (Su *et al.* 2013, Swierczynska *et al.* 2019).

Similarly, gene expression profiling of APA allowed identifying genes and intracellular signaling pathways involved in the pathogenesis and pathophysiology of this tumor. As expected, microarray studies and SAGE (serial analysis of gene expression) showed higher expression of CYP11B2 in APA compared to normal adrenals and adjacent cortical tissue (Assie *et al.* 2005, Williams *et al.* 2010), although other studies report a subgroup of APA with reduced expression of aldosterone synthase (Lenzini *et al.* 2007). However, these data require to be reevaluated in the context of recent work showing that in a certain number of multinodular adrenals some of the larger nodules do not express CYP11B2, which is localized to smaller nodules or APCC (Fernandes-Rosa *et al.* 2015, Nanba *et al.* 2018); this finding may have led to some sample biases in earlier studies. Despite this, APA show a genotype–phenotype correlation indicating that transcriptional signatures may be influenced by APA genotype, although different studies report slightly different results. Indeed, APA with different gene mutations show distinct expression profiles (Azizan *et al.* 2012). While some studies reported a higher CYP11B2 expression in APA with KCNJ5 mutations compared to APA without KCNJ5 mutation (Monticone *et al.* 2012, Seccia *et al.* 2012), a higher expression of CYP11B2 was reported in APA with ATP1A1 and ATP2B3 mutations, compared to KCNJ5-mutated tumors (Akerstrom *et al.* 2015). In a large transcriptome analysis performed on 102 samples, KCNJ5-mutated APA did not show differential clustering compared to tumors not harboring KCNJ5 mutations (Boukroun *et al.* 2012, Zennaro 2019).

Several studies revealed an upregulation of genes encoding transcription factors (NURR1 and NGF1B)

that regulate the transcription of *CYP11B2* (Bassett *et al.* 2004a), genes encoding the transcription factors SF-1 (*NR5A1*) and DAX1 (*NROB1*) that play an essential role in adrenal development and steroidogenesis (Bassett *et al.* 2005) and a target gene of SF-1, *VSNL1*, which increases aldosterone production in the NCI H295R cells (Williams *et al.* 2012). Furthermore, Azizan *et al.* reported higher *CYP17A1* expression in APA composed of cells resembling to the zona fasciculata carrying *KCNJ5* mutations (Azizan *et al.* 2012). This result was also confirmed in *KCNJ5*-mutated tumors compared with *ATP1A1/CACNA1D* mutant adenomas by immunohistochemistry (Tan *et al.* 2017).

Finally, a recent methylome analysis revealed hypomethylation of *CYP11B2* in APA and a higher demethylation of G protein-coupled receptors (GPCRs) and GPCR-related genes (Itcho *et al.* 2018). Thus, this hypomethylation may stimulate aldosterone production in APA through receptors that would regulate gene transcription like ACTH, glucagon, somatostatin, parathyroid hormone and glutamate metabotropic receptors. Remarkably, HTR4, MC2R and PTGER1 (prostaglandin E receptor 1), which all regulate aldosterone biosynthesis, showed hypomethylation and upregulation of their mRNA in APA.

APCC and the development of APA

Different studies showed the existence of APCC in the subcapsular portion of the normal human adrenal gland. These APCC, which are suggested to autonomously produce aldosterone, are characterized by the presence of subcapsular zona glomerulosa-like cells and inner zona fasciculata-like cells (Nishimoto *et al.* 2010) and a uniform *CYP11B2*, but absent *CYP11B1* expression (Nishimoto *et al.* 2016). Therefore, APCC and APA are distinctive in their sizes, cellular arrangements and enzyme expression profiles, as APA may consist of heterogeneous cell types expressing either *CYP11B1* or *CYP11B2*. In the human adrenal gland, aldosterone synthase staining is continuous in the zona glomerulosa at young age between 0 and 11 years, whereas with increasing age, the adrenal cortex loses the continuous staining of aldosterone synthase and acquires a variegated zonation with increased number of *CYP11B2* expressing APCC (Omata *et al.* 2017). The discontinuous *CYP11B2* expression is suggested to be the consequence of a negative feedback produced by the autonomous aldosterone production by APCC.

Even though there is no sex difference in APA prevalence, a higher APCC score was described in female

adrenals (Nishimoto *et al.* 2015). Transcriptome analyses identified markers for APCC in addition to *CYP11B2*, which showed significantly higher transcript expressions in APCC compared to zona glomerulosa or fasciculata. These genes code for proteins playing a role in glucose transport (*SLC35F1*), aldosterone production (*MC2R*) and regulation of intracellular phosphorylation and dephosphorylation (*PPP4R4*) (Nishimoto *et al.* 2015).

Different somatic mutations leading to autonomous aldosterone production were identified in APCC from normal subjects, supporting a pathological behavior of APCC (Nishimoto *et al.* 2015). Next-generation sequencing of 23 APCC samples from FFPE and frozen tissues from normal adrenals showed that 35% of APCC harbored mutations observed in APA and known to cause aldosterone overproduction in *CACNA1D* (26%) and *ATP1A1* (6%). No mutations in *KCNJ5* have been identified in APCC so far. Different explanations are given for this observation, including that APCC with *KCNJ5* mutations may rapidly progress into APA (Nishimoto *et al.* 2015).

Omata *et al.* demonstrated that the accumulation of APCC is a cellular and molecular cause of IHA. Examination of a cohort of 15 adrenals with IHA with *CYP11B2* immunohistochemistry and next-generation sequencing showed that the adrenal cortex of all IHA adrenals harbored at least one *CYP11B2*-positive APCC or aldosterone-producing microadenomas. IHA adrenals had a significantly larger number of APCC than normotensive controls. Somatic mutations were identified in *CACNA1D* in 58% of cases and only in one case in *KCNJ5*. These findings suggest that not only hyperplasia but also the enlargement of APCC with somatic aldosterone driver gene mutations (most likely *CACNA1D*) are responsible for IHA (Omata *et al.* 2018).

Finally, transitional structures were described showing a subcapsular APCC-like portion and inner APA-like portion (Nishimoto *et al.* 2016). These structures are called pAATL, possible APCC-to-APA transitional lesions. These pAATL are characterized by the presence of mutations in APA driver genes, in particular *KCNJ5* and *ATP1A1*, with *KCNJ5* mutations identified exclusively in the APA-like portion of the structure (Nishimoto *et al.* 2016).

Conclusion

Although much progress has been made over the last ten years in our understanding of the molecular mechanisms leading to PA, there still remain a number of open

questions which need to be addressed. In particular, in a certain number of familial cases, diagnosed mainly as FH-II, no genetic abnormality has been identified in known genes. This is also the case for a small proportion of APA, in which even CYP11B2-guided next-generation sequencing of all coding exons of all APA driver genes fails identifying somatic mutations. This suggests that additional genes may be involved in the disease, which remain to be discovered. Another question relates to the mechanisms leading to APA development. Indeed, germline mutations identified in familial forms lead to adrenal cortex hyperplasia, but not to APA. Whether nodule formation requires an additional genetic hit favoring increased proliferation, or whether APA derive from APCC carrying somatic mutations remains to be clarified. A related question is how do APCC develop, in particular how and when do somatic mutations occur and whether there is any difference between APCC with and without somatic mutations. Future work addressing these issues will allow progress in our knowledge on the molecular mechanisms involved in normal aldosterone physiology and development of PA and possibly provide new tools for the development of better diagnostic and therapeutic approaches.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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References

- Aglony M, Martinez-Aguayo A, Carvajal CA, Campino C, Garcia H, Bancalari R, Bolte L, Avalos C, Loureiro C, Trejo P, *et al.* 2011 Frequency of familial hyperaldosteronism type 1 in a hypertensive pediatric population: clinical and biochemical presentation. *Hypertension* **57** 1117–1121. (<https://doi.org/10.1161/HYPERTENSIONAHA.110.168740>)
- Akerstrom T, Willenberg HS, Cupisti K, Ip J, Backman S, Moser A, Maharjan R, Robinson B, Iwen KA, Dralle H, *et al.* 2015 Novel somatic mutations and distinct molecular signature in aldosterone-producing adenomas. *Endocrine-Related Cancer* **22** 735–744. (<https://doi.org/10.1530/ERC-15-0321>)
- Akerstrom T, Maharjan R, Sven Willenberg H, Cupisti K, Ip J, Moser A, Stalberg P, Robinson B, Alexander Iwen K, Dralle H, *et al.* 2016 Activating mutations in CTNNB1 in aldosterone producing adenomas. *Scientific Reports* **6** 19546. (<https://doi.org/10.1038/srep19546>)
- Assie G, Auzan C, Gasc JM, Baviera E, Balaton A, Elalouf JM, Jeunemaitre X, Plouin PF, Corvol P & Clauser E 2005 Steroidogenesis in aldosterone-producing adenoma revisited by transcriptome analysis. *Journal of Clinical Endocrinology and Metabolism* **90** 6638–6649. (<https://doi.org/10.1210/jc.2005-1309>)
- Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, Happerfield L, Marker A, Hoffman GJ & Brown MJ 2012 Microarray, qPCR, and KCNJ5 sequencing of aldosterone-producing adenomas reveal differences in genotype and phenotype between zona glomerulosa- and zona fasciculata-like tumors. *Journal of Clinical Endocrinology and Metabolism* **97** E819–E829. (<https://doi.org/10.1210/jc.2011-2965>)
- Azizan EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, Maniero C, Garg S, Bochukova EG, Zhao W, *et al.* 2013 Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nature Genetics* **45** 1055–1060. (<https://doi.org/10.1038/ng.2716>)
- Bassett MH, Suzuki T, Sasano H, White PC & Rainey WE 2004a The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Molecular Endocrinology* **18** 279–290. (<https://doi.org/10.1210/me.2003-0005>)
- Bassett MH, White PC & Rainey WE 2004b The regulation of aldosterone synthase expression. *Molecular and Cellular Endocrinology* **217** 67–74. (<https://doi.org/10.1016/j.mce.2003.10.011>)
- Bassett MH, Mayhew B, Rehman K, White PC, Mantero F, Arnaldi G, Stewart PM, Bujalska I & Rainey WE 2005 Expression profiles for steroidogenic enzymes in adrenocortical disease. *Journal of Clinical Endocrinology and Metabolism* **90** 5446–5455. (<https://doi.org/10.1210/jc.2005-0836>)
- Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Jossineau C, Damon-Soubeyrand C, Louiset E, Taketo MM, Tissier F, Bertherat J, Lefrancois-Martinez AM, *et al.* 2010 Constitutive beta-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. *Human Molecular Genetics* **19** 1561–1576. (<https://doi.org/10.1093/hmg/ddq029>)
- Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, Samson-Couterie B, Zennaro MC, Plouin PF, Skah S, *et al.* 2014 WNT/beta-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Human Molecular Genetics* **23** 889–905. (<https://doi.org/10.1093/hmg/ddt484>)
- Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, Penton D, Schack VR, Amar L, Fischer E, *et al.* 2013 Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nature Genetics* **45** 440–4, 444e1. (<https://doi.org/10.1038/ng.2550>)
- Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, Mulatero P, Samson-Couterie B, Hahner S, Quinkler M, *et al.* 2012 Prevalence, clinical, and molecular correlates of KCNJ5 mutations in primary aldosteronism. *Hypertension* **59** 592–598. (<https://doi.org/10.1161/HYPERTENSIONAHA.111.186478>)
- Calhoun DA, Nishizaka MK, Zaman MA, Thakkar RB & Weissmann P 2002 Hyperaldosteronism among black and white subjects with resistant hypertension. *Hypertension* **40** 892–896. (<https://doi.org/10.1161/01.HYP.0000040261.30455.B6>)
- Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A, Men CJ, *et al.* 2011 K⁺ Channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* **331** 768–772. (<https://doi.org/10.1126/science.1198785>)
- Connell JM & Davies E 2005 The new biology of aldosterone. *Journal of Endocrinology* **186** 1–20. (<https://doi.org/10.1677/joe.1.06017>)
- Daniil G, Fernandes-Rosa FL, Chemin J, Blesneac I, Beltrand J, Polak M, Jeunemaitre X, Boulkroun S, Amar L, Strom TM, *et al.* 2016 CACNA1H mutations are associated With different forms of primary

- aldosteronism. *Ebiomedicine* **13** 225–236. (<https://doi.org/10.1016/j.ebiom.2016.10.002>)
- Douma S, Petidis K, Doumas M, Papaefthimiou P, Triantafyllou A, Kartali N, Papadopoulos N, Vogiatzis K & Zamboulis C 2008 Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet* **371** 1921–1926. ([https://doi.org/10.1016/S0140-6736\(08\)60834-X](https://doi.org/10.1016/S0140-6736(08)60834-X))
- El Ghorayeb N, Bourdeau I & Lacroix A 2016 Role of ACTH and other hormones in the regulation of aldosterone production in primary aldosteronism. *Frontiers in Endocrinology* **7** 72. (<https://doi.org/10.3389/fendo.2016.00072>)
- El Wakil A & Lalli E 2011 The Wnt/beta-catenin pathway in adrenocortical development and cancer. *Molecular and Cellular Endocrinology* **332** 32–37. (<https://doi.org/10.1016/j.mce.2010.11.014>)
- Fernandes-Rosa FL, Williams TA, Riesters A, Steichen O, Beuschlein F, Boulkroun S, Strom TM, Monticone S, Amar L, Meatchi T, *et al.* 2014 Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* **64** 354–361. (<https://doi.org/10.1161/HYPERTENSIONAHA.114.03419>)
- Fernandes-Rosa FL, Giscos-Douriez I, Amar L, Gomez-Sanchez CE, Meatchi T, Boulkroun S & Zennaro MC 2015 Different somatic mutations in multinodular adrenals with aldosterone-producing adenoma. *Hypertension* **66** 1014–1022. (<https://doi.org/10.1161/HYPERTENSIONAHA.115.05993>)
- Fernandes-Rosa FL, Daniil G, Orozco JJ, Goppner C, El Zein R, Jain V, Boulkroun S, Jeunemaitre X, Amar L, Lefebvre H, *et al.* 2018 A gain-of-function mutation in the CLCN2 chloride channel gene causes primary aldosteronism. *Nature Genetics* **50** 355–361. (<https://doi.org/10.1038/s41588-018-0053-8>)
- Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, Stowasser M & Young WF, Jr 2016 The management of primary aldosteronism: case detection, diagnosis, and treatment: an Endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism* **101** 1889–1916. (<https://doi.org/10.1210/jc.2015-4061>)
- Geller DS, Zhang J, Wisgerhof MV, Shackleton C, Kashgarian M & Lifton RP 2008 A novel form of human Mendelian hypertension featuring nonglucocorticoid-remediable aldosteronism. *Journal of Clinical Endocrinology and Metabolism* **93** 3117–3123. (<https://doi.org/10.1210/jc.2008-0594>)
- Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, Kunstman JW, Korah R, Suttorp AC, Dietrich D, *et al.* 2014 Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nature Genetics* **46** 613–617. (<https://doi.org/10.1038/ng.2956>)
- Grunder S, Thiemann A, Pusch M & Jentsch TJ 1992 Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. *Nature* **360** 759–762. (<https://doi.org/10.1038/360759a0>)
- Hannemann A & Wallaschofski H 2012 Prevalence of primary aldosteronism in patient's cohorts and in population-based studies—a review of the current literature. *Hormone and Metabolic Research* **44** 157–162. (<https://doi.org/10.1055/s-0031-1295438>)
- Itcho K, Oki K, Kobuke K, Yoshii Y, Ohno H, Yoneda M & Hattori N 2018 Aberrant G protein-receptor expression is associated with DNA methylation in aldosterone-producing adenoma. *Molecular and Cellular Endocrinology* **461** 100–104. (<https://doi.org/10.1016/j.mce.2017.08.019>)
- Korah HE & Scholl UI 2015 An update on familial hyperaldosteronism. *Hormone and Metabolic Research* **47** 941–946. (<https://doi.org/10.1055/s-0035-1564166>)
- Lafferty AR, Torpy DJ, Stowasser M, Taymans SE, Lin JP, Huggard P, Gordon RD & Stratakis CA 2000 A novel genetic locus for low renin hypertension: familial hyperaldosteronism type II maps to chromosome 7 (7p22). *Journal of Medical Genetics* **37** 831–835. (<https://doi.org/10.1136/jmg.37.11.831>)
- Lefebvre H, Contesse V, Delarue C, Soubrane C, Legrand A, Kuhn JM, Wolf LM & Vaudry H 1993 Effect of the serotonin-4 receptor agonist zacopride on aldosterone secretion from the human adrenal cortex: in vivo and in vitro studies. *Journal of Clinical Endocrinology and Metabolism* **77** 1662–1666. (<https://doi.org/10.1210/jcem.77.6.8263156>)
- Lefebvre H, Cartier D, Duparc C, Contesse V, Lihmann I, Delarue C, Vaudry H, Fischmeister R & Kuhn JM 2000 Effect of serotonin4 (5-HT4) receptor agonists on aldosterone secretion in idiopathic hyperaldosteronism. *Endocrine Research* **26** 583–587. (<https://doi.org/10.3109/07435800009048575>)
- Lefebvre H, Compagnon P, Contesse V, Delarue C, Thuillez C, Vaudry H & Kuhn JM 2001 Production and metabolism of serotonin (5-HT) by the human adrenal cortex: paracrine stimulation of aldosterone secretion by 5-HT. *Journal of Clinical Endocrinology and Metabolism* **86** 5001–5007. (<https://doi.org/10.1210/jcem.86.10.7917>)
- Lenzini L, Seccia TM, Aldighieri E, Belloni AS, Bernante P, Giuliani L, Nussdorfer GG, Pessina AC & Rossi GP 2007 Heterogeneity of aldosterone-producing adenomas revealed by a whole transcriptome analysis. *Hypertension* **50** 1106–1113. (<https://doi.org/10.1161/HYPERTENSIONAHA.107.100438>)
- Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW & Rossi GP 2015 A meta-analysis of somatic KCNJ5 K(+) channel mutations in 1636 patients with an aldosterone-producing adenoma. *Journal of Clinical Endocrinology and Metabolism* **100** E1089–E1095. (<https://doi.org/10.1210/jc.2015-2149>)
- Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S & Lalouel JM 1992 A chimaeric 11beta-hydroxylase aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* **355** 262–265. (<https://doi.org/10.1038/355262a0>)
- Litchfield WR, Anderson BF, Weiss RJ, Lifton RP & Dluhy RG 1998 Intracranial aneurysm and hemorrhagic stroke in glucocorticoid-remediable aldosteronism. *Hypertension* **31** 445–450. (<https://doi.org/10.1161/01.HYP.31.1.445>)
- Louiset E, Duparc C, Lenglet S, Gomez-Sanchez CE & Lefebvre H 2017 Role of cAMP/PKA pathway and T-type calcium channels in the mechanism of action of serotonin in human adrenocortical cells. *Molecular and Cellular Endocrinology* **441** 99–107. (<https://doi.org/10.1016/j.mce.2016.10.008>)
- MacDonald BT, Tamai K & He X 2009 Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Developmental Cell* **17** 9–26. (<https://doi.org/10.1016/j.devcel.2009.06.016>)
- Mant A, Williams S, Roncoroni L, Lowry E, Johnson D & O'Kelly I 2013 N-glycosylation-dependent control of functional expression of background potassium channels K2P3.1 and K2P9.1. *Journal of Biological Chemistry* **288** 3251–3264. (<https://doi.org/10.1074/jbc.M112.405167>)
- Medeau V, Assie G, Zennaro MC, Clauser E, Plouin PF & Jeunemaitre X 2005 [Familial aspect of primary hyperaldosteronism: analysis of families compatible with primary hyperaldosteronism type 2]. *Annales d'Endocrinologie* **66** 240–246. ([https://doi.org/10.1016/S0003-4266\(05\)81756-1](https://doi.org/10.1016/S0003-4266(05)81756-1))
- Mishunina TM & Kononenko VY 2002 Specific GABA binding in the adrenals and blood corticosteroid levels in stress in intact rats and rats with changes in the functional activity of the hypothalamo-pituitary-adrenal system. *Neuroscience and Behavioral Physiology* **32** 109–112. (<https://doi.org/10.1023/A:1013920722237>)
- Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, Cicala MV, Pezzani R, Auchus RJ, Ghayee HK, Shibata H, *et al.* 2012 Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. *Journal of Clinical Endocrinology and Metabolism* **97** E1567–E1572. (<https://doi.org/10.1210/jc.2011-3132>)
- Monticone S, Burrello J, Tizzani D, Bertello C, Viola A, Buffolo F, Gabetti L, Mengozzi G, Williams TA, Rabbia F, *et al.* 2017 Prevalence and clinical manifestations of primary aldosteronism encountered in primary care practice. *Journal of the American College of Cardiology* **69** 1811–1820. (<https://doi.org/10.1016/j.jacc.2017.01.052>)

- Mulatero P, Tizzani D, Viola A, Bertello C, Monticone S, Mengozzi G, Schiavone D, Williams TA, Einaudi S, La Grotta A, *et al.* 2011 Prevalence and characteristics of familial hyperaldosteronism: the PATOGEN study (Primary Aldosteronism in TORino-GENetic forms). *Hypertension* **58** 797–803. (<https://doi.org/10.1161/HYPERTENSIONAHA.111.175083>)
- Mulatero P, Tauber P, Zennaro MC, Monticone S, Lang K, Beuschlein F, Fischer E, Tizzani D, Pallauf A, Viola A, *et al.* 2012 KCNJ5 mutations in European families with nonglucocorticoid remediable familial hyperaldosteronism. *Hypertension* **59** 235–240. (<https://doi.org/10.1161/HYPERTENSIONAHA.111.183996>)
- Mulatero P, Monticone S, Rainey WE, Veglio F & Williams TA 2013 Role of KCNJ5 in familial and sporadic primary aldosteronism. *Nature Reviews. Endocrinology* **9** 104–112. (<https://doi.org/10.1038/nrendo.2012.230>)
- Nanba K, Omata K, Else T, Beck PCC, Nanba AT, Turcu AF, Miller BS, Giordano TJ, Tomlins SA & Rainey WE 2018 Targeted molecular characterization of aldosterone-producing adenomas in white Americans. *Journal of Clinical Endocrinology and Metabolism* **103** 3869–3876. (<https://doi.org/10.1210/jc.2018-01004>)
- Nanba K, Omata K, Gomez-Sanchez CE, Stratakis CA, Demidowich AP, Suzuki M, Thompson LDR, Cohen DL, Luther JM, Gellert L, *et al.* 2019 Genetic characteristics of aldosterone-producing adenomas in blacks. *Hypertension* **73** 885–892. (<https://doi.org/10.1161/HYPERTENSIONAHA.118.12070>)
- Nishimoto K, Nakagawa K, Li D, Kosaka T, Oya M, Mikami S, Shibata H, Itoh H, Mitani F, Yamazaki T, *et al.* 2010 Adrenocortical zonation in humans under normal and pathological conditions. *Journal of Clinical Endocrinology and Metabolism* **95** 2296–2305. (<https://doi.org/10.1210/jc.2009-2010>)
- Nishimoto K, Tomlins SA, Kuick R, Cani AK, Giordano TJ, Hovelson DH, Liu CJ, Sanjanwala AR, Edwards MA, Gomez-Sanchez CE, *et al.* 2015 Aldosterone-stimulating somatic gene mutations are common in normal adrenal glands. *PNAS* **112** E4591–E4599. (<https://doi.org/10.1073/pnas.1505529112>)
- Nishimoto K, Seki T, Kurihara I, Yokota K, Omura M, Nishikawa T, Shibata H, Kosaka T, Oya M, Suematsu M, *et al.* 2016 Case report: nodule development from subcapsular aldosterone-producing cell clusters causes hyperaldosteronism. *Journal of Clinical Endocrinology and Metabolism* **101** 6–9. (<https://doi.org/10.1210/jc.2015-3285>)
- Oki K, Plonczynski MW, Lam ML, Gomez-Sanchez EP & Gomez-Sanchez CE 2012a The potassium channel, Kir3.4 participates in angiotensin II-stimulated aldosterone production by a human adrenocortical cell line. *Endocrinology* **153** 4328–4335. (<https://doi.org/10.1210/en.2012-1241>)
- Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP & Gomez-Sanchez CE 2012b Potassium channel mutant KCNJ5 T158A expression in HAC-15 cells increases aldosterone synthesis. *Endocrinology* **153** 1774–1782. (<https://doi.org/10.1210/en.2011-1733>)
- Omata K, Anand SK, Hovelson DH, Liu CJ, Yamazaki Y, Nakamura Y, Ito S, Satoh F, Sasano H, Rainey WE, *et al.* 2017 Aldosterone-producing cell clusters frequently harbor somatic mutations and accumulate with age in normal adrenals. *Journal of the Endocrine Society* **1** 787–799. (<https://doi.org/10.1210/js.2017-00134>)
- Omata K, Satoh F, Morimoto R, Ito S, Yamazaki Y, Nakamura Y, Anand SK, Guo Z, Stowasser M, Sasano H, *et al.* 2018 Cellular and genetic causes of idiopathic hyperaldosteronism. *Hypertension* **72** 874–880. (<https://doi.org/10.1161/HYPERTENSIONAHA.118.11086>)
- Pallauf A, Schirpenbach C, Zwermann O, Fischer E, Morak M, Holinski-Feder E, Hofbauer L, Beuschlein F & Reincke M 2012 The prevalence of familial hyperaldosteronism in apparently sporadic primary aldosteronism in Germany: a single center experience. *Hormone and Metabolic Research* **44** 215–220. (<https://doi.org/10.1055/s-0031-1299730>)
- Reimer EN, Walenda G, Seidel E & Scholl UI 2016 CACNA1H M1549V mutant calcium channel causes autonomous aldosterone production in HAC15 cells and is inhibited by Mibefradil. *Endocrinology* **157** 3016–3022. (<https://doi.org/10.1210/en.2016-1170>)
- Rhayem Y, Perez-Rivas LG, Dietz A, Bathon K, Gebhard C, Riester A, Mauracher B, Gomez-Sanchez C, Eisenhofer G, Schwarzmayr T, *et al.* 2016 PRKACA somatic mutations are rare findings in aldosterone-producing adenomas. *Journal of Clinical Endocrinology and Metabolism* **101** 3010–3017. (<https://doi.org/10.1210/jc.2016-1700>)
- Rossi GP, Sacchetto A, Visentin P, Canali C, Graniero GR, Palatini P & Pessina AC 1996 Changes in left ventricular anatomy and function in hypertension and primary aldosteronism. *Hypertension* **27** 1039–1045. (<https://doi.org/10.1161/01.HYP.27.5.1039>)
- Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, Ganzaroli C, Giacchetti G, Letizia C, Maccario M, *et al.* 2006 A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *Journal of the American College of Cardiology* **48** 2293–2300. (<https://doi.org/10.1016/j.jacc.2006.07.059>)
- Ruggiero C & Lalli E 2016 Impact of ACTH signaling on transcriptional regulation of steroidogenic genes. *Frontiers in Endocrinology* **7** 24. (<https://doi.org/10.3389/fendo.2016.00024>)
- Savard S, Amar L, Plouin PF & Steichen O 2013 Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension* **62** 331–336. (<https://doi.org/10.1161/HYPERTENSIONAHA.113.01060>)
- Scholl UI, Nelson-Williams C, Yue P, Grekin R, Wyatt RJ, Dillon MJ, Couch R, Hammer LK, Harley FL, Farhi A, *et al.* 2012 Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. *PNAS* **109** 2533–2538. (<https://doi.org/10.1073/pnas.1121407109>)
- Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, Fonseca AL, Korah R, Starker LF, Kunstman JW, *et al.* 2013 Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nature Genetics* **45** 1050–1054. (<https://doi.org/10.1038/ng.2695>)
- Scholl UI, Healy JM, Thiel A, Fonseca AL, Brown TC, Kunstman JW, Horne MJ, Dietrich D, Riemer J, Kucukkoylu S, *et al.* 2015a Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype. *Clinical Endocrinology* **83** 779–789. (<https://doi.org/10.1111/cen.12873>)
- Scholl UI, Stolting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, Prasad ML, Goh G, Carling T, Juhlin CC, *et al.* 2015b Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. *ELife* **4** e06315. (<https://doi.org/10.7554/eLife.06315>)
- Scholl UI, Stolting G, Schewe J, Thiel A, Tan H, Nelson-Williams C, Vichot AA, Jin SC, Loring E, Untiet V, *et al.* 2018 CLCN2 chloride channel mutations in familial hyperaldosteronism type II. *Nature Genetics* **50** 349–354. (<https://doi.org/10.1038/s41588-018-0048-5>)
- Seccia TM, Mantero F, Letizia C, Kuppusamy M, Caroccia B, Barisa M, Cicala MV, Miotto D & Rossi GP 2012 Somatic mutations in the KCNJ5 gene raise the lateralization index: implications for the diagnosis of primary aldosteronism by adrenal vein sampling. *Journal of Clinical Endocrinology and Metabolism* **97** E2307–E2313. (<https://doi.org/10.1210/jc.2012-2342>)
- Spat A & Hunyady L 2004 Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiological Reviews* **84** 489–539. (<https://doi.org/10.1152/physrev.00030.2003>)
- Stindl J, Tauber P, Sterner C, Tegtmeyer I, Warth R & Bandulik S 2015 Pathogenesis of adrenal aldosterone-producing adenomas carrying mutations of the Na(+)/K(+)-ATPase. *Endocrinology* **156** 4582–4591. (<https://doi.org/10.1210/en.2015-1466>)
- Stowasser M & Gordon RD 2001 Familial hyperaldosteronism. *Journal of Steroid Biochemistry and Molecular Biology* **78** 215–229. ([https://doi.org/10.1016/S0960-0760\(01\)00097-8](https://doi.org/10.1016/S0960-0760(01)00097-8))
- Stowasser M, Gordon RD, Tunny TJ, Klemm SA, Finn WL & Krek AL 1992 Familial hyperaldosteronism type II: five families with a new variety of primary aldosteronism. *Clinical and Experimental Pharmacology and*

- Physiology* **19** 319–322. (<https://doi.org/10.1111/j.1440-1681.1992.tb00462.x>)
- Stowasser M, Bachmann AW, Huggard PR, Rossetti TR & Gordon RD 2000 Treatment of familial hyperaldosteronism type I: only partial suppression of adrenocorticotropic required to correct hypertension. *Journal of Clinical Endocrinology and Metabolism* **85** 3313–3318. (<https://doi.org/10.1210/jcem.85.9.6834>)
- Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D & Marwick TH 2005 Evidence for abnormal left ventricular structure and function in normotensive individuals with familial hyperaldosteronism type I. *Journal of Clinical Endocrinology and Metabolism* **90** 5070–5076. (<https://doi.org/10.1210/jc.2005-0681>)
- Su H, Gu Y, Li F, Wang Q, Huang B, Jin X, Ning G & Sun F 2013 The PI3K/AKT/mTOR signaling pathway is overactivated in primary aldosteronism. *PLOS One* **8** e62399. (<https://doi.org/10.1371/journal.pone.0062399>)
- Sutherland DJ, Ruse JL & Laidlaw JC 1966 Hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone. *Canadian Medical Association Journal* **95** 1109–1119.
- Swierczynska MM, Betz MJ, Colombi M, Dazert E, Jenö P, Moes S, Pfaff C, Glatz K, Reincke M, Beuschlein F, *et al.* 2019 Proteomic landscape of aldosterone-producing adenoma. *Hypertension* **73** 469–480. (<https://doi.org/10.1161/HYPERTENSIONAHA.118.11733>)
- Tan GC, Negro G, Pinggera A, Tizen Laim NMS, Mohamed Rose I, Ceral J, Ryska A, Chin LK, Kamaruddin NA, Mohd Mokhtar N, *et al.* 2017 Aldosterone-producing adenomas: histopathology-genotype correlation and identification of a novel CACNA1D mutation. *Hypertension* **70** 129–136. (<https://doi.org/10.1161/HYPERTENSIONAHA.117.09057>)
- Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, Warth R & Bandulik S 2016 Cellular pathophysiology of an adrenal adenoma-associated mutant of the plasma membrane Ca(2+)-ATPase ATP2B3. *Endocrinology* **157** 2489–2499. (<https://doi.org/10.1210/en.2015-2029>)
- Teo AE, Garg S, Shaikh LH, Zhou J, Karet Frankl FE, Gurnell M, Happerfield L, Marker A, Bienz M, Azizan EA, *et al.* 2015 Pregnancy, primary aldosteronism, and adrenal CTNNB1 mutations. *New England Journal of Medicine* **373** 1429–1436. (<https://doi.org/10.1056/NEJMoa1504869>)
- Vinson GP 2016 Functional zonation of the adult mammalian adrenal cortex. *Frontiers in Neuroscience* **10** 238. (<https://doi.org/10.3389/fnins.2016.00238>)
- Watanabe I, Zhu J, Recio-Pinto E & Thornhill WB 2004 Glycosylation affects the protein stability and cell surface expression of Kv1.4 but not Kv1.1 potassium channels. A pore region determinant dictates the effect of glycosylation on trafficking. *Journal of Biological Chemistry* **279** 8879–8885. (<https://doi.org/10.1074/jbc.M309802200>)
- Williams TA, Monticone S, Morello F, Liew CC, Mengozzi G, Pilon C, Asioli S, Sapino A, Veglio F & Mulatero P 2010 Teratocarcinoma-derived growth factor-1 is upregulated in aldosterone-producing adenomas and increases aldosterone secretion and inhibits apoptosis in vitro. *Hypertension* **55** 1468–1475. (<https://doi.org/10.1161/HYPERTENSIONAHA.110.150318>)
- Williams TA, Monticone S, Crudo V, Warth R, Veglio F & Mulatero P 2012 Visinin-like 1 is upregulated in aldosterone-producing adenomas with KCNJ5 mutations and protects from calcium-induced apoptosis. *Hypertension* **59** 833–839. (<https://doi.org/10.1161/HYPERTENSIONAHA.111.188532>)
- Zennaro MC 2019 From transcripts to proteins. *Hypertension* **73** 284–285. (<https://doi.org/10.1161/HYPERTENSIONAHA.118.11820>)

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