Université de Montréal

Caractérisation de l'implication de β-caténine dans les tumeurs surrénaliennes.

Présenté par

Julien Durand

Département de Sciences Biomédicales

Faculté de Médecine

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Ce mémoire intitulé :
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dans les tumeurs surrénaliennes.

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A été évaluée par un jury composé des personnes suivantes :

Président-rapporteur : DR Pangala.V. Bhat
Directeur de recherche : DR. Isabelle Bourdeau
Membre du jury : DR. Réjean Lapointe

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Abrégé/ Résumé

Les lésions surrénaliennes surviennent dans la population générale à une fréquence d'environ 2-3%. Parmi les anomalies génétiques identifiées jusqu'à présent dans les tumeurs surrénaliennes, les mutations somatiques de β-caténine sont les plus prévalentes. Elles sont présentes dans environ 20% des adénomes et carcinomes cortico-surrénaliens. β-caténine est l'élément central de la voie canonique de WNT qui joue un rôle crucial dans le développement embryonnaire, l'homéostase et la tumourigenèse. Les mutations activatrices de β-caténine conduisent à l'accumulation nucléaire de β- caténine qui interagit avec les TCF/LEF-1 qui active la transcription des gènes cibles. Les gènes cibles de β-caténine, varient et dépendent du contexte cellulaire. Dans la glande surrénale, les gènes cibles de β-caténine sont inconnus. Nous avons effectué des études de microarray qui nous ont permis d'identifier 490 transcrits dérégulés dans les adénomes corticosurrénaliens porteurs de mutations ponctuelles de β-caténine. L'expression aberrante *d'ISM1, RALBP1, PDE2A, CDH12, ENC1, PHYHIP* et *CITED2* dans les adénomes porteurs de mutations de β-caténine a été confirmée par PCR en temps réel. Le traitement des cellules humaines de carcinome cortico-surrénalien H295R (mutation de *CTNNB1*, Ser45Prol) avec les inhibiteurs de β-caténine/TCF (PKF115-584 et PNU74654) ont confirmé l'implication de β-caténine dans la régulation transcriptionelle d'*ISM1, RALBP1*, *PDE2A*, *ENC1* et *CITED2*. En conclusion, nos travaux ont conduit à l'identification de nouveaux gènes cibles de β-catenin impliqués dans la tumourigenèse cortico-surrénalienne.

Mots clés : Glandes surrénales, Cortex surrénalien, Adénome cortico-surrénalien, Voie de signalisation de WNT, Microarray, Tumourigenèse, *CTNNB1*/β-caténine, PKF115-584, H295R

Abstract

Adrenal lesions occur in the general population at a prevalence of about 2-3%. Several mutations have been identified in adrenocortical tumours. β-catenin mutations were recently found to be the most frequent genetic alteration in both sporadic adrenocortical adenomas and carcinomas (20-30%). β-catenin is the central player in canonical Wnt signaling which plays a key role in organ/ gland development, maintenance of homeostasis and tumourigenesis. Activation of Wnt signaling by altered regulation of β-catenin levels evokes β-catenin accumulation in the nucleus, and interaction with the TCF/LEF-1 proteins that activates the transcription of target genes. These target genes are believed to be highly cell and context specific and are linked to developmental and cell cycling functions. β-catenin target genes in adrenocortical tumours are unknown. Using microarray technology, we found 490 transcripts that are deregulated in adrenocortical adenomas harbouring β-catenin activating mutations in comparison to non mutated adenomas and normal adrenal glands. These genes differ highly in function and many are poorly characterized genes. Differential expression of *ISM1, RALBP1, PDE2A, CDH12, ENC1, PHYHIP* and *CITED2* in adenomas with activating β-catenin mutations was confirmed by real-time PCR. Treatment of human adrenocortical carcinoma cells, H295R (*CTNNB1* Ser45Prol), with β-catenin/TCF inhibitors (PKF115-584 and PNU74654) further confirmed the implication of β-catenin on the transcriptional regulation of *ISM1, RALBP1, PDE2A, ENC1* and *CITED2*. In conclusion, we have found new potential β-catenin target genes that may be involved in adrenocortical tumourigenesis.

Key words: Adrenal gland, adrenocortical adenoma, adrenocortical carcinoma, WNT signaling, microarray, tumourgenesis, *CTNNB1*/β-catenin, PKF115-584, H295R

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Abbreviations

AA: Adrenocortical adenoma
ACC: Adrenocortical carcinoma
ACTH: Adrenal Corticotrophic Hormone
AIMAH: ACTH-independant macronodular adrenal hyperplasia
Ang2: Angiotensin 2
ANP : Atrial natriuretic peptide
APC : Adenomatous polyposis coli
BWS: Beckwith-Wiedemann syndrome
cAMP: Cyclic adenosine mono phosphate
CGH: Comparative genomic hybridization
CHIP: Chromatin immupopreciptation
CK1: Cyclin kinase 1
CNC: Carney complex
CRH: Corticotropin-releasing hormone
CS: Cushing's syndrome
DHEA: Dehydroepiandrosterone
DM: Deletion mutation
DNA: Deoxyribonucleic acid
DSH or DVL: Dishevelled genes
EMSA: Electrophoresis mobility shift assay
FAP: Familial adenomatous polyposis coli
FCCM: Fat cell conditioned medium
FISH: Fluorescence in situ hybridization
FOXO:Forkead
GSK3β: Glycogen synthase kinase 3 beta
KO: Knockout
LOH: Loss of heterozygosity
LRP: Low density liprotein receptor
miRNA: Micro ribonucleic acid
NA: Normal adrenal gland
PCP: Planar cell polarity
PI3K : Phosphatidylinositol 3-kinase
PKA: Protein kinase A
PM: Point mutation
PPNAD: Primary pigmented adrenocortical disease
RNA: Ribonucleic acid
RT: Reverse transcriptase
SF-1: Steroidogenic factor 1
siRNA: Small interfering RNA

StAR: Steroidogenic acute regulatory protein
TBE: TCF binding element
TCF: T-cell transcription factor
UPD: Unipaternal disomy
WNT: Wingless
WRE: Wnt responsive element
Wt: Wild type
WT: Wilms tumour
ZF: Zona fasciculate
ZG: Zona glomerulosa
ZR: Zona reticularis

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Chapter 1. Review of literature

Section 1. The Adrenal Gland

1.1. Adrenal gland physiology

The adrenal gland is a small gland (4 grams in adults) situated at the upper extremity of both kidneys in mammals (Figure 1A, page 2). It is composed of two completely distinctive zones, the medulla and the cortex (Figure 1B, page 2) surrounded by a capsule. The medulla is the central part and represents 10-20% of the adrenal gland weight in humans. The medulla is composed primarily of chromaffin cells and secretes epinephrine and norepinephrin. The cortex is yellow in healthy individuals and is itself composed of three zones (zona glomerulosa, zona fasciculata, zona reticucularis) each responsible for the synthesis of different hormones. The cells in each layer have slightly different structures, reflecting their different functions (Figure 1D, page2). Note that the focus of this project was on aspects involving only the adrenal cortex.

The zona glomerulosa is responsible for aldosterone production and is incapable of producing the other hormones secreted by the cortex (see section 1.2). The zona fasciculata is the largest of three zones and makes up to 75% of the total cortex. The cells within this zone are much larger and contain high lipid content. The zona reticularis is a compact layer surrounding the medulla and produces androgens and cortisol along with the zona fasciculata.

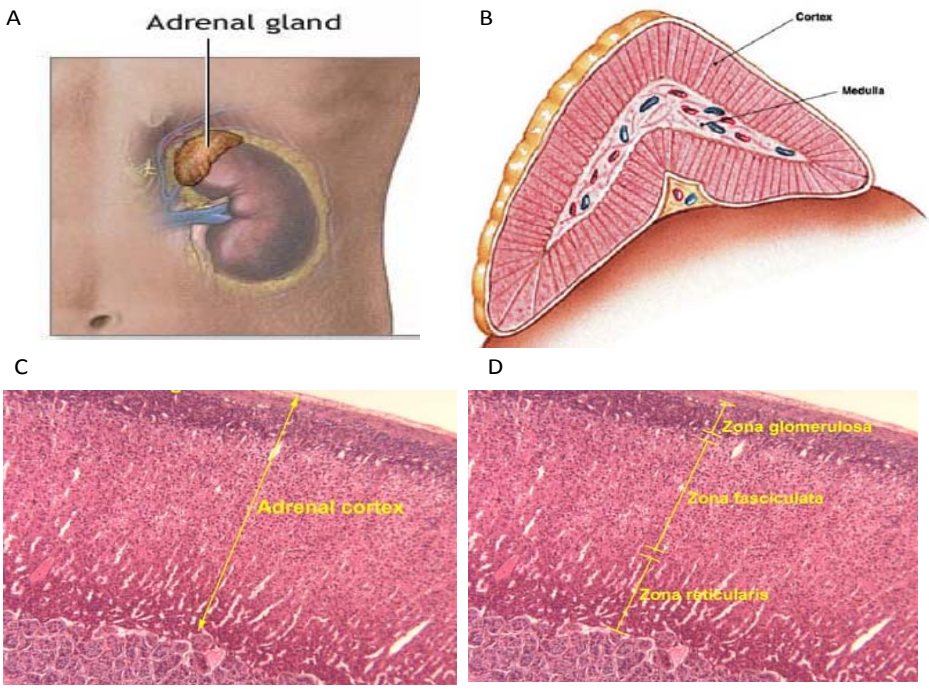

Figure 1 : Adrenal gland and its zones. (A) Picture showing location of the adrenal gland above the kidney. www.nlm.nih.gov (B) Lateral cut of an adrenal gland showing location of the medulla and the cortex www.thyroidinstitute.org/images/adrenal_gland. (C,D) Hematoxylin and eosin staining of the adrenal cortex tissue (C) showing that it is separated into three zones; reticularis; fasciculata (zF); and, glomerulosa (D) http://www.ouhsc.edu/histology

1.2. Adrenocortical Steroidogenesis

Steroids are a type of organic compound that contain a core structure of 4 rings (cyclohexanes and one cyclopentane) that are joined to each other. Steroid structures are highly conserved in both the animal and plant kingdoms. Steroid specificity is derived from diverse modifications of the core structure catalyzed by different enzymes. In mammals the core structure is provided by cholesterol.

1.2.1. Synthesis of Adrenal Steroids

In the adrenal gland, cholesterol can be synthesized *de novo* but the majority of the cholesterol comes from plasma lipoproteins which are absorbed by the cells through membrane receptors (*1*). Once inside the cell, cholesterol is modified by cytochromes P450 enzymes. Steroid synthesis reactions are limited by the steroidogenic acute regulatory

protein (StAR) which controls cholesterol translocation to the mitochondrial outer membrane.

Adrenal steroidogenesis for the production of cortisol, aldosterone and sex hormones is depicted in Figure 2 (page 4). The CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1 or P450SC**)** enzyme which is expressed in all adrenal cells transforms the cholesterol to pregnenolone. Pregnenolone is then converted to progesterone or 17α-Hydroxypregnenolone by 3βHSD (3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase) or CYP17 (17α-hydroxylase**)** respectively. Progesterone can also be converted to 17α-hydroxypregnenolone. In the adrenal zona fasciculata and reticularis cortisol and corticosterone are synthesized respectively from 17α-hydroxypregnenolone and progesterone by the enzymes CYP21 (21-hydroxylase) and CYP11B1 (steroid 11β-hydroxylase). The zona glomerulosa lacks the enzymes required for the production of sex hormones, and cortisol. Instead the zona glomerulosa is responsible for the conversion of corticosterone by CYP11B2 (aldosterone synthase) to aldosterone. CYP11B2 is only expressed in the adrenal zona glomerulosa which makes this zone specific for the production of aldosterone (*2*).

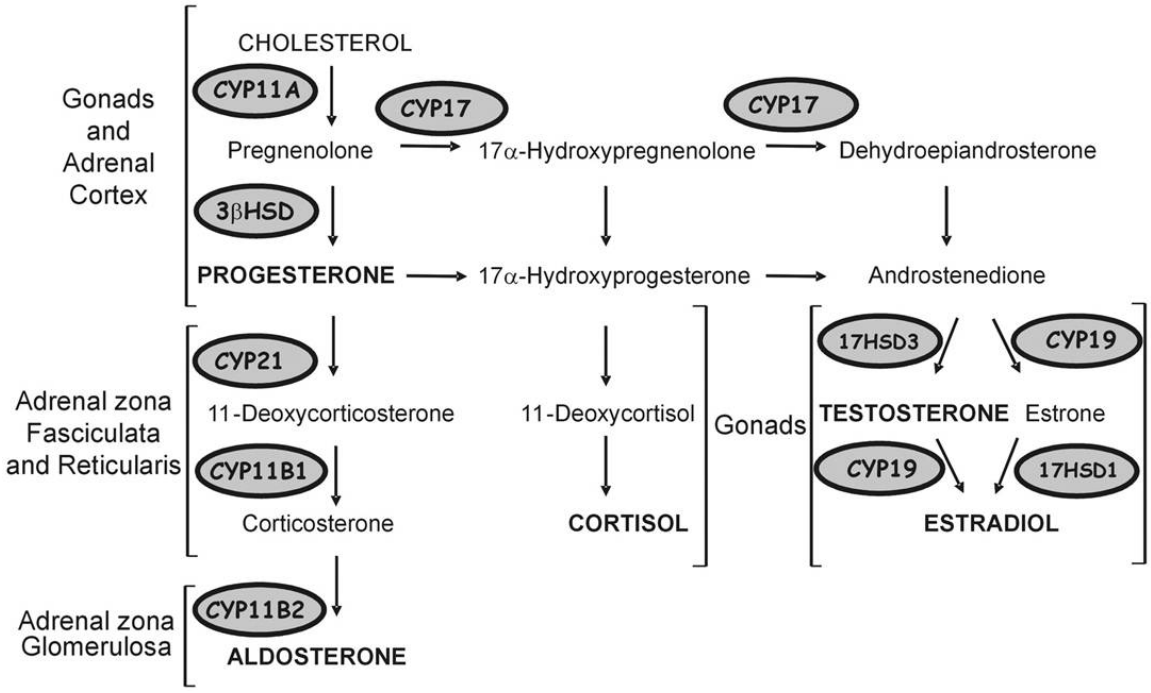

Figure 2: Steroidogenesis pathway in the adrenal gland and gonads. (*2*)

Although most sex hormones are produced by the gonads, the adrenal gland also produces the androgens DHEA, DHEA-S and androstenedione. DHEA is produced by both zona fasciculata and reticularis but only cells of the reticularis express SULT2A1 to produce DHEA-S; the active form of DHEA.

1.2.2. Control of steroid production

Being of pivotal importance to homeostasis, adrenal steroid secretion is under tight control by regulatory pathways. Control of the two main hormones, cortisol and aldosterone will therefore be briefly discussed.

Cortisol, a glucocorticoid hormone is released in response to stress. Its primary functions are to increase blood sugar and glycogen storage in the liver (*3*), aid in fat, protein and carbohydrate metabolism (*4*), and to suppress the immune system (*5*). It is controlled by hypothalamic secretion of corticotropin-releasing hormone (CRH), which in turn

triggers pituitary secretion of Adrenal Corticotrophic Hormone (ACTH); ACTH is carried by the blood to the adrenal cortex where it triggers glucocorticoid secretion. The amount of cortisol produced and secreted in the blood undergoes diurnal variation, with peak production in the early morning and nadir during sleeping hours. Excess cortisol produced by the adrenal gland inhibits further production of CRH and ACTH leading to an inhibitory loop regulation (Figure 3).

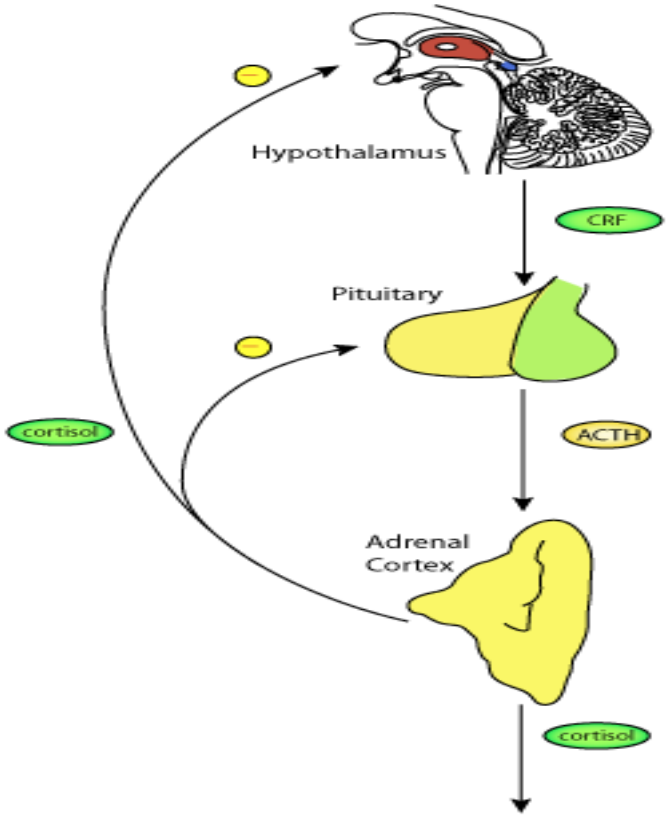

Figure 3: Human cortisol production in the adrenal gland. Neural signals trigger CRF secretion by the hypothalamus. In turn, this signals to the pituitary to release ACTH which stimulates the adrenal gland to release cortisol. Cortisol acts on the hypothalamus and the pituitary to have a negative feedback effect on the release of CRH and ACTH respectively. Figure from http://models.cellml.org

Aldosterone is a mineralocorticoid hormone synthesized exclusively in the adrenal zona glomerulosa. Aldosterone regulates homeostasis by increasing the reabsorption of sodium and water and the secretion of potassium in the kidneys. This increases blood volume leading in part to increased blood pressure. Aldosterone secretion is controlled by the renin-angiotensin-aldosterone system (*6*). In the liver, renin activity (the rate limiting factor of the secretion of aldosterone) leads to the production of angiotensin I. Angiotensin is cleaved by the angiotensin-converting-enzyme to angiotensin II. Angiotensin II increases aldosterone secretion from the zona glomerulosa by increasing transcription of CYP11B2 (*6*). Angiotensin II itself also acts to inhibit renin production creating a negative feedback loop of regulation. ACTH also stimulates aldosterone production indirectly by stimulating the formation of deoxycorticosterone (*7*), a precursor of aldosterone (Figure 2, page 4).

1.3. Adrenal gland development

Adrenocortical cell development in human embryos is due to two independent events. 1) The adrenal cortex arises from adrenogonadal progenitors that first appear in the fourth week of gestation as a thickening of the coelomic epithelium between the urogenital ridge and the dorsal mesentery (*8*). Cells destined to generate the adrenal cortex migrate to the cranial pole of the mesonephros, which creates the foetal adrenal gland by the eighth week of gestation. This fetal adrenal gland contains an inner cluster of large cells, termed the foetal zone (Figure 4, page 7). 2) By week eight (Figure 4, page 7), a second group of cells form a compact outer zone of cells, termed the definitive zone (*9*). Cells in the definitive zone, unlike the foetal zone, lack expression of CYP17 (*9*).

At week nine (Figure 4, page 7), the foetal and definitive zone (foetal adrenal cortex) become encapsulated. The capsule is thought to provide growth factors that are

important in mediating adrenal growth and differentiation, such as insulin like growth factors 1 and 2 (IGF1 and 2) (*9*). Also at nine weeks, neural crest-derived cells migrate into the foetal adrenal cortex and differentiate, hence forming the catecholamine-producing chromaffin cells (*10*), which remain scattered until birth. This differentiation of the neural crest cells is triggered by the glucocorticoids secreted by the cortex (*10*).

At birth the adrenal gland is relatively larger than the adult gland due to the size of the fetal cortex (Figure 4, page 7). Preceding birth, further significant changes of the adrenal gland occurs. First the medulla is formed by compaction of the chromaffin cells (Figure 4, page 7). After three weeks there is full regression of the fetal zone followed by encapsulation of the medulla. Finally, the definitive zone of the human adrenal cortex differentiates into the three zones of the adrenal gland (the outer zona glomerulosa, the middle zona fasciculata, and the inner zona reticularis) (*9*) (Figure 4, page 7). It must also be noted that at this time events controlling adrenal cortex zonation are not fully understood.

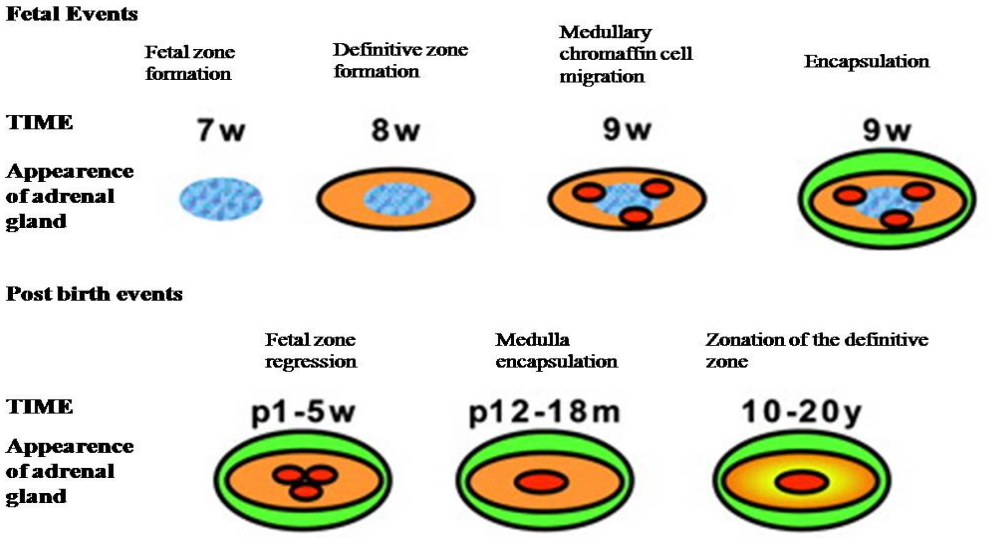

Figure 4: Developmental events of the adrenal gland from embryo to adult. Modified from Hammer, G. D. *et al*.2005 (*11*)

Section 2. Tumours of the adrenal cortex

Adrenocortical adenomas are benign tumours of the adrenal cortex which are extremely common (present in 1-10% of persons at autopsy). They have been detected as incidental findings with increasing frequency in recent years, due to increased use of CT scans and magnetic resonance imaging in a variety of medical settings. As seen in table I, adenomas are the most common of adrenocortical tumours. About 30-50% of adrenal tumors are discovered from endocrine problems (such as in Cushing's syndrome). Other lesions are found by chance with no clinical manifestations and are referred to as incidentalomas. Most of the incidentalomas are benign but about 4% of them are malignant (see table I).

Table I: Classification of adrenal incidentalomas. From a study of patients from le Study Group of the Italian Society of Endocrinology on Adrenal Incidentalomas (*12*)

Non secreting adrenocortical adenomas	74%
Secreting adrenocortical adenomas	
Cortisol-secreting	9,60%
Aldosterone-secreting	1,40%
Pheochromocytomas	4.2%
Adrenocortical carcinomas	4%
Metastases	0,70%
Other lesions	16,40%

2.1. Adrenocortical adenomas

An adenoma is a benign tumour of glandular origin. Adenomas can grow from many organs including the colon, the adrenal glands, the pituitary gland, the thyroid, etc. Although benign, over time they may progress to become malignant in specific tissue such as in the colon, however this malignant transformation has never been described in the adrenal gland. Adrenocortical adenomas have the potential to cause serious health

complications by producing large amounts of hormones in an unregulated, non-feedback-dependent manner. Typically adenomas are well limited, encapsulated nodular lesions of the adrenal cortex ranging from a couple of millimeters to a few centimeters. Histological examination shows that cells in adenomas are similar to those of the normal cortex with high lipid content. Most adenomas are non-functional, meaning that they do not produce any glucocorticoids (cortisol), mineralocorticoids (aldosterone), and/or sex steroids (testoterone, estrogens). In about 15% of cases, adrenocortical adenomas are "functional", and secrete abnormally high levels of the glucocorticoid cortisol or the mineralcorticoid aldosterone which results respectively in endocrine disorders such as Cushing's syndrome and Conn's syndrome (hyperaldosteronism).

2.1.2. Cortisol-secreting adrenocortical adenomas

Overproduction of cortisol is the most common characteristic of functional adrenocortical adenomas. Elevated cortisol levels are the cause of Cushing's syndrome which leads to high blood pressure, diabetes, osteoporosis, skin atrophy, infection and, many other symptoms. In most cases Cushing's syndrome is due to elevated ACTH levels caused by improper functioning of the pituitary gland (pituitary tumour). Elevated ACTH levels can lead to overproduction of cortisol and an enlargement of the adrenal gland (*13*). In other cases, adrenocortical adenomas can produce hypercortisolism independently of ACTH (ACTH-independent Cushing's syndrome) (*14, 15*).

2.1.3. Aldosterone-secreting adenomas

Adenomas, which secrete abnormally high levels of aldosterone cause primary aldosteronism. Hyperaldosteronism can be asymptomatic or associated with hypertension and hypokaliemia (*16*).

2.2. Adrenal Hyperplasias

Adrenal hyperplasias are much less common than adenomas. There are 2 main subtypes of primary bilateral ACTH-independant hyperplasias; 1) ACTH-independent macronodular adrenal hyperplasia (AIMAH) and 2) micronodular adrenal hyperplasia which includes primary pigmented nodular adrenocortical disease (PPNAD). Bilateral adrenal hyperplasias cause approximately 10 to 15% of adrenal Cushing's syndrome (*17, 18*)

2.2.1. ACTH-independent micronodular adrenal hyperplasias and PPNAD

PPNAD is diagnosed mainly in young adults (*19*) and is characterized by normal sized adrenal glands containing several small cortical pigmented nodules (*20*). PPNAD may be isolated or associated with a multiple neoplasia syndrome, Carney complex (CNC), which can include spotty skin pigmentation, heart and skin myxomas and various endocrine tumours. PPNAD is the most frequent endocrine manifestation of Carney complex (*19*). Germline mutations of the *PRKAR1A* gene encoding for the types 1-α regulatory subunit of cAMP dependent protein kinase A are found in more than 50% of cases of CNC and are also frequent in isolated PPNAD (*21-23*). More recently, using a genome-wide scan approach, Horvath et al., identified germline mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in patients with CS secondary to micronodular adrenocortical hyperplasia (*24*).

2.2.2. ACTH-independent macronodular adrenal hyperplasia

AIMAH may rarely develop during the first year of life associated with McCune-Albright syndrome (*25*), but the majority of cases are detected during the fifth or sixth decade of life (*26*). The most frequent presentation of AIMAH is with clinical or

subclinical CS (*18*). Usually, computed tomography reveals clear enlargement of both adrenal glands. Histological examination is characterized by non-pigmented nodules composed of two types of cells, those with clear cytoplasm (lipid-rich) that form cordon nest-like structures, and those with a compact cytoplasm (lipid-poor) that form small nest or island-like structures (*27*).

AIMAH was most often reported as sporadic cases, but there have been increasing reports of familial AIMAH suggesting an autosomal dominant pattern of transmission (*28*). AIMAH may be rarely associated with syndromes where genetic defects have been identified such as MEN 1 (menin), familial adenomatous polyposis (APC) and hereditary leiomyomatosis and renal cell cancer disorder (fumarate hydratase) (*27, 29*). In addition, AIMAH is found in a subgroup of patients with Mc Cune-Albright syndrome, where activating mutations of the Gsα subunit occur in the adrenal gland during embryogenesis and lead to constitutive activation of the cAMP signaling and CS. Fragoso *et al*, identified gsp mutations in 3 of 5 patients with AIMAH and CS without any manifestations of Mc Cune-Albright syndrome (*30*). In a patient with AIMAH an ACTH receptor (MC2R) mutation was identified which lead to impaired desensitization and internalization of the receptor associated to apparent constitutive activity (*31*). Recently, two mutations in the same allele of MC2R was identified in a patient with clinical hypersensitivity to ACTH (*32*).

Although, there is some evidence of cylic AMP-dependent signaling aberrations, no mutations were found in *PRKAR1A* gene in AIMAH (*33*). However, somatic losses of the 17q22-24 region which contains *PRKAR1A* gene was found frequently in AIMAH samples (*33*).

2.2.3. Congenital hyperplasia (CAH)

Congenital hyperplasia (CAH) represents another type of adrenal hyperplasia and is one of the most frequent adrenal disorders observed in children at birth. CAH is characterized by deficiency in 21-hydroxylase expression or other adrenal specific steroidogenic enzymes in less frequent cases. Deficiency in cortisol production leads to excess of ACTH secretion which stimulates adrenal hyperplasia growth (*34*).

2.3. Adrenocortical carcinoma (ACC)

Adrenocortical carcinoma (ACC) is a rare, highly aggressive cancer of adrenal cortical cells, which may occur both in children and adults. ACC may be "functional", producing steroid hormones and consequent endocrine dysfunction similar to that seen in many adrenocortical adenomas, or non-functional. Due to their location deep in the retroperitoneum, most ACCs are not diagnosed until they have grown quite large. They frequently invade large vessels, such as the renal vein. ACC metastasizes via the lymphatics and through the blood, to the lungs and other organs. The overall prognosis of the disease is poor and present treatments are limited leading to poor survival. ACCs like adenomas are often sporadic of origin and can also be associated with familial syndromes like Li-Fraumeni (*35*) and Beckwith-Weidemann (*36*) syndrome. In the recent years, progress to determine key genes and pathways involved in the pathogenesis of ACCs has been slow, in part due to the rarity of these tumours.

2.4. Genetic and pathway alterations of adrenocortical tumours

Genetic alterations either hereditary or sporadic are the key to cancer development. Alterations that affect either tumour supressors or cause overexpression of oncogenes can

cause alterations of signaling pathway, resulting in uncontrolled cell growth and tumour formation. A number of hereditary syndromes with known genetic alterations are associated with adrenocortical tumours (Table II). Interestingly, a number of these genetic alterations may also be found in sporadic adrenocortical tumours like in the *MEN*, *TP53* and *PRKAR1A* genes. The following section will discuss several pathways/genes thought to play a role in adrenal tumour development and progression, summarized in table II.

Table II: Genes /and associate familiale syndromes in adrenocortical tumours.

Gene/Protein	Type	Chromosomal location	Syndromes	Types of adrenal tumours	Familial/ sporadic	References
TP53/p53	Tumour supressor	17p13.1	Li-Fraumeni	ACC	Both	(*37*)
IGF2/IGFII	Growth factor	11p15.5	Beckwith-Weideman syndrome	ACC	Sporadic	(*38, 39*)
MEN1/ Menin	Tumour supressor	11q13	Multiple endocrine neoplasia type 1	ACC/AA/AIMAH	Both	(*40, 41*)
CTNNB1/B-catenin	Transcription factor	3p21	None	ACC/AA/PPNAD	Sporadic	
ras/RAS (HRAS, KRAS,NRAS)	small GTP binding proteins	11p15.5, 12p12.1, 1p13.2	None	ACC/AA (N and K-ras only)	Sporadic	(*42, 43*)
PRKAR1A/ PRKAR1A protein kinase	PKA regulator, Tumour supressor	17q22-24	Carney Complex	PPNAD/AA	Both	(*44, 45*)
PDE11A / Phosphodiesterase 11A *PDE8B*/ Phosphodiesterase 8B	Phosphodiesterase	2q31-35	None	Micronodular adrenal hyperplasias	Familial	(*24, 46*)
GNAS1/GNAS	G protein signaling	20q13.2	McCUne-Albright syndrome	AIMAH	Both	(*30*)

2.4.1. Alterations of *TP53*

The *TP53* tumour supressor gene codes for the protein p53. The p53 protein controls cell proliferation by controlling the G1/S cell cycle check point and can initiate program cell death in presence of DNA damage. *TP53* mutations are highly common in human cancers (*47*). A hereditary germline mutations of TP53 located at 17p13.1 are the underlying genetic abnormality in 70% of Li-Fraumeni cancer syndrome cases (*37*). The Li-Fraumeni syndrome is a syndrome linked to an increased risk of breast cancer, soft tissue sarcoma, brain cancer, leukemia as well as ACC (*48*). ACC associated with Li-

Fraumeni syndrome occurs at an early age (children and young adults). In pediatric ACC, about 50-80% of sporadic cases are also associated with germline *TP53* mutations (*49, 50*). In adults, 1/3 of sporadic ACCs have somatic *TP53* mutations while in adenomas somatic mutations are uncommon suggesting that these alterations happen late in tumourogenesis (*51-54*). Furthermore ACC with somatic but not germline TP53 mutations are associated with poor prognosis and are considered to represent a more malignant phenotype.

2.4.2. Insulin Growth Factor 2 (IGF2)

The IGF pathway involves two ligands (IGF1 and IGF2), two receptors (IGFR1 and 2) as well as 6 IGF binding proteins (1 to 6)(*55*). IGF-2 is expressed during the embryonic and foetal stages of adrenal development and contributes to growth and differentiation, whereas expression is kept low in the adult adrenal (*56-58*). IGF-I and IGF-II, can both act as adrenocortical mitogens by promoting progression through the G1/S phase of the cell cycle via activation of phosphatidylinositol 3-kinase (PI3K) and the AKT protein kinase (*59, 60*).

Transcriptional analysis by microarray technology has shown *IGF2* as the most over-expressed gene in human ACC compared with adrenocortical adenomas or normal tissues (*61-63*). IGF1 has not been observed to be overexpressed in ACC (*38*). In human ACC alterations at chromosome 11p15 by genetic or epigenetic, are present in about 90% of cases (*64, 65*) and this is believed to be the cause of overexpressed IGF2.

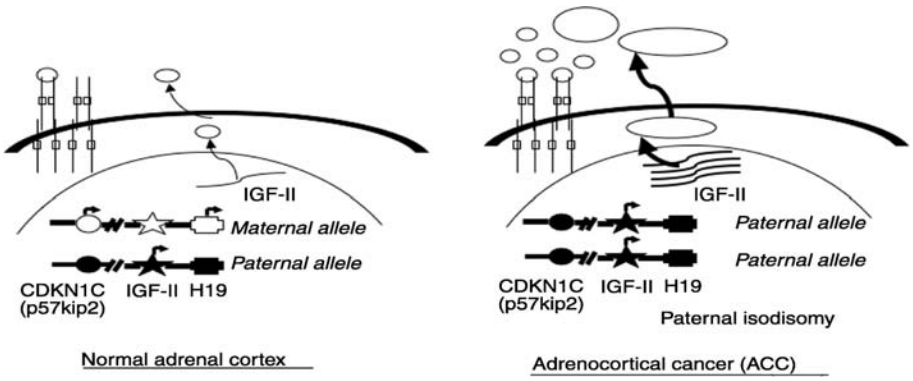

Figure 5 : Alterations of 11p15 locus and IGF-II overexpression in ACC. The imprinted 11p15 locus contains the *CDKN1C* (p57kip2), *IGF-II,* and *H19* genes. In normal differentiated tissue (on the left), only the paternal allele of the *IGF-II* gene is expressed, whereas only the maternal alleles of *CDKN1C* and *H19* are expressed. Paternal isodisomy is usually observed in adrenal cancers (on the right) with loss of the maternal allele at 11p15. This leads to the overexpression of IGF-II and decreased expression of *CDKN1C* and *H19.* Modified from Libe *et al* . 2007(*65*)

The *IGF2* locus (11p15) (Figure 5) is parentally imprinted, and normally only the paternal allele is expressed (*66*). Two other imprinted genes in this region, *H19* and *p57KIP2*, are expressed only from the maternal allele. The H19 mRNA is not translated and serves to inhibit *IGF2*expression. The *p57KIP2* gene encodes a cyclin-dependent kinase inhibitor involved in the G1/ S phase of the cell cycle. Mutation and/or aberrant expression of imprinted genes in the *IGF2* locus causes Beckwith-Wiedemann syndrome (BWS), a syndrome associated with adrenal hyperplasia and ACC (*67*).

Due to the significance and frequency of IGF-II upregulation in sporadic ACC, this growth factor and its receptors are considered candidates for molecularly targeted drug therapy. Overexpression of the IGF-1 receptor (IGF1R) has also been observed in ACC (*39, 68*). IGF1R antagonists cause growth inhibition *in vitro* and of human ACC xenografts in nude mice (*60*). Early clinical trials with humanized IGF1R antibodies have also shown to be promising in PHASE1 trials for ACC treatment (*69*)

2.4.3. *PRKAR1A* and other cAMP pathway alterations

The regulatory R1A subunit of protein kinase A (PRKAR1A) is the main regulator of protein kinase A (PKA) activity and a key component of the cAMP signaling pathway that has been the subject of many studies in endocrine tumourigenesis (*70, 71*). The *PRKAR1A* gene is located at the 17q22–24 locus and is, as previously mentioned implicated in the Carney complex (CNC; (*21, 23*) and PPNAD (*44, 45*)). Heterozygous inactivating germline mutations of *PRKAR1A* have been demonstrated in about 45 to 65% from two studies in CNC families (*23*), (*72*). Somatic *PRKAR1A* mutations have also been found in sporadic secreting adrenocortical adenomas, with similar characteristics to those of PPNAD (*73*). Mutations of *PRKAR1A* are not found in ACC although LOH at 17q is found in adenomas. In adenomas, LOH is restricted to the *PRKAR1A* locus (17q22–24) but in ACC LOH effect a larger area of 17q (*65*). This suggests that *PRKAR1A* alterations may play a role at the early onset of tumoural growth but that this effect is minimized in malignant growth.

More recently inactivation germline mutations in two phosphodiesterases (PDE) genes *PDE11A* and *PDE8B* have been found (*24, 46*) in adrenal micronodular adrenal hyperplasias. *PDE11A* is a dual specificity PDE which catalyzes hydrolysis of cAMP and cGMP (*74*). Of the four known isoforms of *PDE11A* (A1 to A4) only A4 is reported to be expressed in the adrenal gland (*74*). *PDE11A* is located at 2q31-35 and fluorescent in situ hybridization (FISH) studies of tumours have demonstrated 2q allelic loss in patients with *PDE11A* inactivating mutations. Strangely, inactivation mutations of *PDE11A* are also found in the general population although at a lower frequency. This suggests low penetrence of *PDE11A* mutations and that other PDE's must be present in the adrenal gland to compensate for the lack of PDE11A activity.

The *PDE8B* gene codes for the PDE with the highest cAMP affinity (*75*). It is highly expressed in the adrenal gland and is harboured in the 5q13 locus, a region thought to be associated with micronodular adrenocortical disease (MAD)(*46*). In HEK293 cells, cAMP levels are significantly increased following transfection with mutant *PDE8B* (*76*).

Another type of cAMP pathway alteration involves activating somatic mutations in the gene coding for *GNAS* (*Gsα*). GNAS is a member of the G protein family, which form a heterotrimers with β, γ subunits that participate in signal transduction. GNAS has intrinsic GTpase activity and stimulates cAMP production (*77*). Mutants found of *GNAS,* prevent downregulation of cAMP signaling (*27*). *GNAS* mutations are associated with AIMAH and the McCune-Albright syndrome (MAS) which is primarily associated with fibrous dysplasias (*78*).

Up to date, alterations in multiple players of cAMP pathway leading to increased cAMP signaling appear to be the main cause of nodular adrenocortical disease. Clearly the adrenal gland seems highly susceptible to changes in the cAMP pathway with adrenal hyperplasias being the most predominant clinical manifestation in humans with germline mutations in *PDE11A, PDE8B* and *PRKAR1A*. In contrast, cAMP pathway alterations are not found in ACC and thus are not believed to lead to malignant growth, but may be associated with early tumor formation.

2.4.4. Ras pathway alterations

Ras proteins are membrane associated small GTPases proteins and are referred to molecular switches in signaling pathways that control proliferation, differentiation, death and mobility. The three ras proteins (H, N, and K) are the most commonly mutated oncogenes in human cancers (*79*). Controversial data is present in the literature; Lin et al, (*42*) found K-ras mutations in about 50% of tumoural tissues of Conn's adenomas and

Yashiro *et. al*, who found N-ras mutations in 7 of 56 (12.5%) of all tumours tested (3 of 24 (12.5%) carcinomas and 4 of 32 (12.5%) adenomas (Yashiro et al 1994), while Moul *et al.* (*80*) and Ocker *et al.*(*81*) did not identify any Ras mutations. In a more recent study K-ras and N-ras mutations were each found in one out of 35 ACCs (*43*). In this study, 2 (5.7%) and 4 ACCs (11.4%) were also found to carry BRAF and EGFR TK domain mutations respectively (*43*). K-ras mutation found by Lin *et al*. were active in ACC (Lin, Hsu et al. 2000) and lead to increases in steroidogenic enzyme expression and an increase in cortisol production when transfected into normal adrenal cells (*82*). These data suggest that EGFR-Ras-Raf-MEK-ERK pathway alterations are present in only a subset of ACC.

2.4.5. *MEN 1*

Adrenal tumours are also associated with mutations in the *MEN 1* gene which codes for the Menin protein. A heterozygous inactivating germline mutation of *MEN 1* can be found in about 90% of families affected by multiple endocrine neoplasia type 1 (MEN 1). Menin Endocrine Neoplasia type 1 (MEN1) is a rare autosomal dominant hereditary cancer syndrome presented mostly by tumors of the parathyroids, endocrine pancreas and anterior pituitary. About 25–40% of MEN 1 patients are affected by adrenocortical tumours and/or hyperplasias (*41, 83*). In most cases, these tumours are non-functional adenomas and are rarely seen in ACC. Somatic mutations in the *MEN 1* gene are known but are very rare in all adrenocortical tumours (*40, 83*). The 11q13 locus containing the *MEN1* gene frequently losses heterozygosity at a greater frequency in ACC's (90%) compared to adenomas (20%), however expression of the *MEN1* gene does not differ between them (*83*), suggesting that the *MEN1* gene does not play a role in the progression of sporadic adrenal tumours.

2.5. Adrenocortical tumourigenesis

2.5.1. Clonality of adrenocortical tumours

Determining tumour clonality is important to establish the origin as well as the mechanisms dictating tumour progression. Mono-clonality indicates that tumour progression is the end result of a or multiple genetic mutations, whereas polyclonality suggests that tumour cells are affected by local or systemic stimuli (*59*). Three X-chromosome inactivation studies have up to date been performed to determine the clonality of adrenocortical tumours. All three studies are in agreement and show that ACC consists of monoclonal populations of cells, adenomas tumours are both monoclonal as well as polyclonal (*64, 84, 85*). Hyperplasias on the other hand are only polyclonal (*84*). Variation observed in adenomas tumours may be explained by different mechanisms or are due to observation of a multiple steps, within a common mechanism. These observations have been suggestive of a multistep (clonal) genetic model of adrenocortical tumour development.

2.5.2. Models of adrenocortical tumour development

Unlike other cancers, the factors and steps leading to adrenocortical tumour development is still under debate. In colon cancer progression of adenoma to carcinoma was proposed, however this sequence is not recognized in adrenocortical tumourogenesis.

Adrenocortical tumours comparative genomic hybridization (CGH) studies have shown a positive correlation between adrenocortical tumour size and the number of chromosomal alterations, supporting the hypothesis that chromosomal changes accumulate during tumour progression (*65, 86*). In benign lesions the average number of chromosomal

aberrations per tumour cell is low (*87*), with chromosomal gains occurring often of 9q (14%-22%) (*87*) (*88*) and 17q (26-35%) (*88*). Chromosomal losses also occur, although less often, with the most common being at chromosomal 1p (5%)(*87*) or 6q (9%) (*88*). In hyperplasias gain of complete or partial chromosome 17q is the most common chromosomal alteration. In hyperplasias similar chromosomal abnormalities to those in adenomas (e.i., gain of chromosome 17q), provides evidence that hyperplasias may evolve into adenomas (*88*). The hyperplasia-adenoma transition is also supported by clinical observation of adenomas presence within hyperplasias like PPNAD (*89*). In contrasts to benign adrenocortical neoplasms multiple chromosomal alterations in nearly all ACCs is observed (*86-88, 90*). In addition to deletions or amplifications, a high percentage of ACC cells exhibit loss of heterozygosity (LOH) at key loci which can result in an altered TP53 pathway of unipaternal disomy (UPD) causing IGF2 overexpresssion.

In adrenocortical tumourogenesis, only β-catenin mutations are found commonly in both benign and malignant neoplasm. In contrast, cAMP pathway genetic alterations are present in adrenocortical hyperplasias and some adenomas while the presence of IGF2 locus alteration is more specific to ACCs. In addition to these differing genetic alterations, clinically no clear progression of adenomas or hyperplasias to ACC has been observed. These facts support that different mechanisms lead to adrenocortical adenoma or ACC formation and that adenoma do not progress to malignant tumours.

On the other hand, some evidence refutes the latter. In mice model, overexpression of IGF2 does not lead to tumour formation (*68, 91*), suggesting that it plays a role later in tumourogenesis and serves as a pro-proliferative factor and not as an initiating element. Although this remains to be proven in humans, it suggests that IGF2 overexpression which is seen in the majority of ACCs does not causes tumour formation and that other genetic

alterations are involved in ACC formation. As mentioned earlier, somatic mutation of TP53 genes are found only rarely in non malignant adrenocortical neoplasm suggesting that these mutations may be gained during tumour progression and are not initiating events. Clonality status of adrenocortical tumours also suggests a multi-stage process of adrenocortical development. Finally, a case report described in 2003 supports the hypothesis of multistep adrenocortical tumourigenesis where an adrenocortical tumour had a central component of ACC surrounded by benign adrenocortical tissue (*92*). Unfortunately, clonal studies to determine if both parts originated from the same population of cells were not possible since the patient was a male. Although this is not proof of a multistep model it does suggest that a multistage process can occur, though rarely.

Section 3. Wnt/β-catenin signaling

3.1 Wnt signaling pathways

Wnt signaling is a highly complex and versatile process leading to a multitude of cellular effects. Wnt signaling is driven by a family of secreted glycoprotein called WNTS. Variability of Wnt effects are in part caused by the 19 different Wnt proteins present in humans. Wnt's activate Wnt signaling by interaction with the frizzled family of membrane receptors (FZD1-10) which then activate dishevelled proteins (DVL1-3) to activate downstream events. There are three branches to the Wnt signaling pathway: 1) the canonical or Wnt/β-catenin pathway, 2) the non-canonical or the planar cell polarity pathway and the 3) Wnt/Ca^{2+} pathway. The canonical pathway involves stabilization of β-catenin leading to transcription activation of target genes while the other two pathways exert their effect independently of β-catenin. Since our studies involved Wnt/β-catenin, we will only briefly discuss the other two pathways.

3.1.1 Non-canonical or planar cell polarity (PCP) signaling

In PCP signaling (Figure 6, page 23), Wnt signaling is transduced through Frizzled independent of LPR5/6 (LOW density lipoprotein receptor related protein) like in the canonical pathway. Utilizing the PDZ and DEP protein domains of DVL, this pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac instead of leading to β-catenin stabilization. Hence, the PCP signaling pathway controls tissue polarity and cell migration/movement. Aberrant activation of Wnt/PCP signaling pathway in human cancer (i.e.WNT5a overexpression) leads to more malignant phenotypes (*93*). This pathway is also involved in organ development (*94*).

3.1.2. The Wnt-Ca^{2+} pathway

In the Wnt-Ca^{2+} pathway (Figure 6), Wnt signaling via Frizzled mediates activation of heterotrimeric G-proteins, which engage DVL, phospholipase C (PLC; not shown), calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC), which modulates cell adhesion and motility (*95*).

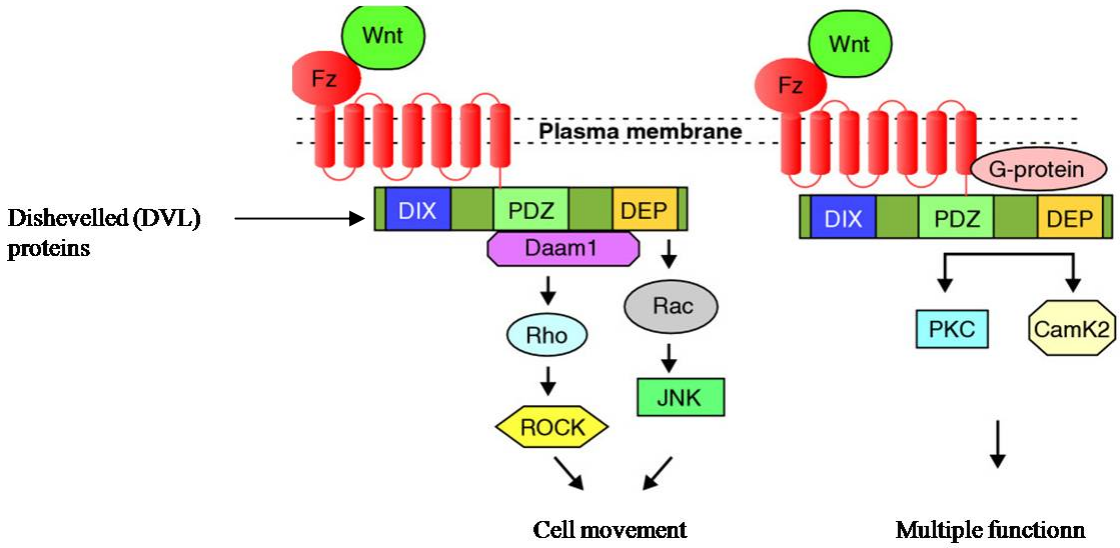

Figure 6: Wnt pathways independant of β-Catenin. On the left, planar cell polarity signaling. On the right, WNT Ca^{2+} signaling.(*95*)

3.1.3. The Wnt/ β-catenin pathway

This pathway is commonly referred to as either being on (Figure 7-IB page 25), or off (Figure 7-IA, page 25). Normally the pathway is off, and cytosolic β-catenin is constitutively degraded by the cytoplasmic degradation complex, which comprises axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and casein kinase1 (CK1). This degradation complex triggers SCF (SKP1, Cullin, F-box)/β-TrCP-mediated polyubiquitylation leading to proteosomal degradation of β-catenin.

The events are triggered through phosphorylation control of the of β-catenin NH2 terminus by multiple kinases (depicted on Figure 7II page 25). Initially β-catenin is bound by an Axin: APC complex. APC and Axin can be hyperphosphorylated by CK1α and GSK3β which increase the affinity to bind β-catenin. Once bound Axin recruits CK1α to the complex to phosphorylate β-catenin at serine 45 (S45). This acts a primer for further phosphorylation events by GSK3β at residues S37, S33, and T41. Phosphorylation of all four sites at the NH2 terminus are essential for proper degradation of β-catenin. APC, which acts as a scaffold like Axin, may also cleave the NH2 phosphorylated end and transfer β‑catenin to proteosomal complex (*96*). Ultimately the destruction complex maintains low levels of β-catenin when no activation signal is present.

Upon activation, WNTs interacts with Frizzled receptors and the co-receptor LRP5/6. DVL proteins are activated and cause dissociation of the destruction complex and thus β-catenin stabilization. CK1 also synergizes with DVL to mediate this event (*97*). CK2 another kinase also seems required to stabilize β-catenin by phosphorylation at residue T393 to inhibit the axin: β-catenin interaction (*98*). Upon stabilization β-catenin enters the nucleus and acts as a transcriptional activator via interaction of with T-cell transcription factor family members (Figure 7I, page 25).

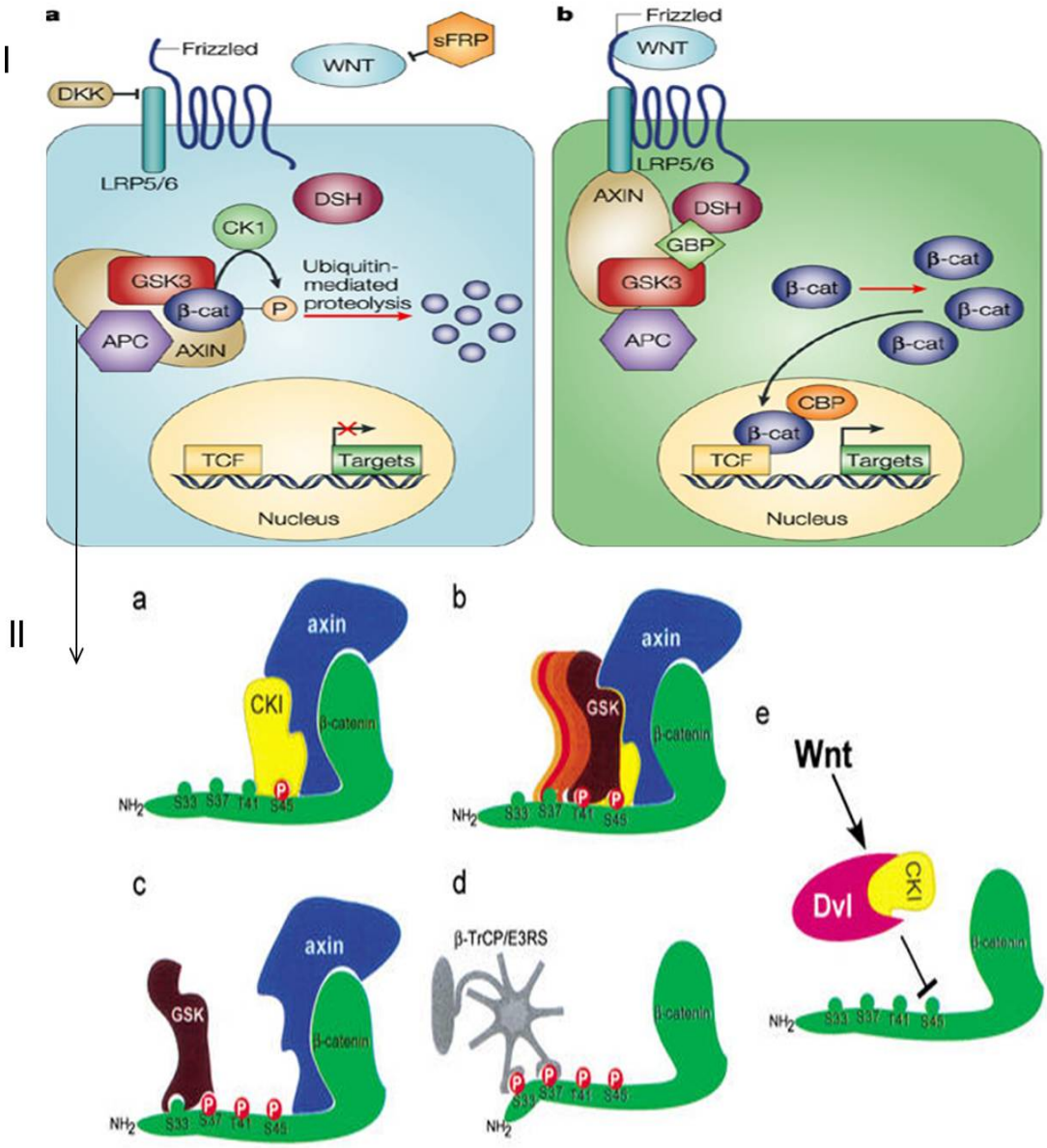

Figure 7: (I) Wnt/β-catenin pathway. a) When pathway is off β-catenins is recruited to the destruction complex and targeted for degradation by ubiquitin mediated proteolysis. b) When pathway is activated via WNT interaction with frizzled recpetors, DSH/Dvl proteins interaction with the frizzled receptors and inhibit recrutement of β-catenin to the destruction complex which is termed β-catenin stabilization or active β-catenin. β-catenin is then exported to the nucleus and interacts with the TCF transcription factor and activates transcription. **(II) A model depicting the β-catenin phosphorylation–degradation cascade by the destruction complex.** (*a*) Axin recruits CKI to phosphorylate β-catenin at S45. (*b,c*) S45 phosphorylation primes β-catenin for the succeeding GSK-3β phosphorylation cascade, finally hitting the S33/37 sites. (*d*) Phosphorylation at S33/37 creates a docking site for β-TrCP/E3RS, promoting the ubiquitination and degradation of β-catenin. (*e*) Wnt signaling, possibly through Dvl and CKI, regulates S45 phosphorylation. Figure from Amit et al. (*99*)

3.2. β-catenin

In humans β-catenin is a protein encoded by the *CTNNB1* gene. β-catenin is a member of the armadillo family of proteins. These proteins have multiple copies of the so-called armadillo repeat domain which is specialized for protein-protein binding.

As mentioned previously, β-catenin is the centre player of the Wnt/β-catenin pathway. β-catenin itself exists in three states within the cell (Figure 8). 1) Complexed within the destruction complex. 2) Complexed with E-cadherin and other proteins that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. 3) In an active state (unphosphorylated at S45, T41, S37, S33) where it is either in the cytoplasm or the nucleus bound to transcription factors such as TCF/LEF (Figure 8) and SF1. Proper control of β-catenin levels and its state is essential for development and maintaining homeostasis. Improper control can lead to disease such as cancer (see Section 5).

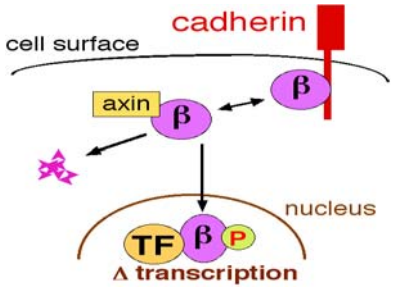

Figure 8: The 3 cellular states of β-catenin. Within the cell β-catenin (β) exist within three states; bound by Cadherins (top right), complexed with the destruction complex (axin only is shown here, middle), or in the nucleus. TF= TCF; β= β-catenin; P= phosphorylated residues.

3.2.1. β-catenin in the nucleus

β-catenin has been involved in the transcriptional control of a multitude of genes mainly by co-activating gene transcription with the T-cell specific transcription factor (TCF) family of transcription factors (TCF-1, LEF-1(formally TCF-2), TCF-3, and TCF4 (TCF7L2). What is of great interest is that activation of genes by β-catenin/TCF is usually cell and context specific. TCF proteins contain a DNA binding domain but no trans-activation domain and can therefore not drive transcription by itself. TCF serves a dual role as both a repressor and activator. When unbound to β-catenin, TCF is still found bound to WRE (WNT responsive elements)/TBE's (TCF binding elements) were it can act as a repressor upon interaction with the transcriptional repressor Groucho (Figure 9, left panel). When β-catenin enters the nucleus, it replaces Groucho and binds TCF. β-catenin can also interact with TCF that is yet unbound to DNA which then leads to DNA binding (Figure 9 right panel).

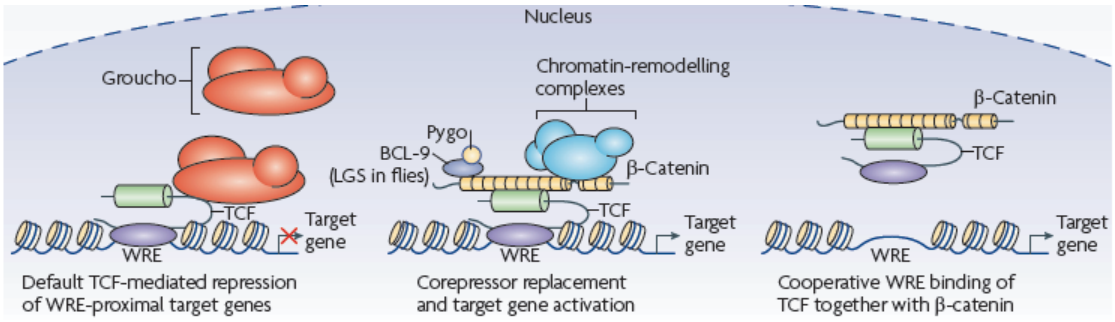

Figure 9: β-catenin dependent gene transcription. This pathway centres on β-catenin, which, together with the DNA-binding T cell factor/lymphoid enhancer factor (TCF/LEF) family proteins, functions as a transcription factor to control Wnt target genes. A subset of these target genes are constitutively inhibited by pioneering nuclear TCF, which recruits transcriptional corepressors (left panel) to Wnt response elements (WREs). Once β-catenin is exported the nucleus it replaces TCF-bound corepressors (such as groucho; middle panel) or co-imports additional TCF to occupy WREs (right panel). Once bound to WREs through TCF, β-catenin functions as a scaffold to recruit an auxiliary machinery of co-activators that are involved in chromatin remodelling and control of RNA polymerase II to induce Wnt target gene expression.(*100*).

It is yet unclear if these two methods for the activation of β-catenin/TCF transcription are gene specific of or if both can occur for the expression of the same gene. Once bound to WREs through TCF, β-catenin functions as a scaffold to recruit an auxiliary machinery of co-activators that are involved in chromatin remodelling (i.e. BRG-1(*101*)) and control of RNA polymerase II to induce Wnt target gene expression. Regulation of β-catenin activity is quite complex inside the nucleus. In fact, there are over 25 non transcription factor proteins that are known to interact with β-catenin that act as either antagonist or agonist of its transcriptional activity (*102*). This supports the hypothesis that nuclear accumulation of β-catenin alone is not sufficient to state activity. Transcriptional activity hence, what genes are expressed is affected by the variation of co-factors present.

3.2.2. Regulation of β-catenin target genes

Multiple genes are known to be directly regulated by β-catenin/TCF (Table 3, page 29) and the list keeps growing. Most genes regulated by β-catenin/TCF were discovered in colon cancer cells. Two of the most studied genes in relation to β-catenin/TCF regulation have been *CCND1* (CyclinD1) and *AXIN2*. *CCND1* regulation by β-catenin/TCF was originally proven by reporter assays (*103*). Its regulation by β-catenin/TCF in colon has since been refuted as CyclinD1 reporter constructs do not appear to reflect endogenous gene regulation (*104*). It is still considered a target of β-catenin/TCF activity although caution should be warranted for its use as a marker of β-catenin/TCF activity.

One of the highly important genes regulated by β-catenin/TCF is *AXIN2*. Similarly to Axin1, the Axin2 protein reduces β-catenin stability leading to a feedback inhibition on Wnt-signaling (*105*). *AXIN2* contains the most known TCF sites (6 in the first intron and 2 in the 5'flanking site) (*105*) and is suggested by leaders in the WNT field, to be the most

conserved gene to determine activation of Wnt/β-catenin signaling between different cell types and signals (*100, 105*). Its regulation by β-catenin/TCF differs in that it contains TCF binding sequences (n=6) within the first intron, while other genes, *CCND1, MYC* and *ENC1* which are likely to be non universal targets, contain sites only within the 5' flanking region of the transcriptional start site (*103, 106, 107*).

Table III: Genes that are upregulated directly by β-catenin/TCF in Humans

Genes	Tissues/cell lines	References
c-myc	Colon cancer	He, Sparks et al. 1998)(*106*)
Cyclin D	Colon cancer	Tetsu and McCormick 1999) (*103*)
Tcf-1	Colon cancer	Tetsu and McCormick 1999 (*103*)
PPARdelta	Colon cancer	He, Chan et al. 1999(*108*)
c-jun	Colon cancer	Mann, Gelos et al. 1999 (*108*)
fra-1	Colon cancer	Mann, Gelos et al. 1999) (*108*)
MMP-7	Colon cancer	Brabletz, Jung et al. 1999 (*109*)
CD44	Colon cancer	Wielenga, Smits et al. 1999 (*110*)
Gastrin	Colon cancer	Koh, Bulitta et al. 2000 (*111*)
ENC-1	Colon cancer	Fujita, Furukawa et al. 2001 (*107*)
Claudin-1	Colon cancer	Miwa, Furuse et al. 2001) (*112*)
Id2	colon cancer	Rockman, Currie et al. 2001 (*113*)
Axin-2	Colon cancer	Yan, Wiesmann et al. 2001; Lustig, Jerchow et al. 2002 (*105, 114*)
ITF-2	Colon cancer	Kolligs, Nieman et al. 2002 (*115*)
Nr-CAM	Colon cancer	Conacci-Sorrell, Ben-Yedidia et al. 2002 (*116*)
BMP4	Colon cancer	Kim, Crooks et al. 2002 (*117*)
Frizzled 7	EC cells	Willert, Epping et al. 2002 (*118*)
LEF1	Colon cancer	Filali, Cheng et al. 2002 (*119*)
Follistatin	EC cells, ovary	Willert, Epping et al. 2002 (*118*)
FGF18	Colon cancer	Shimokawa, Furukawa et al. 2003 (*120*)
c-myc binding protein	Colon cancer	Jung and Kim 2005 (*121*)
LGR5/GPR49	Intestine	Barker, van Es et al. 2007 (*122*)

Gene list was obtained from http://www.stanford.edu/~rnusse/wntwindow.html (WNT homepage). Only genes with substantial evidence for implication of β-catenin in their transcription are described.

It is now well recognized that β-catenin target genes are cell specific. The use of microarray technology for transcriptome studies highly strengthens this point. Microarray

studies comparing gene expression profile of wild-type and tissues carrying β-catenin mutations in Wilms tumours (*123, 124*) and ovarian endometrioid adenocarcinomas (*125*) have shown a very different list of deregulated genes in comparison to table III (page 29). Two different studies of Wilms tumours (*123, 124*) even showed significant differences despite large cohorts studied (n=36, n=73 respectively).

Transcriptome studies have also highlighted differences in response to WNT molecules from different cells. In a study by Railo *et al.* (*126*), it was shown that upon WNT3a stimulation in mice NIH3T3 and rat PC12, only the expression of two genes were commonly activated and that only one (Disabled-2) was a direct target. The common target cyclinD1 (*CCND1*) was not observed to be activated by WNT3a in this study although cyclinD1 upregulation by WNT3A was demonstrated in human embryonic carcinoma cells (*118*). In another study with mice immature $CD34^+$ thymocytes (*127*), only four genes were commonly activated between three methods to activate Wnt signaling; WNT3a stimulation, expression of a constitutive β-catenin mutant and inhibition of GSK3-β by lithium. This demonstrated that different methods of β-catenin activation lead to unique transcriptional effects. There are presently, three types of control of β-catenin gene expression, hypothesized.

1) Alternative isoforms of TCF: Alternative splicing of TCF7L2 (TCF4) often occurs and as shown in neonatal mice tissues these various transcripts lead to different gene promoter activation (*128*). In the mice developing pituitary gland, a TCF variant without DNA binding domain (TCF-4N) is expressed and reduces transcription of TCF/LEF dependent promoters by binding to β-catenin. Furthermore, TCF-4N redirects β-catenin, to activate non TCF/LEF dependent promoters by interaction with SF1 and the adipogenic transcription factor CCAAT/enhancer binding protein α (C/EBPα) (*129*). What controls the

expression of variants involved remains unknown, but it does appear that variants of TCF can be expressed at key times especially during developing to alter β-catenin target genes.

2) Nuclear antagonist: There exists several antagonist of β-catenin TCF transcriptional activity such as Chibby and ICAT proteins that appear to have a dedicated role in disrupting β-catenin interactions with TCF and promoting its nuclear export (*130, 131*). Other transcription factors such as members of the FOXO family or the vitamin D receptor can bind β-catenin which consequently inhibits β-catenin/TCF driven transcription (*132, 133*). For more details on β-catenin interaction with transcription factors, please see section 3.2.3.

3) Post translation modification of TCF. Post translation modification such as acetylation, phosphorylation, sumoylation, and ubiquitination/degradation of TCF family members can also occur and can either alter β-catenin/TCF transcription in an agonistic or antagonistic fashion (*134, 135*).

These factors are in part responsible for the variations of genes expression mediated by β-catenin but these factors cannot fully explain the observation made. Cell specific factors determining the variability seen in β-catenin target genes remain to be determined.

3.2.3. β-catenin transcription factors and alternative activation

Throughout literature the main interest in β-catenin driven gene transcription is with the transcription factor TCF/LEF. It is now clear that β-catenin can interact with a multitude of other transcription factors. Therefore, TCF proteins compete with several other transcription factors to bind to the limited pool of β-catenin in the nucleus (table IV).

Further regulatory pathways must be present in order to maintain balance, as interactions of β-catenin with other transcription factors leads to quite specific effects. Regulation of these pathways is not fully understood and beyond the scope of this review, but it is important to highlight that transcription factors other than TCF may interact with β-catenin (Table IV) to understand the complexities of β-catenin's roles.

Table IV: Transcription factors other than TCF family members that may use β-catenin as a co-activator or co-repressor.

Transcriptional Factors	Effect on β-catenin/ TCF	Role of β-catenin	References
AR(androgen receptor)	-	Enhances AR transcriptional activity in prostate cancer in response to androgens	Truica, Byers et al. 2000 (*136*); Yang, Li et al. 2002 (*137*)
ERα	+	Cross-talk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and Erα	Kouzmenko, Takeyama et al. 2004 (*138*)
Foxo3a, Foxo1,Foxo4	-	Stress response	Essers, de Vries-Smits et al. 2005 (*139*); Hoogeboom, Essers et al. 2008 (*132*)
HIF1a	-	Enhance HIF-1-mediated transcription, promoting cell survival and adaptation to hypoxia	Kaidi, Williams et al. 2007 (*140*)
c-Jun	+	Tumour development. c-Jun and TCF4 cooperatively activated the c-jun promoter in reporter assays in a β-catenin-dependent manner.	Nateri, Spencer-Dene et al. 2005 (*141*)
Mitf	-	Self-renewal and maintenance of melanocyte stem cells, Activate transcription of Mitf-specific target promoters.	Schepsky, Bruser et al. 2006 (*142*)
MyoD	UNK	Essentiel for MyoD transcription which is a Transcription factor essential for muscle differentiation	Kim, Neiswender et al. 2008 (*143*)
p50	UNK	Inflammatory response. Acts with p50 to activate CRP expression	Choi, Hur et al. 2007(*144*)
Pitx2	+	Activation of specific cell cycle gene . Activates CylinD1 and cyclinD2 with and without LEF respectively	Kioussi, Briata et al. 2002 (*145*)
Prop1	+ and -	Activates PIT1 (critical lineage-determining transcription factor,) expression, Supresses Hesx1 (lineage-inhibiting transcription factor)	Olson, Tollkuhn et al. 2006 (*146*)

RAR	-	The activity of RA on RAR-responsive promoters was also potentiated by β-catenin. RA influences development, cell differentiation and cancer	Easwaran, Pishvaian et al. 1999 (*147*)
Runx2	-	Control development and maturation. Enhances LEF1-dependent repression of Runx2	Kahler and Westendorf 2003 (*148*)
Sox9	-	Control of chondrocyte differentiation	Akiyama, Lyons et al. 2004 (*149*)
VDR vitamine D receptor	-	Enhances VDR activity. Involved in differentiation.	Palmer, Gonzalez-Sancho et al. 2001 (*133*)
Steroidogenic factor 1 (SF1)	UNK	Hormone producing cells only, Synergies SF1 driven expression of StAR	(Hossain and Saunders 2003 (*150*); Kim, Reuter et al. 2008 (*151*)

Alternative transcription factors (non TCF) that are associated with β-catenin can be divided into two main groups. 1) Factors that lead to redirection of β-catenin resulting in decreased β-catenin/TCF transcription such as RAR, FOXO and AR. 2). Factors leading to increased β-catenin/TCF transcription such as c-jun, SF1, and PITX2. Both groups of transcription factors also lead to activation of other genes which are not direct TCF targets. Note that the interaction with SF1 has been observed with both decrease and increase in β-catenin/TCF transcription (*150, 151*).

Section 4. WNT implications in normal adrenal function and development

4.1. WNT in the adult adrenal gland

Wnt/β-catenin signaling has been poorly studied in the normal adrenal gland function with little to no direct studies of the players involved in this pathway. In the normal adrenal gland β-catenin the central player of the pathway is restricted in location and activity to the outer cortex (capsule and ZG) (*152-154*). In 2003, Suwa *et al*., reported that DKK3 an agonist of WNT pathway and WNT4 expression was much higher in the ZF/ZG cells then in ZR cells (*155*). Further study of Wnt pathway demonstrated that FZD1,2, DVL 3 were also expressed in the adrenal cortex but other WNTs, FZDs, and DVL were not expressed at the mRNA levels. WNT4 is known to be a non transforming WNT (*156*) which means that it does lead to accumulation of β-catenin. In fact it has been recently shown that WNT4 redirects β-catenin to the cell membrane and thus inhibits β-catenin/TCF transcriptional activity (*157*). In human pituitary adenomas, WNT4 plays a role in cell survival/proliferation independently of β-catenin (*158*). This suggests that WNT4 expression may help to reduce levels of β-catenin transcriptional activity in the adrenal gland even upon activation by other WNT ligands. Furthermore, *in vitro* studies have demonstrated that WNT4 antagonizes the interaction between SF-1 and β-catenin which halts the synergistic activation of the StAR (*159*). In normal adrenal glands, it would thus appear that basal Wnt/β-catenin signaling is quite low but that the adrenal cortex cells can respond to WNT signals due to expression or the Wnt/β-catenin pathway proteins. In adults, adrenal cortex effects of WNT signaling and β-catenin driven transcription are largely unknown, however some studies have linked this pathway to steroidogenesis both in a canonical and non canonical fashion (*151, 160-162*).

4.2. WNT signaling in adrenal steroidogenesis

Wnt signaling also plays a role in adrenal steroidogenesis. Of the WNTs expressed in the adrenal gland, WNT4 has been linked to aldosterone synthesis. The initial evidence of involvement of WNT4 in adrenal glands was demonstrated by knock out studies of WNT4 that lead to a normally apparent adrenal gland (size and shape) but with an abnormal ZG associated with lower aldosterone levels (*160*). WNT4 overexpression in primary adrenocortical cells, stimulates production of aldosterone by increasing transcriptional levels of the enzymes CYP17, CYP21 (in a cAMP independent manner) and, CYP11B2 (aldosterone synthase) (*163*). Furthermore, WNT4 expression in the adrenal gland is stimulated by cAMP, ACTH, and slightly by AngII (*161*).

As discussed earlier in section 3.2.3, β-catenin can directly interact with several transcription factors including the steroidegenic factor-1(SF-1) (*150*). Studies of adipocytes effects on adrenal glands demonstrated that fat cells conditioned medium (FCCM) containing WNT10b and WNT3a, stimulated WNT transcriptional activity (β-cat/TCF activity) and StAR promoter in the adrenocortical cancer cell line H295R. In addition, cortisol and aldosterone secretion was stimulated (*162*). Overexpression of β-catenin levels (without FCCM) lead to similar effects on hormone production and was apparently synergistic with SF1. This study overexpressed β-catenin with an activating mutation (Serine45 deletion) in the H295R cells to determine β-catenin effects on StAR, SF-1 activity and aldosterone, cortisol secretion. However, it is now well known that the H295R cells carry a S45P (Serine 45 to proline) mutation in the exon 3 of β-catenin gene (*153, 164*) which lead to intrinsic constitutive activation of β-catenin (*164*) and its cytoplasmic and nuclear accumulation (personal observations). Moreover, this study showed no direct

evidence of altered β-catenin stability a hallmark of Wnt/β-catenin activation. Very recently, Berthon *et al* (*152*), demonstrated that constitutive activation of β-catenin in the adrenal cortex of transgenic mice resulted in adrenal hyperplasia as well as dysplasia of the cortex and medulla. Over a 17 month time period, transgenic adrenals developed malignant characteristics and primary hyperaldosteronism. Interestingly, the hyperaldosteronism observed did not involve increased expression of StAR or CYP11B2 mRNA levels but protein levels of CYP11B2 were increased, suggesting post translational effects. Several types of stabilizing β-catenin mutations in humans (see section 5.2.1 table VII) are found in non-secreting as well as cortisol and aldosterone secreting adenomas and carcinomas (*153, 164*).

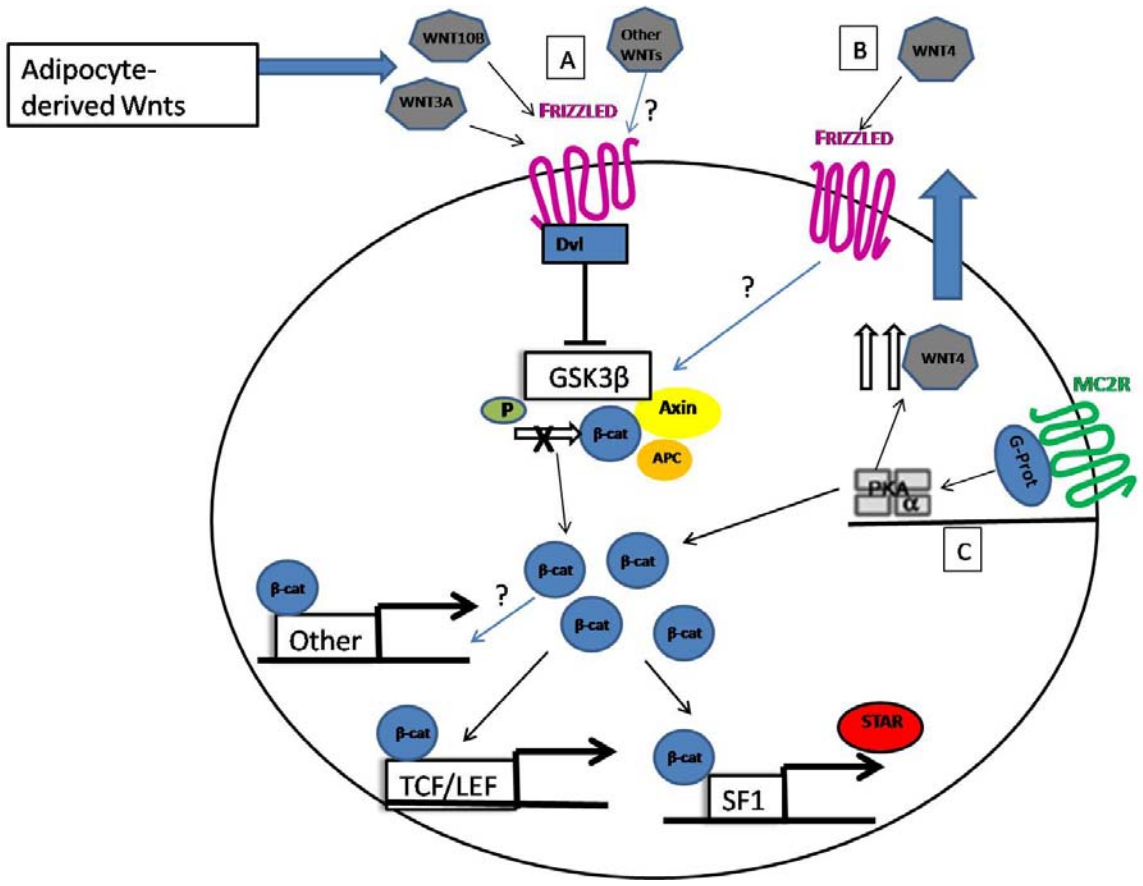

Figure 10: WNT/β-catenin in adrenal function.

Figure 10 illustrates Wnt-actions in adrenocortical cells. **A)** On one hand, adipocyte-derived Wnt10b and Wnt3a can induce canonical Wnt-signaling in adrenocortical cells which can stimulate steroidogenesis causing an increase in aldosterone and cortisol levels. WNT10b and WNT3a bind to frizzled receptors which lead to disassembly of the destruction complex and β-catenin accumulation and nuclear export. Once inside the nucleus β-catenin interacts with SF1 to increase StAR production thus increasing steroidogenesis. TCF/LEF transcription has also been proposed to be activated but to unknown effects. There is also the possibility of β-catenin interaction with other transcription factors leading to other cellular effects. **B**) On the other hand WNT4 which

regulates aldosterone synthesis by increasing CY11B2 (aldosterone synthase) via an unknown mechanism though frizzled interaction. It is yet unknown if WNT4 can activate canonical WNT signaling but WNT4 does block SF-1 β-catenin association. **C**) Constitutive activation of the cAMP protein kinase A pathway promotes β-catenin stabilization although transient effects are unknown. PKA alpha activation via bromo-8-cAMP also increases WNT4 levels along with ACTH although through these types of activation direct cell effects were not determined.

Recent evidence has shown that 1alpha, 25-dihydroxyvitamin D(3) suppresses levels of corticosterone and aldosterone in the H295R cell line(*165*). 1alpha, 25-dihydroxyvitamin D3 can also inhibit nuclear translocation of β-catenin.(*166*). Whether or not, 1alpha, 25-dihydroxyvitamin D3 effects β-catenin to cause this effect on steroid production is unknown.

4.3. Wnt/β-catenin in adrenal development

Although the involvement of Wnt/β-catenin signaling is well known in development its role in the development of the adrenal gland has been described only recently. Initial studies were in WNT4 knockout mice where decreased aldosterone secretion was observed with abnormal formation of the ZG. Mice after 18 weeks could not survive due to aldosterone deficiency. Whereas, β-catenin levels were not described in this study, thus the effects of WNT4 on β-catenin levels remain unknown.

Unlike what is found in the adult adrenal gland, nuclear β-catenin is observed at varying times during human adrenal gland development (table V). The variability of levels suggests that β-catenin plays a role in the control of adrenal development.

Table V: Nuclear β-catenin immunoreactivity in adrenal development

	10-15 weeks (n=2)	16-18 weeks (n=2)	22 weeks	35weeks	1.5 years	44 years
Adrenal	-	+[h]	-	++[h]	++[h]	+/-[i]

[h] Outer cortex, [i] Glomerulosa cells. Table modified form Charles Eberhart, C,G and Argani (*154*)

Later studies showed that disruption of β-catenin driven by the *Sf1/Crehigh* transgene causes the complete absence of adrenal glands in mice (*151*). This effect was different than that seen in *WNT4* KO mice (*160*) perhaps owing to potential roles of WNT4 in noncanonical Wnt signaling (*158, 167, 168*) and to the activation of the canonical pathway in the adrenal gland by additional Wnt ligands, or to Wnt-independent actions of β-catenin. Furthermore, mice with *Sf1/Crelow*-mediated β-catenin KO (*n*=4) exhibited histological disorganization and thinning of the adrenal cortex by 45 weeks (*151*). These experiments demonstrated requirement of β-catenin in mouse adrenal development and

places β-catenin in a small group of transcriptional regulators, which includes Sf1, Dax1, Wt1 Pbx1 and Cited2, to which deficiency causes complete adrenal absence (*151*).

Section 5. Wnt/β-catenin in adrenal tumourogenesis

5.1. Wnt/β-catenin signaling alterations in cancers

Over the last decade Wnt signaling has been found to be increasingly important in cancer and as such has been under extensive study to understand its implication in cancer development and tumour maintenance. Wnt/β-catenin signaling alterations have been found to some extent in almost all cancers studied. Alterations leading to uncontrolled regulation of Wnt/β-catenin are caused in three ways (see figure 11 for diagram).

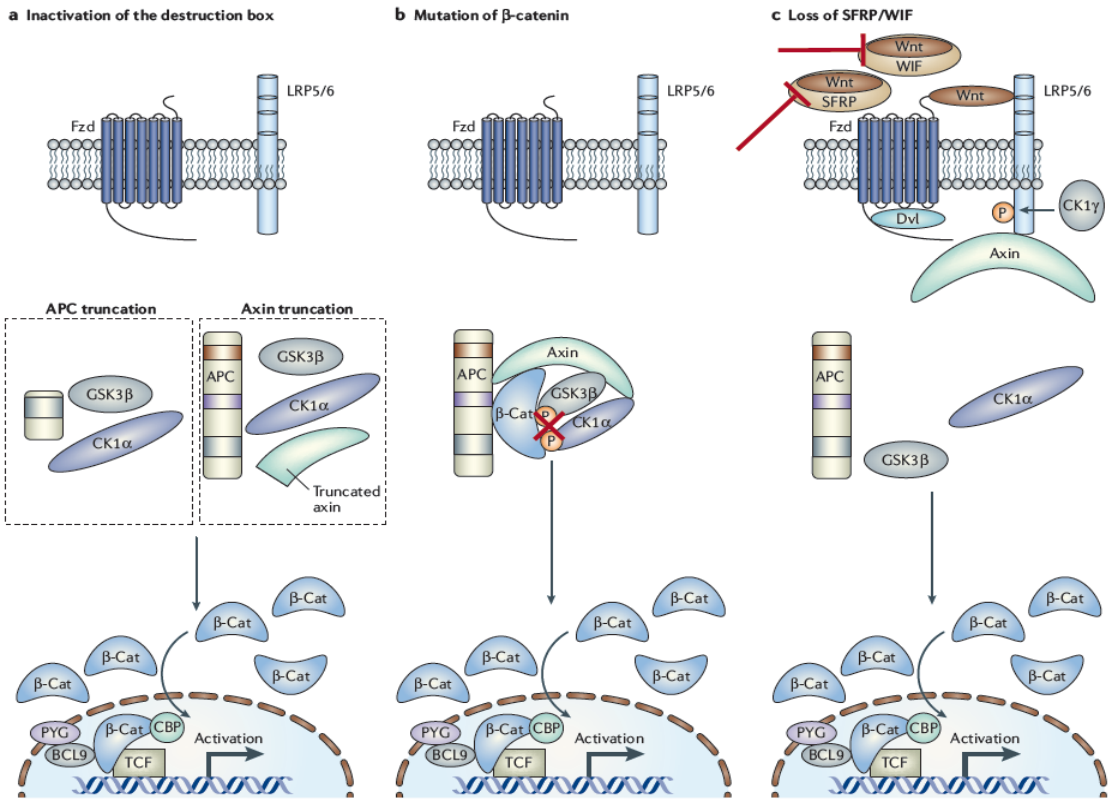

Figure 11: Common causes of Wnt signaling aberrant activation in cancer cells. (A) Mutations in APC or axin 1 genes causing expression of truncated protein. (B) Mutation of the conserved serine/threonine phosphorylation sites at the amino terminus of β-catenin blocks its phosphorylation within the destruction complex, allowing accumulation of β-catenin and formation of an active transcription factor complexes with Tcf/Lef in the nucleus. (C) Loss of natural Wnt inhibitors such as SFRP or WIF through epigenetic silencing of the corresponding genes allows Wnt proteins produced by the cancer cells to activate the pathway at the membrane. (*169*)

A) Mutations of axin or APC genes leading to absence of binding of β-catenin. β-catenin remains free of the destruction complex leading to constitutive activity of β-catenin.

B) Mutations of exon 3 of β-catenin gene coding for serine/threonine residues which are normally phosphorylated. The mutations lead to alterated phosphorylation of β-catenin and constitutive activity of β-catenin as well. Although mutations in each of the components of the complex may cause abnormal cytosolic stabilization of β-catenin, mutations of the β-catenin gene itself appear to be the most common cause of stabilization of pathological situations. These mutations predominantly affect residues at positions Ser33, Ser37, Thr41 or Ser45 (Figure 12) located in exon3 of the human β-catenin gene that are the targets of priming phosphorylation by CKIα (S45) or subsequent phosphorylation by GSK3β (S33, S37 and T41). The other mutations (Figure 12) that have been found are thought to affect the recognition sites of the enzymes that regulate β-catenin. However, it is yet unclear if these mutations lead to β-catenin constitutive activity.

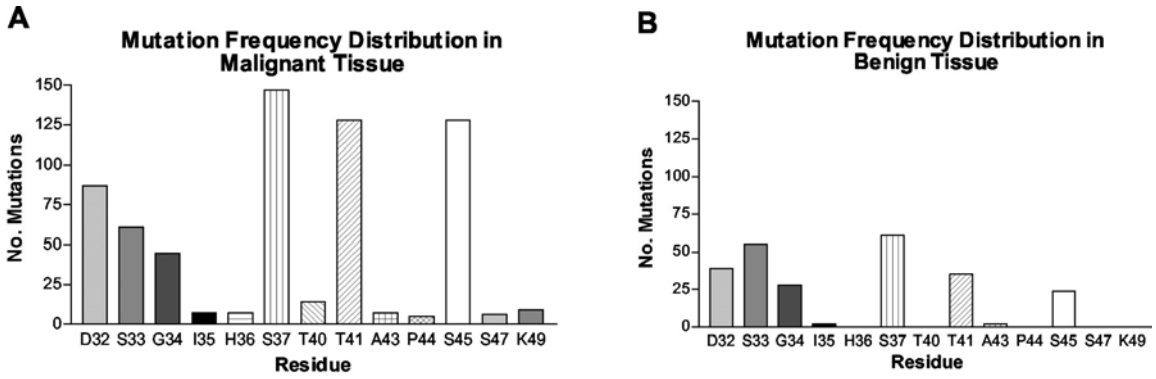

Figure 12 : Mutations of exon 3 of β-catenin in the literature. A) Graph showing thespectrum of β-catenin mutations in human malignant tumors Only amino acids reported as mutated more than five time in the literature were included. Ser37, Thr41, Ser45, Asp32, Ser33, and Gly34 were identified as the more prevalent mutations. B) Spectrum of mutations in benign tumors. The same six mutations were dominant, but in a different order of frequency: Ser37, Ser33, Asp32, Thr41, Gly34, and Ser45. The only other mutations identified in benign lesions were Ile35 (two cases) and Ala43 (two cases). Figure from (*170*).

C) Although less common, loss of SFRP or WIF proteins which normally inhibit WNT molecules can also lead to constitutive activity of β-catenin.

Activation of β-catenin finally leads to transcriptional activation of various genes (Table III, page 29). Up to now, in the literature the role of these genes in tumor development remains mainly speculative, although the predominant mutations lead to increased stability of β-catenin, the downstream effects may vary. Thus implications of β-catenin may also change depending on the alterations of the pathway present. In a study performed in MDCK cell line, cells were transfected with β-catenin S45, 37, 33 and T41 mutants (*170*). All mutants contained some level of transforming ability but mutations did lead to varying cellular effects. Transcription of cyclinD1/*CCND1* was also increased but only with the S45 mutant (*170*). Studies of two non serine/threonine mutations (Asp32 and Gly34) in MCDK cells demonstrated that only the Asp32 mutation had cell transformation potential but this mutation did alter expression of the genes normally activated by β-catenin such as *CCND1* (*171*). Another study in Hek293 cells demonstrated using reporter assays that transfection of Asp32 substitution mutations (D32G, D32N and D32Y) activated β-catenin/TCF transcriptional activity (*172*). However, expression of specific genes were not reported in this study. Furthermore, they also showed that various types of substitutions of D32 differentially affect efficiency of β-catenin phosphorylation at serine33 with D32Y showing the greatest reduction. Interestingly, there were no differences in β-catenin stability between the different substitutions (*172*).

5.2. Implication of Wnt signaling alterations in adrenal tumors.

Although most cases of adrenocortical lesions are sporadic a small percentage may be associated with hereditary syndromes such as familial adenomatous polyposis (FAP). FAP is primarily associated with high risk of colon cancer. In 1997, Marchesa et al., reported that patients with FAP more frequently developed adrenal tumors (7.4%) in comparison to the general population (0.6%-3%) (*173*). In an another study, the prevalence of adrenal masses in 107 FAP patients was 13% (*174*). Patients with FAP carry a germline mutation in APC, which can lead to constitutive activation of β-catenin (*175, 176*), and increased levels of β-catenin/TCF target genes (*177*). Most adrenal masses in FAP patient are benign. In 2000, a first study was interested into the possibility of Wnt/β-catenin alterations in adrenocortical tumors. A cohort of 26 adrenocortical tumours, 13 neuroblastoma and 12 pheochromocytomas was studied however any nuclear accumulation of β-catenin was found thus no genetic alterations of β-catenin were searched (*178*). Following this work, several transcriptome studies using microarray technology have demonstrated aberrant expression of several genes known to be regulated by Wnt/β-catenin in adrenocortical tumors (summarized in table V1, page 45). Gene array analysis of macrodular adrenal hyperplasia (AIMAH) by Bourdeau *et al.,* demonstrated that WISP2 and connexin43 were up-regulated in GIP-dependant AIMAH (*179*). Both the expression of WISP2 and connexin43 are known to be regulated by β-catenin (*180, 181*). Serial analysis of gene expression (SAGE) analysis of PPNADs with *PRKAR1A* inactivating mutations also revealed overexpression of multiple genes implicated in Wnt pathway (see table V1, page 45) (*182*). However the latter includes negative regulators such as *AXIN1* and *GSK3β* which could inhibit additional effects provided by overexpression of *CTNNB1* or *DVL2*.

Giordano *et al*., reported aberrant expression of *ENC1* and *TOP2A* (*62*) in ACCs. *ENC1* is regulated by β-catenin/TCF in Lovo cells (colon cancer cells) and may contribute to colorectal carcinogenesis by suppressing colonic cells differentiation (*107*). The *TOP2A* gene codes for topoisomerase IIα. Topoisomerase IIα is recruited by β-catenin and acts as a transcriptional enhancer (*183*). Studies have shown that overexpresing of β-catenin increased catalytic activity of Topoisomerase IIα and that overexpression of Topoisomerase IIα increases β-catenin/TCF transcriptional activity (*183*). It is therefore plausible that in ACC, increased *TOP2A,* could contribute to the high amount of cases with β-catenin accumulation which are not accounted by presence of mutations (section 5.2.2, Figure 13, page 48)

Table VI: Deregulation of Wnt signaling/target genes in adrenocortical

Genes	Type of study/diseases	Deregulation	Validated by real-time PCR	References
TOP2A, ENC1	Microarray study of ACC vs normal and ACA	Upregulated in ACC	Validated (TOP2A only)	Giordano, Thomas et al. 2003. (*62*)
Connexin43, WISP2	AIMAH	Upregulated in AIMAH	Validated (WISP2 only)	Bourdeau, Isabelle et al. 2004. (*179*)
DVL2, Axin1, CTNNB1, CSNK1E, GSK3β, CTNNAL1, WISP2	SAGE analysis of PPNAD vs normal adrenal gland	Upregulated in PPNAD	Unvalidated	Horvath, Mathyakina et al. 2006.(*182*)
WNT4, TOP2A	Microarray study of pediatric ACC	Downregulated in children ACT's	Validated (TOP2A only)	West, Neale et al. 2007.(*184*)
WISP2	MiRNA array study of PPNAD.'S	Upregulated in PPNAD	Validated	Iliopoulos, Bimpaki et al. 2009. (*185*)
APC	ACC vs ACA	Upregulated in ACC	Not validated	Laurell, Ceilia et al.2009. (*186*)
ENC1, TOP2A	ACC vs ACA and NA	Upregualted in ACC	Not validated	Giordano, Thomas et al. 2009.(*187*)

5.2.1. Implication of β-catenin mutations

Currently β-catenin mutations are the most frequent genetic alteration found in both sporadic adrenocortical adenomas and ACC. Our laboratory reported for the first time the presence of β-catenin mutations in adrenocortical adenomas at the 55th ASHG Annual Meeting (*188*) which was published in 2008 (*89*). Similarly, Tissier *et al (164)* reported the presence of beta-catenin mutations in 7 out of 26 (27%) adrenocortical adenomas and 4 out of 13 adrenocortical carcinomas (30%). We found similar results in a cohort of 33 adrenocortical adenomas (5/33) 15% but did not find any beta-catenin mutation in ACC, although only four samples were studied (*89*). Sixteen ACTH independent hyperplasia's were not found to carry any β-catenin mutation in this study. Both of latter studies also found an S45P mutation in the human adrenocortical cancer cell line H295R which was shown to activate β-catenin/TCF transcription (*164*). A third study by Masi *et al* (*189*) also found mutations in 3 of 15 (20%) ACCs and in 8 of 41 (19.5%) adrenocortical adenomas. Most β-catenin mutations are present at the critical serine and tyrosine residues of exon3 of the β-catenin gene which leads to cytoplasmic and nuclear accumulation of β-catenin. In accordance to the nature of the mutations all mutants were found to have elevated nuclear and cytoplasmic levels of β-catenin suggestive of constitutive β-catenin activity. Note that immunohistochemistry to localize β-catenin was not performed in the study by Masi *et al (189)*. All mutations found in adrenal tumours are summarized in table VII (page 47).

Table VII: Summary of β-catenin mutations found in adrenocortical tumors

Studies	**Adenomas**		**Carcinomas**		**PPNAD**	
	Prevalence %	Mutations (n : number of cases)	% Prevalence	Mutations(n : number of cases)	% Prevalence	Mutations(n : number of cases)
Tissier et al, 2005.(*164*)	7/26 (26,9%)	S45P (n : 3); S45Y (n : 1); S45F (n : 1); 26,813 del 376 bp (n : 1); DinsS33S(n : 1)	4/13 (30,8%)	S45F (n :1); del S45 (n :1); T41A (n :1); 26,993 del 489 bp(n :1)		
Tadjine et al, 2008.(*153*)	5/33 (15%)	S37C (n : 1); S45F (n : 1) ; 26 943 del55 bp (n : 1); 27 127 del6 bp (n : 1); 26 995 del271 bp(n :1)	0/4 (0%)			
Tadjine et al, 2008.(*89*)					2/18 (11,1%)	T41A (n :1) S45P (n :1)
Gaujoux et al, 2008. (*190*)	1/3 (33,3%)	S45F (n :1)	1/1 (100%)*	p.Tyr30X (n :1)	2/9 (22%)	c.236_349 191del304 (n :1) T41A (n :1)
Masi G et al, 2009. (*189*)	8/41 (19,5%)	S45P (n : 5); P44A + S45P (n :2) ; A43_R53Del (n:1)	3/15 (20%)	H36P (n :1) ; T41P (n :1); D32H (n :1)		
Pusantisampan T, 2010. (*191*)			1/1 Childhood ACC *	Del S45		
Prevalence of mutations	21/103 (20%)		7/32 (20%)		4/27	

*This case study was not included in the calcul of total prevalence.

In total 32 β-catenin mutations in adrenal disorders have been reported and 19 of them (59%) involved some alteration affecting the serine 45 residue. Both Tissier *et al (164)* and Tadjine *et al (89)* studies also revealed elevated β-catenin levels in tumors with no β-catenin mutations. This suggests that other factors of the Wnt/β-catenin may be affected. This is mainly observed in ACC where 75% of the tumours showed β-catenin accumulation (*164,* and personal observations). This is in accordance with the

overexpression of *ENC1* seen in the majority of ACCs. Aberrant nuclear localization of β-catenin without genetic alterations in *CTNNB1* or *AXIN* genes was also found in oesophageal cancer (*192*). Mutations in pigmented nodular adrenocortical disease (PPNAD) have also been found in 2 of 18 (*89, 193*) and 2 of 9 (*190*) cases studied. Mutation status in APC was also studied in our laboratory but no mutants were found (unpublished results). The total amount of *CTNNB1* mutations and observation of β-catenin accumulation are graphically represented in figure 13 (page 49).

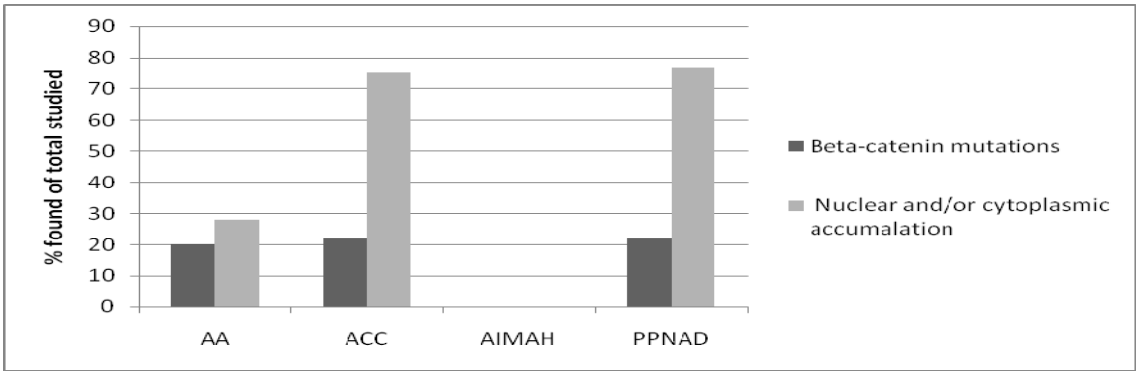

Figure 13: Summary of β-catenin mutations and aberrant accumulation of beta-catenin in adrenocortical diseases. AA= adrenocortical adenomas (21 mutations out of 103). ACC=adrenocortical carcinomas (7 mutations out of 32). AIMAH=ACTH independent macronodular adrenocortical hyperplasia (0 mutations out of 13, unpublished data). PPNAD= primary pigmented nodular adrenocortical disease (4 mutations out of 27). Data from (*89, 164*),(*153, 190*). Note at this time data is insufficient to correlate mutation type to adrenal tumour type.

5.2.2. Wnt/ β-catenin and cAMP pathway cross talk

In a study by Gaujoux *et al,* patients carrying a germline genetic alterations of the type 1-α-regulatory subunit of cAMP-dependent protein kinase A (PRKAR1A) had abnormal β-catenin accumulation in their PPNAD tissues (*190*). These mutations cause abnormal activity of PKA which alters cAMP cellular levels. Therefore the cAMP pathway in the adrenal may also regulate Wnt/β-catenin signaling. It is yet unproven in this context but protein kinase A (PKA) has been shown to directly phosphorylate β-catenin at S675 *in*

vitro and promote its stability by inhibiting its ubiquination in L cells and Hek-293 cells (*194*) leading to increased cell proliferation (*195*). In accordance to the increased β-catenin levels observed in PPNAD, especially in those with PRKAR1A mutations (*190*), several genes implicated in WNT signaling are overexpressed in PPNAD (see section 5.2.1). A microRNA (miRNA) profiling study in PPNAD's by miRNA array comparative analysis demonstrated that WISP2, like in GIP dependant-AIMAH is up-regulated, due to downregulation of miR449 (*185*). PKA activity was directly shown to affect MiR449 levels (*185*). Therefore PKA may also affect the Wnt signaling pathway, by regulation of miRNA's.

5.2.3. Model of β-catenin driven adrenal tumourogenesis

Steps involved in tumor development in adrenocortical tumourogenesis are still under extensive study. Moreover, progress has been slow in part to the rare prevalence of ACC. Up to now there is no direct evidence of progression of adrenocortical adenomas to ACC. The main recognised hypothesis is that ACC is a separate identity then adenomas and hyperplasias. β-catenin mutations are to date the only mutations found commonly in ACC as well as in adenomas. This is suggestive that β-catenin could be involved in adrenocortical tumour progression. Studies from mice expressing β-catenin with a stabilizing mutation specifically in the adrenal gland, have shown that this mutation causes aldosterone producing hyperplasias as well as dysplasia of the cortex and medulla (*152*). After 17 months expression of the mutants can lead to the presence of malignant tumors (*152*). This evidence is highly suggestive that not only β-catenin is involved in tumor progression but that ACC can evolve from hyperplasias. In this study β-catenin/TCF transcription was confirmed to occur by observation of moderate (1.5fold) *AXIN2* overexpression in adrenal tumors expressing the β-catenin mutations. Mechanistically it is

still unclear on how β-catenin triggers/participate in malignant tumor formation. Gene targets of β-catenin transcriptional activity in adrenal gland and its associated tumors remain unknown and therefore it not yet possible to determine how β-catenin mutations alter the transcriptome to cause tumor progression. Transcriptome of malignant and benign adrenocortical tumors clearly differ (*62, 63*) but this is based on evidence which does not consider β-catenin mutational status. The actual difference between the transcriptome of benign and malignant tumors with WNT/β-catenin signaling alterations remains unknown. β-catenin mutations have been associated with genomic instability in colon cancer (*196*) and hepatocellular carcinomas (*197*). Therefore β-catenin mutations could also possibly cause the increase in genomic instability observed from hyperplasia to adenoma to ACC in a subset of tumors, but this remains speculative. In other models such as colon cancer, β-catenin mutations are not considered sufficient to cause malignancy therefore a secondary event such as chromosome alteration/mutations are required. In ACC, genetic events such as overexpression of IGF2 or TP53 alterations would be essential for the development of malignancy and or to accelerate tumor formation. The prevalence of somatic β-catenin mutations in ACC carrying TP53 (somatic or germline) mutations remains to be determined. While IGF2 overexpression is wide spread in ACC and surely occurs in presence or absence of Wnt/ β-catenin signaling alterations.

5.3. β-catenin and human Adrenocortical cell lines.

Permanent and stable cell lines are of great importance in molecular biology and scientific research in general. Unlike for colon or breast cancer there are few human ACC cell lines available with only two commercially available; the human adrenocortical carcinoma cell lines SW13 (ATCC# CCL-105) and H295R (ATCC# CRL-2128). Two

other adrenal cell lines have been generated, the human pediatric adrenocortical cancer cell line HAC15 and the PPNAD cell line described by Palmar *et al*. (*198*) and Iliopoulos *et al* (*185*) that are not commercially available.

5.3.1. H295R cells.

The H295R cell line is the most commonly used and has been useful for defining the cellular mechanisms regulating steroid production as its produces both aldosterone and cortisol. In addition, H295R cells are responsive Ang2. As mentioned earlier, H295R cells carry the S45P β-catenin mutation (*164, 199*) which leads to constitutive β-catenin/TCF transcriptional activity (*164*). This cell line also contains an F338L mutation in the *TP53* gene (*200*). Treatment of H295R cells with the β-catenin/TCF inhibitor PKF115-584 results in decreased proliferation and increased apoptosis demonstrating the importance of β-catenin/TCF for H295R cell survival when the Wnt signaling pathway is active.

5.3.2. SW13 cells

The SW13 cell line was isolated from a 55 year old female with stage IV adrenocortical carcinoma in 1973 (*201*). This cell line is non-functional (non steroid producing) and therefore is not often used in adrenal research. It is considered to represent a model of ACC much more aggressive than the ACC H295R cells. SW13 cells do not contain any known mutations in APC, Axin1/2, β-catenin (unpublished results) and is therefore considered to not have any aberrant Wnt/β-catenin activity. It could therefore be thought to be a good wild-type model to study β-catenin activation. However the major population of SW13 do not express BRG-1 a critical factor in the SWI/SNF complex required in chromatin remodelling. β-catenin recruits the SWI/SNF complex via interaction with BRG-1 to activate expression of at least a subset of genes like *CCND1*. This cell line

could therefore potentially represent a non WNT responsive cell line. This cell line also does not produce active p53 (*202*).

5.3.3. HAC15 cells

HAC15 is a recently developed cell line in the Rainey's laboratory from a human adrenocortical carcinoma (HAC) removed from an 18-month old girl presenting with virilization and hypertension (*198*). HAC15 is the first ACTH responsive cell line. Although very few studies with this cell line have been done, this cell line is now known to us to also carry a β-catenin S45P mutation such as the one found in the H295R cells (unpublished data). This cell line allowed us to distinguish β-catenin effects in childhood ACC (HAC15cell line) and adult ACC (H295R cell line).

Chapter II. Research Project

1. Basis for Research

Broad application of radiological techniques have led to frequent detection of adrenal lesions (adenomas, hyperplasias or carcinomas) in the general population (2-3%) (*203*). Currently, there are no reliable diagnostic or prognostic markers to clinically (before surgery) and histologically determine the benign or malignant potential of the lesions (*204, 205*). Estimation of the likelihood of malignancy is clinically important since adrenocortical carcinoma is an aggressive tumor and the overall 5-year survival rate is less than 40% (*204, 206, 207*). Surgery is the only efficient treatment since the response to chemotherapy and the adrenolytic agent mitotane is poor (*204*). A better understanding of the molecular mechanisms with improved diagnostic and prognostic markers as well as treatment strategies is required to assist clinicians in the management of adrenocortical tumours.

Adrenocortical tumours and hyperplasias may be associated with familial syndromes: Li-Fraumeni (*p53*, 17p13), familial adenomatous polyposis (*APC*), and Carney complex (*PRKAR1A*) (*15, 24*) (*27, 28*). However, the genetic alterations remain unknown in most sporadic adrenocortical tumours. Using cDNA microarray analysis, studies a number of genes related to the Wnt/β-catenin signaling pathway have been found altered, in hyperplasias (AIMAH and PPNAD), adenomas and carcinomas, suggesting the involvement of the Wnt pathway in adrenocortical tumourigenesis ((*179*) and unpublished data).

The genes for β-catenin (*CTNNB1)*, *APC*, and *AXIN* in the Wnt signaling pathway are often mutated in human cancers, which results in the activation of transcription by β-catenin (*208-210*). *APC* gene is mutated in the majority of sporadic colorectal cancers. β-

catenin mutations also occur commonly in sporadic cancers outside of the gut such as in endometrioid ovarian cancer (*211*), hepatoblastoma (*212-214*) and Wilms' kidney tumour (*215*). Wnt signaling pathway alterations are highly linked to tumor progression in cancers such as in the colon (*216*) and hepatoblastoma (*217*).

Recently β-catenin mutations have been demonstrated to be highly prevalent in adrenocortical hyperplasias (PPNAD only), adenomas and carcinomas (*89, 153, 164, 189*) with a prevalence of about 20% (Figure 13, page 48). β-catenin mutations are the most commonly found mutation in benign and malignant tumors. Studies implicating β-catenin in adrenal tumors have been largely descriptive and the fundamental changes brought upon by β-catenin mutations remains unknown.

The principal effects brought upon by β-catenin activation are changes in gene transcription. β-catenin gene targets are believed to be largely context dependant and in the adrenal gland gene targets and subsequent cellular effects remain unknown. Transcriptome studies by cDNA microarray analysis allows determination of the gene expression profile of all genes/transcripts from the human genome. Comparisons between two or more groups can therefore be performed to determine changes in gene expression profiles. cDNA microarray analysis has been extensively used in the study of adrenal tumors/hyperplasias in order to determine prognostic factors, specific diseases markers, and pathway alterations. These studies have lead to the discovery of *IGF2* and *TOP2A* overexpression in ACC, *WISP2* overexpression in AIMAH and others. Microarray studies have not been performed yet to distinguish expression profiles brought upon by changes of mutations in a specific gene.

2. Research Plan and objectives

Due to the common occurrence of β-catenin mutations in adrenal lesions it is imperative to better understand the effects brought upon by these mutations. β-catenin is considered a legitimate cancer therapeutic target however its essential nature in normal cell functioning throughout the body provides certain challenges. Genes which are abnormally activated by β-catenin like *CCND1* and *MYC* have direct functional role in tumourigenesis (*103, 106*). Therefore, it is pertinent to first study the impact of the presence of β-catenin mutations on gene expression profile of tumours which may lead to the identification of new potential therapeutic targets. Taking into account that a number of genes affected by β-catenin can most likely themselves modify expression of another set of genes, it is expected that total gene expression analysis of tumours harbouring β-catenin mutations would reveal deregulation of genes that may not be directly regulated by β-catenin. However, transcriptome studies may provide a global picture of transcriptional changes and may help to distinguish genetic changes specific to tumours with or without β-catenin mutations.

We therefore designed a microarray experiment to determine transcriptional differences in adenomas with known β-catenin activating mutations (n=3) and confirmed β-catenin nuclear accumulation compared to adrenocortical adenomas with wild-type β-catenin gene (n=4) and no nuclear accumulation of β-catenin protein, as determined in Tadjine *et al*. 2008 (*153*). Seven genes were selected for validation by real-time PCR analysis in an expanded cohort of adrenocortical samples (3 adenomas with β-catenin missense mutations, 3 adenomas with β-catenin deletion mutations, 13 Wt adenomas (5 aldosterone-secreting adenomas and 8 cortisol-secreting adenomas), 1 ACC with β-catenin missense mutations, 4 Wt ACCs and 10 normal adrenal glands. Given the small number of

tumor harbouring β-catenin mutations available to us, we used β-catenin/TCF inhibitors to treat the human adrenocortical carcinoma cell line H295R, which harbour β-catenin activating mutation to determine if inhibition of β-catenin/TCF would alter expression of our selected genes. This allowed us to determine further links between β-catenin and expression of genes found in the microarray analysis.

3. Article 1

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Characterization of adrenocortical tumours harbouring β-catenin mutations identifies new candidate genes

Julien Durand, Antoine Lampron, Tania L. Mazzuco, Audrey Chapman,

Isabelle Bourdeau[¶]

Division of Endocrinology, Department of Medicine, Research Centre, Centre hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada

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[¶]To whom correspondence should be addressed:

Isabelle Bourdeau, MD
Division of Endocrinology, Department of Medicine
CRCHUM − Hôtel-Dieu
3850, Saint Urbain Street
Montréal, Québec, Canada H2W 1T7
Telephone: (514) 890-8000 Ext. 14086

ABSTRACT

Background. Mutations of β-catenin gene (*CTNNB1*) are frequent in adrenocortical adenomas (AA) and carcinomas (ACC). However, the target genes of the de-regulated Wnt/β-catenin signaling pathway have not yet been identified in adrenocortical tumours.

Objective. To identifiy genes specifically de-regulated in adrenocortical tumours harbouring *CTNNB1* genetic alterations.

Methods. We compared gene expression profiles of AA with (n: 3) and without (n: 4) *CTNNB1* mutations using Affymetrix Human Genome U133 Plus 2.0 oligonucleotide arrays. The mRNA expression of 7 selected genes was validated in 6 AA with *CTNNB1* mutations, 13 AA with wild-type (WT) *CTNNB1,* 1 ACC with *CTNNB1* mutation, and 4 WT ACC in comparison to 10 normal adrenal glands. We then studied the effects of suppressing β-catenin transcriptional activity with the β-catenin/TCF (T-cell factor) inhibitors PKF115-584 and PNU74654 in H295R and SW13 adrenocortical cell lines.

Results. One hundred and twenty-eight probe sets had a minimal mean expression ratio of 2, and 407 probe sets had a mean ratio of 0.5 or less in tumours with *CTNNB1* mutations compared to WT tumours. Real time polymerase chain reaction analysis confirmed the over-expression of *ISM1, RALBP1* and *PDE2A* as well as the down-regulation of *PHYHIP* and *CITED2* in 5/6 AA harbouring *CTNNB1* mutations compared to WT AA (n: 13) and normal adrenal glands (n: 10). *CDH12* and *ENC1* were specifically over-expressed in AA harbouring *CTNNB1* point mutations. mRNA expression of *ISM1, RALBP1, PDE2A* and *ENC1* was decreased in a dose-dependent manner in H295R cells after treatment with the TCF/β-catenin antagonists PKF115-584 and PNU74654 whereas *CITED2* levels were increased.

Conclusion. This study identified new candidate genes specifically de-regulated in *CTNNB1*-mutated adrenocortical tumours that may lead to a better understanding of the role of Wnt-β-catenin pathway in adrenocortical tumourigenesis.

INTRODUCTION

Adrenocortical tumours may be associated with familial syndromes, however, the genetic alterations remain unknown in most sporadic cases (1). Recent microarray studies described the aberrant expression of a number of genes related to the WNT-β-catenin pathway in adrenocortical hyperplasias, adenomas and carcinomas (2-6). In addition, β-catenin (*CTNNB1*) mutations were found recently in 15 to 26.9% of adrenocortical adenomas (AA) (7, 8, 9) and in up to 30.8% of adrenocortical carcinomas (ACC) (7) (9).

Wnt signaling plays a key role in development and tumourigenesis (10-13). WNT proteins bind to a complex containing frizzled and low-density lipoprotein receptor-associated proteins (LRP5/6) at the cell surface. This mobilizes signaling protein dishevelled, which, in turn, inhibits the activity of glycogen synthase kinase-3beta (GSK-3β). GSK-3β forms a complex with 2 β-catenin-binding proteins, APC, and axin (AXIN). In the absence of WNT's, β-catenin is recruited into the complex which facilitate its phosphorylation by casein kinase 1 and activate GSK-3β, making β-catenin available for degradation (13). Thus, β-catenin level is normally low in the cell cytoplasm and nucleus. In contrast, activation of Wnt, inhibits GSK-3β activity; consequently, β-catenin accumulate and form complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins in the nucleus.

CTNNB1 mutations affect specific serine and threonine residues localized in exon 3 that are essential for the targeted degradation of β-catenin (14). Doghman et al., reported that the TCF/CTNNB1 antagonist PKF115-584 inhibits proliferation of H295R ACC cells (15). Very recently, Berthon et al. observed that constitutive activation of β-catenin in the

adrenal cortex of transgenic mice leads to benign aldosterone-secreting tumour development and promotes malignancy (16).

When it is translocated to the nucleus, β-catenin functions as an oncogene, binding to TCF/LEF family members, and trans-activating its target genes. c-Myc (17) and cyclin D1 (18) have been found to be critical TCF-regulated target genes in colon cancer. However, this aspect has never been explored in adrenocortical tumours. To identify candidate genes linked to *CTNNB1* mutations in adrenocortical tumourigenesis, we investigated by microarray analysis the differential gene expression profiles of adrenocortical tumours with mutational defects in *CTNNB1* gene and tumours with intact regulation of β-catenin activity.

MATERIALS AND METHODS

Adrenocortical tissues

We studied 34 adrenocortical tissues, including 10 normal adrenal glands (NA), 13 AA with wild-type (WT) *CTNNB1* gene, 6 AA with *CTNNB1* genetic alterations (3 harbouring missense mutations and 3 with deletion mutations), 4 ACC with WT *CTNNB1* and 1 with *CTNNB1* missense mutation. Adrenal glands were surgically removed from patients and snap frozen. A pool of commercially-available RNA (Clontech Laboratories, Palo Alto, CA, USA), isolated from NA of 62 Caucasian subjects aged 15-61 yr, was also studied. The study was approved by the Institutional Ethics Committee of Centre hospitalier de l'Université de Montréal (CHUM), and all patients provided informed, written consent.

Human adrenocortical cancer cell lines

The human adrenocortical carcinoma cell lines SW13 and H295R were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured according to ATCC. H295R cells harbour an activating missense mutation in exon3 of *CTNNB1* gene (S45P) (7, 8), along with a heterozygous F338L mutation in the *TP53* gene.

Microarray sample labelling and hybridization

To evaluate differential gene expression related to β-catenin, we studied 3 β-catenin-mutated tissues with point mutations showing higher translocation of nuclear β-catenin protein: 2 cortisol-secreting AA (S45P, S37C) and 1 aldosterone-secreting adenoma (T41A). The gene expression profile was compared to 3 cortisol-secreting and 1

aldosterone-secreting AA with WT *CTNNB1* and no known nuclear accumulation of β-catenin protein. The commercially-available pool of 60 NA described earlier served as control.

Total RNA was isolated with TriZOL reagent (Invitrogen, Carlsbad, CA, USA), and all RNA used had RNA integrity number (RIN) values above 8 [values ranging from 10 (totally intact) to 1 (totally degraded)]. Microarray experiments were performed with human expression Human Genome U133 Plus 2.0 gene chip arrays (Affymetrix, Santa Clara, CA, USA). Briefly, 5 μg total RNA were reverse transcribed with T7-(dT) 24 oligonucleotide as primer, labelled with biotin, and fragmented with Affymetrix reagents. Ten μg of the resulting cRNA were loaded onto each chip. After washing and staining with streptavidin-phycoerythrin (Invitrogen), the chips were scanned with a Genechip Scanner 3000 workstation (Affymetrix).

Microarray data analysis and statistics

Probe set intensity levels were extracted from scanned oligochips with the gene chip operating system (version 1.2, Affymetrix) and normalized with all probe sets and a target value of 500. For normalization, each oligochip's dataset was centered on its median intensity and each probe set was normalized to control RNA. Probe sets that were statistically different from adenomas with *CTNNB1* mutations compared to AA with WT *CTNNB1* were identified by Student's 2-tailed heteroscedastic *t* test with an alpha of 0.05. For up-regulated genes, we retained only probe sets flagged as present in at least 2 of the 3 adenomas with *CTNNB1* mutations. For down-regulated genes, we kept the probe sets flagged as present in the control and in at least 2 of the 4 WT AA. Finally, the probe sets

were filtered according to differences between the 2 adenoma groups represented by a minimum 2-fold increase in intensity.

Validation of gene expression by real time-polymerase chain reaction (RT-PCR) analysis.

To validate gene expression, RT-PCR was performed on 3 AA with *CTNNB1* missense mutations, 3 AA with *CTNNB1* deletion mutations, 13 WT AA (5 aldosterone-secreting adenomas and 8 cortisol-secreting adenomas), 5 ACC (4 WT and 1 with *CTNNB1* S45P mutation), and 10 NA. One μg of RNA was processed for cDNA synthesis. PCR amplification mixture was prepared with Quantitect SYBR green RT-PCR kit (QIAGEN), and amplifications were conducted in a Rotor-Gene 6000 cycler (Corbett Research, Sydney, NSW, Australia). Primer sequences are described in Supplemental Table 1. An initial denaturation step of 5 min at 95^{o}C was followed by 45 cycles of 95^{o}C for 30s, 60°C for 30s and 72^{o}C for 30s. Standard curves for each primer set reaction were charted to confirm 100% efficiency and melting curve analysis was conducted. mRNA levels were normalized to 18S rRNA. Relative expression ratios were determined by the Pfaffl method (19) by normalizing expression values with the pool of commercially-available adrenal gland RNA (Clontech Laboratories). For each tissue sample studied, RT-PCR was repeated at least twice for 2 independent RNA extractions, or three times when only 1 RNA extraction was available. Averages were calculated for each group, and error was computed from standard deviation. One-way ANOVA and Bonferroni's multiple comparison test ascertained statistical significance with a cut off p-value of 0.05.

Treatment of cells with antagonists of TCF4/β-catenin protein complex

PKF115-584, generously provided by Novartis (Basel, Switzerland), was diluted at a stock concentration of 30 mM in DMSO. PNU74654 (Sigma-Aldrich, Saint Louis, MO, USA.) was diluted at stock concentrations of 100 mM and 30 mM. H295R cells were plated at 700,000 cells/well in 6-well plates. The cells were allowed to attach overnight and serum-starved for 24 h. They were then treated with PKF115-584 (1, 2.5, 5 and 10 μM) or PNU74654 (3, 30 and 100 μM), at a final DMSO concentration of 0.1%, or with DMSO alone (vehicle). After 24 h of treatment, the cells were collected and RNA or protein was extracted from the cells.

Protein extraction/immunoblotting

Cell pellets were re-suspended in RIPA protein lysis buffer and incubated on ice for 40 min, then spun at 15,000 rpm for 15 min. For tissue protein extraction, RIPA buffer was supplemented with 10% glycerol for storage. 10-40 μg of total protein were heated to 95°C for 5 min in 1X sample buffer and run on 10% SDS-polyacrylamide gel at 150 V for 1 h. Protein was then transferred onto PVDF for 1 h at 100 V. The blots were blocked with 5% milk Tris-buffered saline-Tween 0.1% (TBS-T). To detect β-catenin, β-actin and RalA-binding protein 1 (RALBP1), the blots were probed with mouse anti-β-catenin (1:2,000) (BD Transduction Laboratories, Franklin Lakes, NJ, USA), mouse anti-actin (1:2,000) (Sigma), or mouse anti-RALBP1 (1:500) (Abnova, Taipei City, Taiwan) overnight at 4°C. The blots were washed 3X with 1X TBS-T, and probed with anti-mouse horseradish peroxidase (Sigma) at 1:5,000-10,000 for 1 h. They were developed with ECL™ Western blotting detection reagent (GE Healthcare, London, UK) and exposed on X-ray film for varying time periods to achieve the best signal.

RESULTS

Differential expression profile of AA with CTNNB1 mutations

We compared the gene expression profile of 3 AA with *CTNNB1* point mutations to 4 AA WT *CTNNB1*. 1,509 probe sets showed differential expression profiles between the 2 groups with a t-test p-value of at least 0.05. Within the dataset, 128 probe sets corresponding to 114 genes/transcripts had a minimal mean expression ratio of 2 in tumours with *CTNNB1* mutations. Forty-five probe sets (corresponding to 43 genes) with a minimal 3-fold difference in expression values are listed in Table 1. Furthermore, 407 probe sets, corresponding to 376 genes/transcripts, had a mean ratio of 0.5 or less. Eighty-one probe sets (Table 2), corresponding to 75 genes, had a significant 4-fold decrease of expression in adrenal tumours with *CTNNB1* mutations relative to WT adenomas.

We deployed the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org) (5) which classifies genes by their function. Among the 1,509 statistically significant probe sets, PANTHER recognized 22 biological processes including over-expression of genes related to "developmental processes" (p=0.003) and "systems development" (p=0.003) (Supplemental Table 2). Moreover, the PANTHER system revealed over-representation of genes involved in Wnt signaling (Supplemental Table 3).

We then analyzed the 1,509 probe sets, focusing on genes known to be related to Wnt/β-catenin signaling available from the WNT homepage http://www.stanford.edu/~rnusse/wntwindow.html. Interestingly, 2 probe sets corresponding to *ENC1* (ectodermal-neural cortex 1), known as a downstream target of β-catenin/TCF complex which is upregulated in colon cancer, were over-expressed in

tumours carrying *CTNNB1* missense mutations with a ratio of 6-fold ($p<0.05$) and 5.4-fold ($p=0.1$) (20). GJA1 (connexin 43) probeset, known to be regulated by β-catenin/TCF, was down-regulated in tissues with *CTNNB1* mutations (0.243; $p=0.008$).

RT-PCR validation of genes expressed differentially in adrenocortical tumours harbouring CTNNB1 mutations

To validate the microarray data, 7 genes were selected for confirmation by RT-PCR. *ISM1* (isthmin1, zebrafish homolog), *RALBP1* (RalA-binding protein 1), *PDE2A* (phosphodiesterase 2A, cGMP-stimulated), *CDH12* (cadherin-12), *ENC1, PHYHIP* (phytanoyl-CoA 2-hydroxylase interacting protein) and *CITED2* (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2) were significantly de-regulated in AA with *CTNNB1* point mutations (n=3), compared to NA (n=10) and WT AA (n=13) ($p<0.05$) (Figure 1).

The 2 highest over-expressed probes (28- and 32-fold, Table 1) on microarray analysis were C20orf82, now known as *ISM1* gene. RT-PCR studies confirmed the up-regulation of *ISM1* in AA with point mutations, giving an average ratio of 65.6 ± 12.2-fold compared to either WT adenomas ($p<0.001$) and NA ($p<0.001$) (Figure 1). *CDH12* over-expression was confirmed in AA with *CTNNB1* point mutations for a ratio of 9.4 compared to NA ($p<0.001$) and 9.8 ± 2.4 for WT AA ($p<0.001$) (Figure 1). Interestingly, although mean *CDH12* expression in WT AA was not significantly different from NA, 6 out of 13 AA showed higher expression, and 6 of 13 under-expression, compared to NA. *RALBP1* gene was over-expressed in AA with *CTNNB1* point mutations by a mean factor of 4.7 ± 0.3-fold and 4.8 ± 0.4-fold, compared to NA and WT AA ($p<0.001$) (Figure 1). *PDE2A* was over-

expressed compared to NA (10.1±1.6) and WT AA (10.6±2.2) ($p<0.001$) (Figure 1). *ENC1* and *PHYHIP* genes were also over-expressed in 4 out of 5 and under-expressed in 5 of 5 ACC, respectively. RT-PCR validated the under-expression of *PHYHIP* and *CITED2* (21) (Figure 1) in tumors with *CTNNB1* mutations compared to NA tissues (-28±5.9-fold, $p<0.001$ and -4.2±0.9-fold, $p<0.001$, respectively) and WT AA (-21.8±5.9-fold, $p<0.001$ and -3.9±0.8-fold, $p<0.001$).

We then studied the expression of the 7 selected genes in 3 AA harbouring deletion mutations localized in exon 3 of *CTNNB1* gene including 26,943 del 55 bp, 26,995 delExon3(271 bp) and 27,127 del 6 bp (8). Similarly, as in AA with point mutations, significant increased expression of *ISM1*, *RALBP1* and *PDE2A* was found in 2/3 AA with deletion mutations compared to NA and WT AA (Figure 1). *ENC1* and *CDH12* levels showed expression similar to NA and WT AA. Decreased *PHYHIP* and *CITED2* expression was respectively observed in 2/3 and 3/3 AA with deletions. Tissue with the 26,943 del 55 bp presented an opposing trend of expression for the 7 genes except for *CITED2* (Figure 1).

The only ACC sample with *CTNNB1* point mutations available over-expressed *ISM1, PDE2A, RALBP1* and *ENC1* compared to WT AA and ACC and NA with relative expression ratios of 63.0, 4.5, 2.24 and 18.0, respectively (Figure 1). However, this ACC showed decreased *CHD12* expression compared to NA (23.0-fold). *PHYHIP* levels were reduced in ACC harbouring *CTNNB1* point mutation compared to WT AA (137-fold) and WT ACC (36.7-fold). *CITED2* was significantly down-regulated by 3.1- and 3.7-fold in 2/4 WT ACC and disclosed 1.5-fold under-expression in ACC with *CTNNB1* point mutation.

Antagonists of TCF4/β-catenin complex dose-dependently inhibit the expression of selected genes in human adrenocortical cell lines

To support the hypothesis that the 7 selected genes were functionally related to *CTNNB1* mutations, we studied the effects of suppressing β-catenin transcriptional activity with antagonists of TCF4/β-catenin complex, PKF115-584 and PNU74654 SW13 (22) and H295R (23) cell lines. H295R cells carry the *CTNNB1* mutation S45P (7, 8) which elicit nuclear accumulation of β-catenin and β-catenin/TCF interaction (Supplemental Figure 1). SW13 cells were considered as controls because they harbour no *CTNNB1* mutation (personal data) and express low amounts of β-catenin (Supplemental Figure 1). RT-PCR demonstrated that *ISM1, RALBP1, PDE2A, CDH12* and *ENC1* genes were up-regulated in the H295R ACC cell line (40.6-, 4.8-, 5.3-, 41.7- and 3.6-fold, respectively) compared to NA (Supplemental Figure 2). Moreover, these genes were not over-expressed in non-mutated SW13 cells. *CITED2* was found to be down-regulated in H295R cells (5-fold) but not in SW13 cells. *PHYHIP* was very poorly expressed in both cell lines (data not shown) and was not further studied.

RT-PCR was conducted to examine the effects on gene expression after treatment with PKF115-584, PNU74654 or vehicle for 24 h in H295R and SW13 cells. As expected, the pattern of gene expression was linked to β-catenin activity as seen in adrenocortical tumours with *CTNNB1* mutations, except for *PHYHIP* and *CDH12* (results not shown). The most potent effect was observed on *ISM1* expression: 88$\pm$1.1% dose-dependent decrease of *ISM1* expression after PKF115-584 and 74.1$\pm$4.3% after PNU74654 treatments in H295R cells (Figure 2). Similarly, PKF115-584 led to reduced expression of *PDE2A* (by 35.9$\pm$5.0%) and *RALBP1* (by 51.7$\pm$3.0%) at 10 µM. With PNU74654, *PDE2A* expression

levels were diminished by up to 50±14% whereas R*ALBP1* expression declined only slightly (by 25%) (Figure 2). mRNA *ENC1* levels were significantly decreased by PKF115-584 (45±13%) and PNU74654 (55.1±6.8%). In contrast, *CITED2* levels increased after PKF115-584 treatments in H295R cells (194±28%) (Figure 3). As controls, we studied *AXIN2* which is considered to be the most faithful gene for determining β-catenin/TCF activity (24, 25). As expected, *AXIN2* levels were reduced by PKF115-584 (41.3±4.9%) and PNU7654 (47.3±8.2%) (Figure 3). In SW13 cells, *ISM1, PHYHIP, PDE2A* and *CDH12* expression was not determined because of their very low to undetectable levels (Supplemental figure 2).

Validation of RALBP1 protein over-expression in adrenocortical tumours

RALPB1 is a drug-transporter that can mediate drug-resistance (26) in lung and kidney cancers (27). RT-PCR confirmed *RALBP1* over-expression in 3/3 AA and 1/1 ACC with *CTNNB1* point mutations, disclosing significant increases of 3.59±1.57-fold and 2.25±0.48-fold, respectively, compared to NA (n=10, p=0.02) and WT *CTNNB1* tumours (n=13; p=0.019) (Figure 1). Moreover, heightened expression of *RALBP1* was noted in 2/3 tumours with deletion mutations (Figure 1).

Western blot analysis confirmed RALBP1 protein over-expression in 4/6 AA with *CTNNB1* mutations (3.9±0.7-fold) and in 1/1 ACC with *CTNNB1* point mutation (4.7±0.3-fold). Interestingly, RALBP1 protein levels were elevated in 4/4 WT ACC (3.1±0.7-fold) (Figure 4) and detected at lower levels in WT AA (2±0.6-fold, n=5) compared to NA (n=6).

mRNA *RALBP1* expression decreased after treatment with PKF115-584 achieving an inhibition of 52%±2% ($p<0.001$) in H295R cells at 10 μM (Figure 2B). However, in WT

SW13 cells, there was no significant variation of *RALBP1* or *AXIN2* expression after treatment (results not shown). Furthermore, we found 61.4% reduction of RALBP1 protein expression after treatment with 5 μM of PKF115-584 in H295R cells (Figure 4D). As seen at the mRNA level, RALBP1 protein was only slightly diminished (by 19 +/- 4.6%) after 24 h of treatment with 100 μM of PNU74654.

DISCUSSION

In this study, we searched for new target genes of the Wnt pathway specifically de-regulated in adrenocortical tumours with *CTNNB1* mutations. We compared the expression profiles of these tumours to those without *CTNNB1* mutations. Our approach was previously validated in Wilms tumours (28) and endometrioid adenocarcinomas (29) and identified new target genes of Wnt/β-catenin specifically in these cancers. De-regulation of β-catenin results in the constitutive formation of β-catenin/TCF complexes and in the altered expression of TCF-regulated target genes, such as *MYC* (17) and *CCND1* (18), as reported in colon cancer. However, it is strongly believed that the target genes of β-catenin are tissue-specific. Although *CTNNB1* mutations are encountered in about 20% of adrenocortical tumours, no studies evaluating presumptive target genes of β-catenin in human adrenocortical tumourigenesis have yet been performed.

We recognized 114 genes/transcripts that were significantly over-expressed in tumours with *CTNNB1* mutations, compared to WT tumours. *ISM1* had the highest over-expression and was up-regulated specifically in 6/7 adrenocortical tumours with *CTNNB1* mutations, supporting β-catenin involvement in *ISM1* transcription, as shown previously in zebrafish (30). *CDH12* which is known to interact with the MCP1-Induced Protein; a mediator of angiogenesis was the second most up-regulated gene (31). We confirmed the up-regulation of *ENC1* gene in 3/3 AA with point mutations and 4/5 ACC by RT-PCR. However, tumours with deletion mutations did not show *ENC1* over-expression. Giordano et al. previously reported *ENC1* over-expression in ACC by micorarray analysis as well (5, 32). RALBP1 which mediate drug-resistance in cancers was over-expressed in 5/6 AA and 1/1 ACC with *CTNNB1* mutations (26). *RALBP1* down-regulation leads to a decrease of drug

resistance but also to apoptosis and regression of lung and prostate tumours in xenograft models (26, 33). *RALBP1* has never been studied before in adrenocortical tumours.

PDE2A is a dual-function phosphodiesterase capable of both cGMP and cAMP hydrolysis (34). Its over-expression was validated in 6/7 tumours with *CTNNB1* mutations, compared to the 27 NA tissues and WT *CTNNB1* adrenocortical tumours. Mainly expressed in glomerulosa cells in the adrenal gland (35, 36), *PDE2A* is stimulated by atrial natriuretic peptide (37, 38) which culminates in the inhibition of aldosterone production (36).

We confirmed the under-expression of *PHYHIP* gene in all adrenocortical tumours with *CTNNB1* mutations. Moreover, we observed *PHYHIP* down-regulation in all ACC. This finding was not surprising since, very recently, Giordano et al. reported *PHYHIP* down-regulation in ACC compared to NA and AA, in a transcriptome-profiling study (32).

The transcription co-factor *CITED2* was specifically under-expressed in all AA harbouring *CTNNB1* genetic alterations. Targeted disruption of *CITED2* in mice results in adrenal agenesis (39). Moreover, *CITED2* is activated by SF-1 in a dose-dependent manner in NCI-H295R cells (40). Our study is the first to suggest a potential link between *CITED2* and the Wnt/β-catenin pathway in adrenocortical tumourigenesis.

Although candidate genes reported here may not be direct targets of β-catenin, *ENC1* is known to be regulated by TCF/β-catenin and has recognized β-catenin/TCF-responsive upstream regulatory sequences (20). Moreover, *CDH12*, *PDE2A* and *ISM1* were found to have 7, 5 and 4 potential putative binding sites for LEF-1/TCF, respectively, according to the general consensus sequence WWCAAWG/CTTWGWW (41).

One of the limitations of our data is the small number of samples studied. We present complete data on 6 AA with *CTNNB1* mutations, which are about 30% of all the AA

reported as mutated in the literature so far (n=21) (7-9, 42). Moreover, to identify target genes specifically related to β-catenin activation, we focused our microarray analysis on tissues harbouring point mutations because they showed higher β-catenin nuclear translocation than tissues with deletion mutations. In addition, among all *CTNNB1* mutations reported so far in adrenocortical tumours, point mutations were more frequent than other types of mutations with a prevalence of 72% (23/32) (7-9, 42). Nevertheless, we examined whether our results in tumours with *CTNNB1* point mutations were similar to those in tumours with *CTNNB1* deletion mutations. *ISM1, RALBP1*, *PDE2A* and *PHYHIP* over-expression was confirmed in 2/3 tissues with deletion mutations. The third sample, which harboured *CTNNB1* 26,943 del 55 bp, showed an opposing trend of expression for the 7 genes, except for *CITED2,* which was under-expressed in 3/3 deleted mutant adenomas. We found no over-expression of *ENC1* gene in AA with deletion mutations. This absence of up-regulation in tissues with deletion mutations may be explained by the fact that various types of mutations do not elicit equivalent stabilization of β-catenin and transcriptional activation of Wnt target genes (43). Interestingly, *ENC1* and *PHYHIP* were also aberrantly expressed in 3/4 and 4/4 WT ACC, respectively, supporting common expression characteristics of tumours with *CTNNB1* missense mutations and ACC.

We then tested PKF115-584 and PNU74654 compound. PKF115-584 has previously been shown to alter β-catenin/TCF transcriptional activity in H295R cells (15) and other cancer models (44, 45), by interfering with β-catenin/TCF interaction which, consequently, affects proliferation and apoptosis. We demonstrated that both antagonists down-regulated the over-expressed *ISM1, RALBP1, PDE2A* and *ENC1* gene. In addition, PKF115-584 up-regulated the under-expressed *CITED2* gene.

This study is a first attempt to identify genes specifically de-regulated in *CTNNB1*-mutated adrenocortical tumours. Our data led to the identification of new candidate genes that may be involved in adrenocortical tumourigenesis. Further investigations are needed to determine the functional significance of these genes in the development of AA and ACC. Nevertheless, our data may contribute to a better understanding of the role of Wnt-β-catenin in adrenocortical tumourigenesis.

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FIGURE LEGENDS

Figure 1. Validation of Microarray results by RT-PCR. mRNA expression of **(A)** *ISM1*, (**B)** *ENC1*, **(C)** *RALPB1*, **(D)** *PDE2A*, **(E)** *CDH12*, **(F)** *PHYHIP* and **(G)** *CITED2* in adrenocortical tissues. The results represent the mean of 2 experiments performed with 2 different RNA extractions from each tissue. Real-time reactions were performed in triplicate. The results were subjected to 1-way ANOVA, followed by the Bonferroni test to determine statistical significance ($p \leq 0.05$) between groups and WT adenomas. NA: NA, AA: adrenocortical adenoma, WT: wild-type *CTNNB1,* PM: tissue with *CTNNB1* point mutation, DM: tissue with *CTNNB1* deletion mutation, ACC: adrenocortical carcinoma. Arrows denote data of the tissue with *CTNNB1* 26 943 del 55 bp.

Figure 2. Effects of β-catenin/TCF inhibitors on gene expression of **(A)** *ISM1*, **(B)** *RALBP1*, **(C)** *PDE2A* and **(D)** *ENC1* in H295R cells by RT-PCR. The cells were treated with PKF115-584 (left panels) (1, 2.5, 5 and 10 μM), PNU74654 (right panels) (3.3, 30 and 100 μM) or vehicle (0.1% DMSO) for 24 h. Bars represent the means of 2 independent experiments, each in triplicate with real-time reactions performed in duplicate. Errors are shown as % standard deviation. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 3. Effect of β-catenin/TCF inhibitors on gene expression of **(A)** *CITED2* and **(B)** *AXIN2* in H295R cells. The cells were treated with PKF115-584 (left panels) or PNU74654 (right panels) or with vehicle (0.1% DMSO) for 24 h. Bars represent the means of 2 independent experiments, each in triplicate with real-time PCR reactions performed in duplicate. Errors shown as %standard deviation. ***$p \leq 0.001$.

Figure 4. **(A)** Western blot disclosing RALBP1 over-expression at the protein level in adrenocortical adenomas (AA) with *CTNNB1* point mutations (PM) and deletion mutations (DM) compared to wild-type adrenocortical adenomas (WT AA) and normal adrenal glands (NA). **(B)** Western blot analysis of RALBP1 in adrenocortical carcinomas (ACC) compared to NA. **(C)** Quantitative RALBP1 levels relative to β-actin in normal adrenal glands (NA), wild-type AA (WT AA) and ACC, and AA and ACC with *CTNNB1* mutation (Mutant AA and Mutant ACC). **(D)** Western blot analysis of RALBP1, CTNNB1 (β-catenin) and β-actin after 24-h treatment with PKF115-584 and PNU74654 in H295R cells (left panel). Graph presenting the means of quantitative RALBP1 and β-catenin levels relative to β-actin in H295R cells from 2 independent experiments, each in triplicate (right panel). Errors are shown as % standard deviation.

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Table 1. Summary of 45 probe sets corresponding to 43 genes with an intensity ratio of at least 3-fold in AA with *CTNNB1* mutations ("Mutant") compared to AA with WT *CTNNB1* gene ("WT") ($p<0.05$). Genes selected for validation are highlighted in bold.

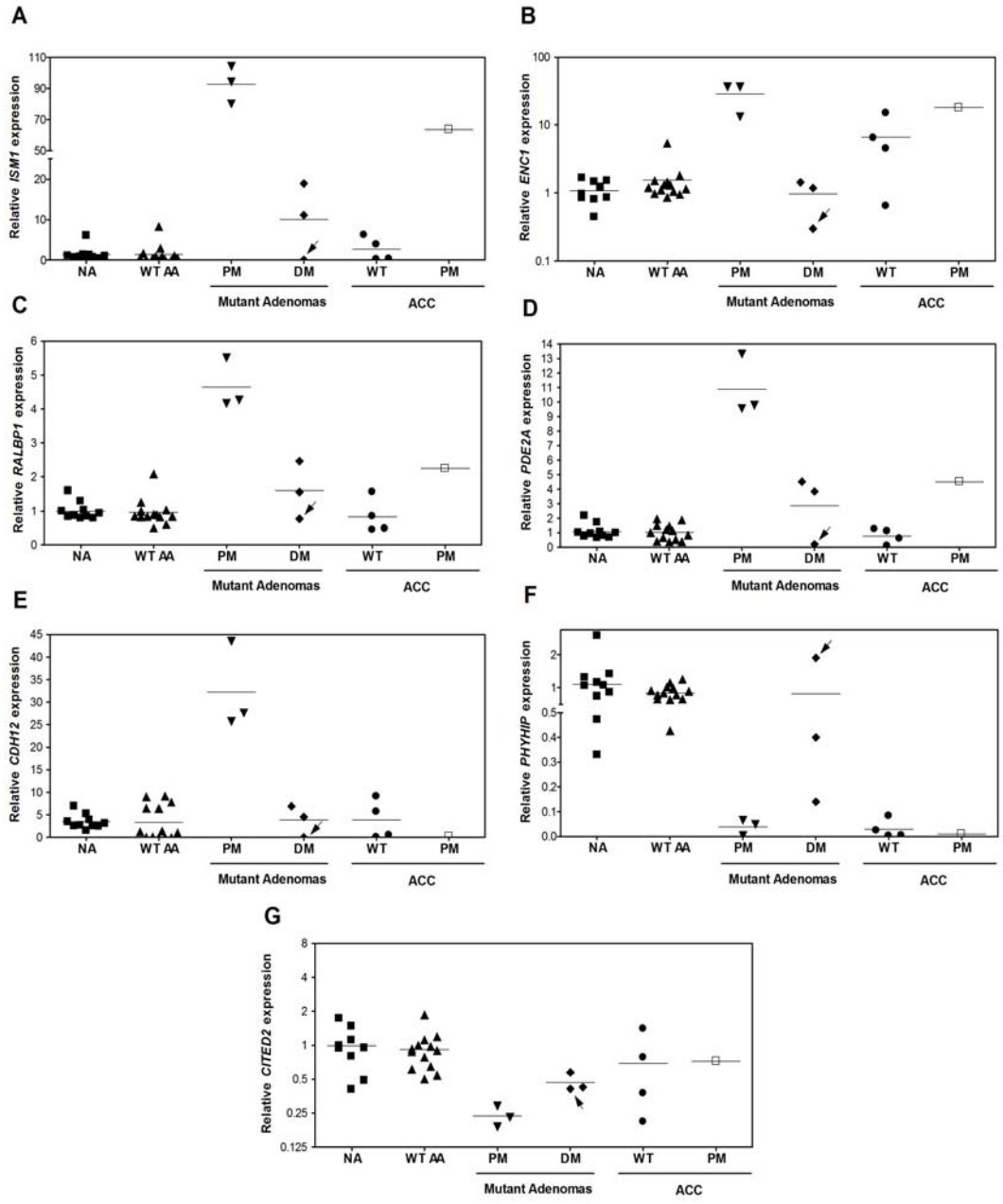
Affymetrix ID	Gene title	Gene symbol	Mutant/WT ratios	p-value
235182_at	**chromosome 20 open reading frame 82/ ISM1**	**C20orf82**	**30.07**	**0.005**
232888_at	**chromosome 20 open reading frame 82/ISM1**	**C20orf82**	**28.33**	**0.014**
207149_at	**cadherin 12, type 2 (N-cadherin 2)**	**CDH12**	**25.79**	**0.039**
1563933_a_at	phospholipase D family, member 5	PLD5	8.52	0.035
221577_x_at	growth differentiation factor 15	GDF15	8.40	0.044
201341_at	**ectodermal-neural cortex (with BTB-like domain)**	**ENC1**	**6.04**	**0.035**
223557_s_at	transmembrane protein with EGF-like and 2 follistatin-like domains 2	TMEFF2	5.78	0.035
232286_at	AF4/FMR2 family, member 3	AFF3	5.57	0,004
204134_at	**phosphodiesterase 2A, cGMP-stimulated**	**PDE2A**	**5.44**	**0.048**
201579_at	FAT tumour suppressor homolog 1 (Drosophila)	FAT	4.95	0.041
203699_s_at	deiodinase, iodothyronine, type II	DIO2	4.83	0.026
241954_at	farnesyl-diphosphate farnesyltransferase 1	FDFT1	4.59	0.025
220707_s_at	FAD-dependent oxidoreductase domain containing 2	FOXRED2	4.50	0.027
1566880_at	AF4/FMR2 family, member 3	AFF3	4.37	0.003
204428_s_at	lecithin-cholesterol acyltransferase	LCAT	4.33	0.019
206361_at	G protein-coupled receptor 44	GPR44	4.28	0.009
244696_at	AF4/FMR2 family, member 3	AFF3	4.04	0.000
234040_at	helicase, lymphoid-specific	HELLS	4.00	0.025
242549_at	protein kinase D3	PRKD3	3.92	0.011
1555382_at	premature ovarian failure, 1B	POF1B	3.91	0.010
239455_at	transcribed locus	---	3.87	0.016
1553229_at	zinc finger protein 572	ZNF572	3.84	0.039
238139_at	homo sapiens, clone IMAGE:4523945, mRNA	---	3.58	0.005
212750_at	protein phosphatase 1, regulatory (inhibitor) subunit 16B	PPP1R16B	3.57	0.021
201911_s_at	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1	FARP1	3.43	0.041
220640_at	casein kinase 1, gamma 1	CSNK1G1	3.42	0.001
233055_at	protein kinase D3	PRKD3	3.40	0.045
233884_at	human immunodeficiency virus type I enhancer-binding protein 3	HIVEP3	3.26	0.001
216228_s_at	WD repeat and HMG-box DNA-binding protein 1	WDHD1	3.26	0.015
1554910_at	protein kinase D3	PRKD3	3.25	0.021
239358_at	farnesyl-diphosphate farnesyltransferase 1	FDFT1	3.22	0.043
201596_x_at*	keratin 18	KRT18	3.21	0.010
1552390_a_at	chromosome 8 open reading frame 47	C8orf47	3.14	0.024
203069_at	synaptic vesicle glycoprotein 2A	SV2A	3.09	0.031
235488_at	RAS-like, family 10, member B	RASL10B	3.03	0.031
222361_at	similar to tubulin, β8	LOC64322	3.02	0.002
202844_s_at	**ralA-binding protein 1**	**RALBP1**	**3.00**	**0.000**
232027_at	spectrin repeat containing, nuclear envelope 1	SYNE1	3.00	0.017

Table 2. Summary of 81 probe sets corresponding to 75 genes with an intensity ratio of 4-fold and less in AA with *CTNNB1* point mutations compared to WT AA ($p<0.05$). Genes selected for validation are highlighted in bold. *Probes that were of poor quality or were unspecific for 1 sequence. IDEM la modification de table 1 si vous êtes d'accord Mutant: AA with *CTNNB1* point mutations. WT: AA with WT *CTNNB1* gene

Affymetrix ID	Gene title	Gene symbol	Mutant/WT ratio	p-value
240312_at	similar to CG4768-PA	LOC389895	0.007	0.013
203924_at	glutathione S-transferase A1	GSTA1	0.008	0.031
232636_at	SLIT and NTRK-like family, member 4	SLITRK4	0.019	0.031
1557146_a_at	hypothetical protein FLJ32252	FLJ32252	0.025	0.035
213247_at	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	SVEP1	0.039	0.025
219932_at	solute carrier family 27 (fatty acid transporter), member 6	SLC27A6	0.05	0.041
211748_x_at	prostaglandin D2 synthase 21-kDa (brain)	PTGDS	0.065	0.038
204777_at	spectrin repeat-containing nuclear envelope 2	SYNE2	0.07	0.050
212444_at	CDNA clone IMAGE:6025865	---	0.072	0.030
217767_at	complement component 3 /// similar to complement C3 precursor	C3 /// LOC653879	0.074	0.022
200872_at	S100 calcium-binding protein A10	S100A10	0.088	0.034
202995_s_at	fibulin 1	FBLN1	0.089	0.024
201787_at	fibulin 1	FBLN1	0.091	0.028
209981_at	cold shock domain containing C2, RNA-binding	CSDC2	0.093	0.005
228214_at	transcribed locus	---	0.094	0.012
202994_s_at	fibulin 1	FBLN1	0.095	0.030
206252_s_at	arginine vasopressin receptor 1A	AVPR1A	0.097	0.042
1569785_at	CDNA clone IMAGE:5287047	---	0.1	0.003
227826_s_at	sorbin and SH3 domain containing 2	SORBS2	0.106	0.039
202437_s_at	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	0.106	0.045
202920_at	ankyrin 2, neuronal	ANK2	0.121	0.024
220794_at	gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis)	GREM2	0.131	0.011
207213_s_at	ubiquitin-specific peptidase 2	USP2	0.134	0.011
206410_at	nuclear receptor subfamily 0, group B, member 2	NR0B2	0.135	0.005
205999_x_at	cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4	0.144	0.000
206638_at	5-hydroxytryptamine (serotonin) receptor 2B	HTR2B	0.145	0.020
206742_at	c-fos induced growth factor (vascular endothelial growth factor D)	FIGF	0.146	0.031
208607_s_at	serum amyloid A1 /// serum amyloid A2	SAA1 /// SAA2	0.147	0.013
229461_x_at	neuronal growth regulator 1	NEGR1	0.147	0.014
242524_at	cerebellin 4 precursor	CBLN4	0.15	0.013
217292_at	myotubularin-related protein 7	MTMR7	0.153	0.049
227498_at	CDNA FLJ11723 fis, clone HEMBA1005314	---	0.157	0.032
232081_at	ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	0.158	0.004
223582_at	G protein-coupled receptor 98	GPR98	0.159	0.001
202435_s_at	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	0.162	0.047
209159_s_at	NDRG family member 4	NDRG4	0.165	0.008
204174_at	arachidonate 5-lipoxygenase-activating protein	ALOX5AP	0.171	0.042
227271_at	fibroblast growth factor 11	FGF11	0.178	0.029
226213_at	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	0.182	0.039
205325_at	**phytanoyl-CoA 2-hydroxylase interacting protein**	**PHYHIP**	**0.187**	0.002
206698_at	x-linked Kx blood group (McLeod syndrome)	XK	0.196	0.022
203837_at	mitogen-activated protein kinase kinase kinase 5	MAP3K5	0.196	0.043
238673_at	transcribed locus	---	0.199	0.004
227598_at	chromosome 7 open reading frame 29	C7orf29	0.203	0.000
208189_s_at	myosin VIIA	MYO7A	0.203	0.036
222247_at	putative X-linked retinopathy protein	DXS542	0.205	0.013
215602_at	FYVE, RhoGEF and PH domain containing 2	FGD2	0.207	0.016
227506_at	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	SLC16A9	0.208	0.000
219610_at	Rho-guanine nucleotide exchange factor	RGNEF	0.208	0.009
201189_s_at	inositol 1,4,5-triphosphate receptor, type 3	ITPR3	0.211	0.012
203836_s_at	mitogen-activated protein kinase kinase kinase 5	MAP3K5	0.213	0.019
224339_s_at	angiopoietin-like 1 /// angiopoietin-like 1	ANGPTL1	0.215	0.037
204955_at	sushi-repeat-containing protein, X-linked	SRPX	0.215	0.032
209496_at	retinoic acid receptor responder (tazarotene induced) 2	RARRES2	0.219	0.003
203662_s_at	tropomodulin 1	TMOD1	0.22	0.013
231773_at	angiopoietin-like 1	ANGPTL1	0.221	0.021
236550_s_at	zinc finger protein 311	ZNF311	0.221	0.012
202308_at	sterol regulatory element-binding transcription factor 1	SREBF1	0.221	0.004
205727_at	telomerase-associated protein 1	TEP1	0.222	0.016
211864_s_at	fer-1-like 3, myoferlin (C. elegans)	FER1L3	0.224	0.039
229378_at	storkhead box 1	STOX1	0.224	0.028

230144_at	---	---	0.227	0.035
213849_s_at	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), β isoform	PPP2R2B	0.227	0.038
229975_at	transcribed locus	---	0.229	0.037
228195_at	hypothetical protein MGC13057	MGC13057	0.229	0.027
212884_x_at	translocase of outer mitochondrial membrane 40 homolog (yeast)	TOMM40	0.229	0.016
206645_s_at	nuclear receptor subfamily 0, group B, member 1	NR0B1	0.23	0.039
209613_s_at	alcohol dehydrogenase IB (class I), β polypeptide	ADH1B	0.23	0.018
211726_s_at	flavin containing monooxygenase 2 (non-functional)	FMO2	0.234	0.030
210073_at	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	ST8SIA1	0.236	0.010
1558388_a_at	family with sequence similarity 77, member D	FAM77D	0.236	0.009
1568190_at	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	GOT1	0.238	0.031
201010_s_at	thioredoxin-interacting protein	TXNIP	0.239	0.049
201667_at	gap junction protein, alpha 1, 43-kDa (connexin 43)	GJA1	0.243	0.011
207195_at	contactin 6	CNTN6	0.245	0.012
206171_at	adenosine A3 receptor	ADORA3	0.246	0.014
203424_s_at	insulin-like growth factor-binding protein 5	IGFBP5	0.246	0.017
218487_at	aminolevulinate, delta-, dehydratase	ALAD	0.247	0.008
239183_at	angiopoietin-like 1	ANGPTL1	0.248	0.005
217771_at	golgi phosphoprotein 2	GOLPH2	0.249	0.025

Figure 1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Relative ISM1 expression
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C
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Relative CITED2 expression
NA
WT AA
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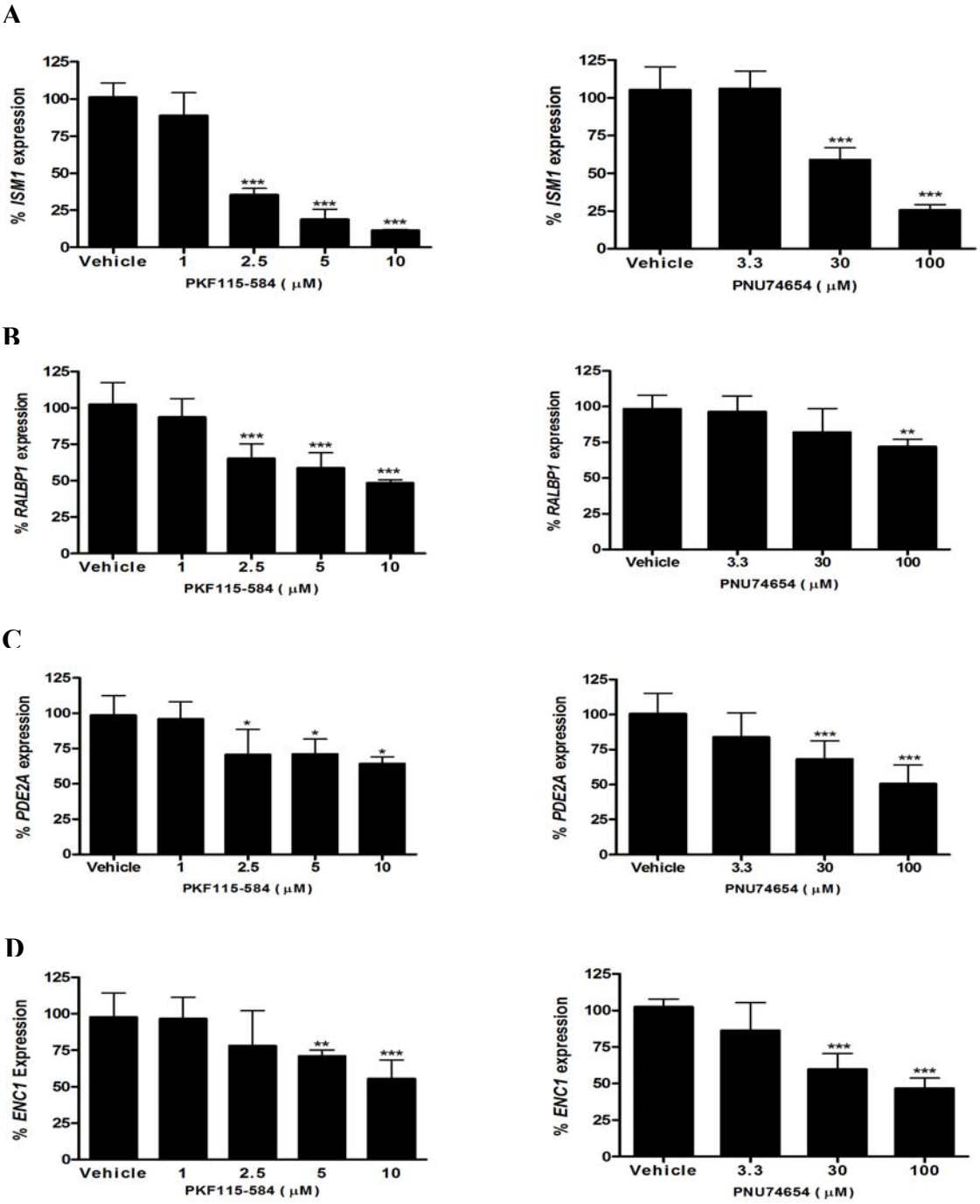
PM
Mutant Adenomas
ACC

Figure 2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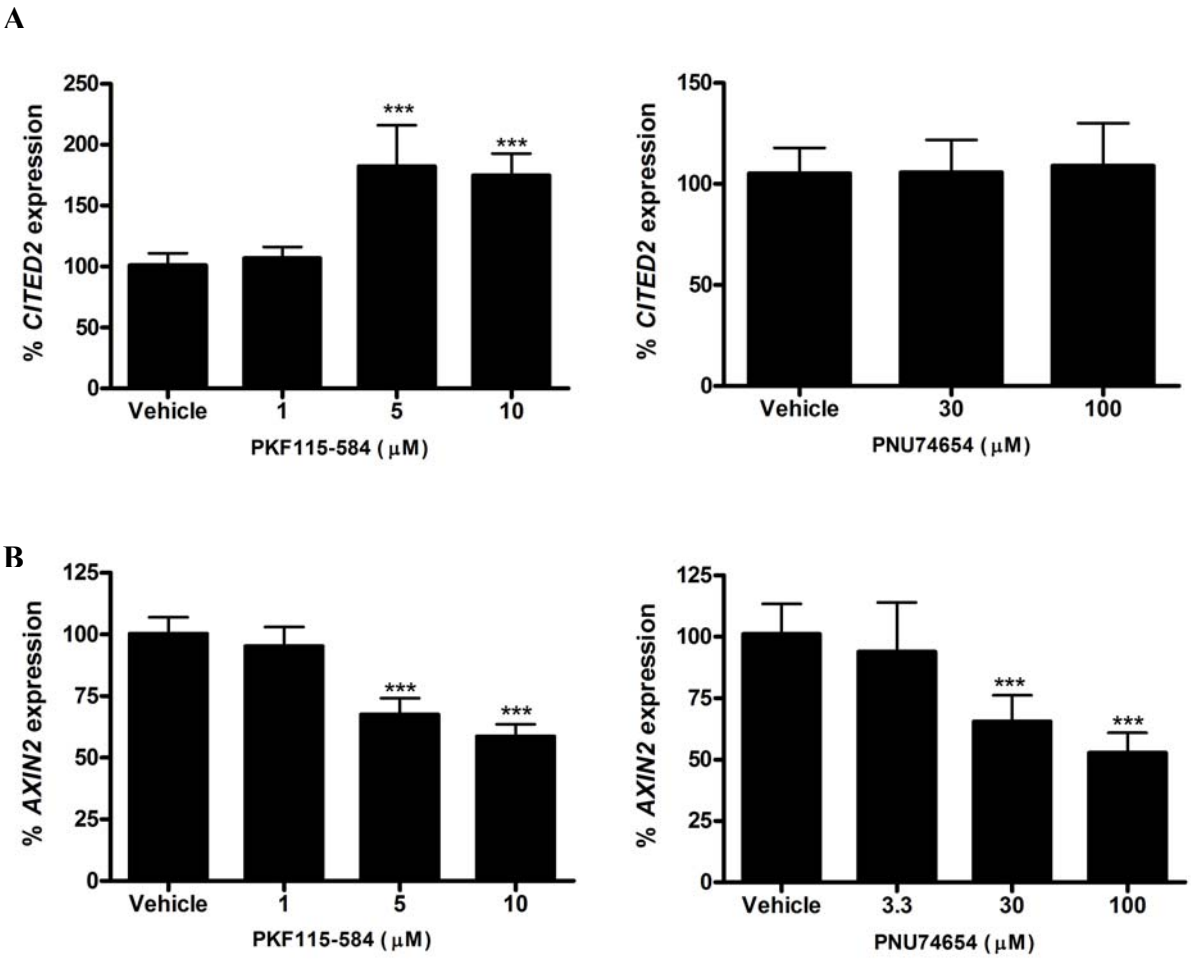
Vehicle
3.3
30
100
PNU74654 (μM)

Figure 3


A
% CITED2 expression
250
200
150
100
50
0
***
***
Vehicle
1
5
10
PKF115-584 (μM)
% CITED2 expression
150
100
50
0
Vehicle
30
100
PNU74654 (μM)
B
% AXIN2 expression
125
100
75
50
25
0
***
***
Vehicle
1
5
10
PKF115-584 (μM)
% AXIN2 expression
125
100
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25
0
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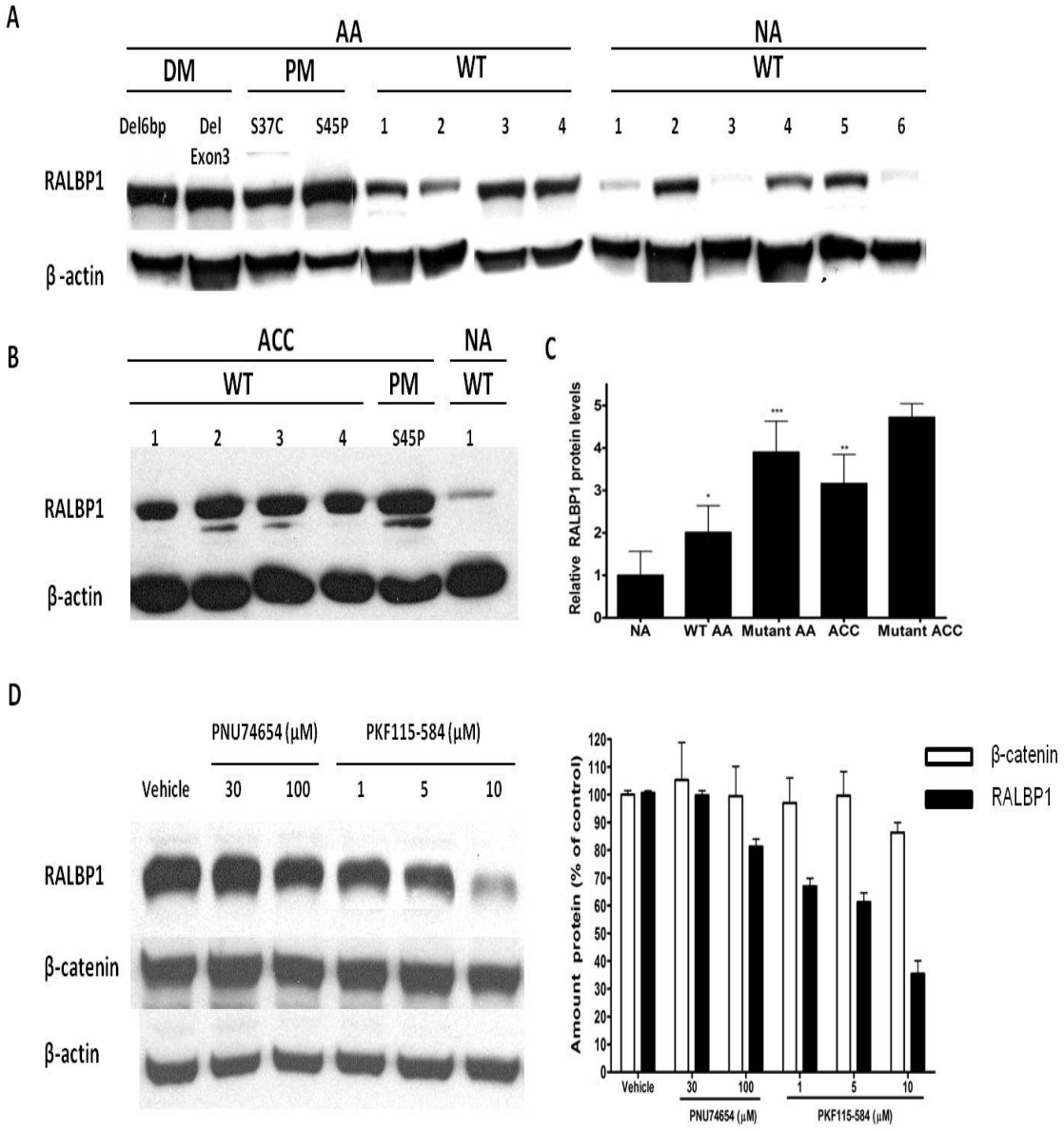
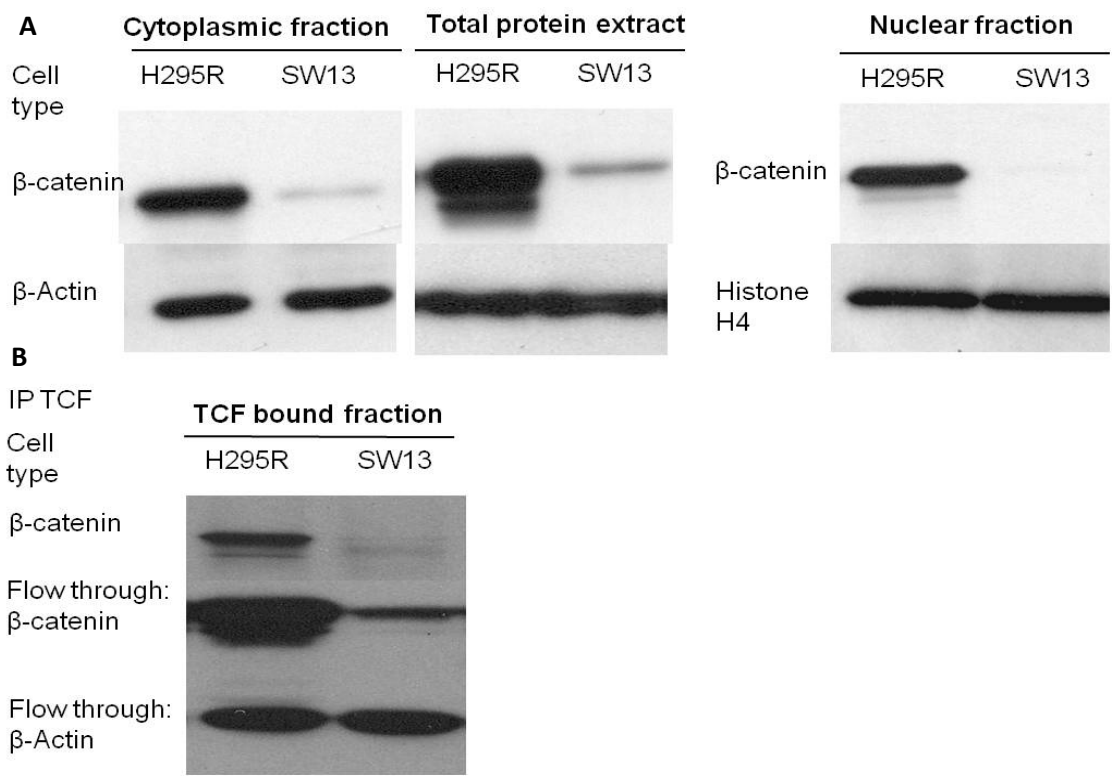
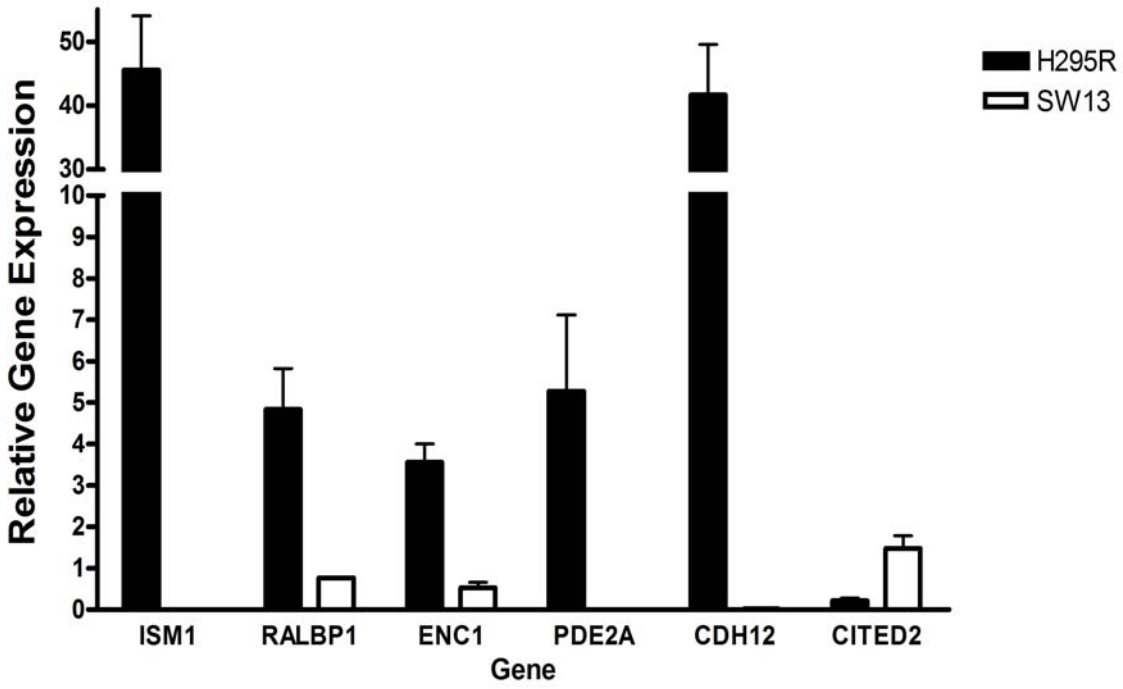
***
Vehicle
3.3
30
100
PNU74654 (μM)

Figure 4


A
AA
NA
DM
PM
WT
WT
Del6bp
Del Exon3
S37C
S45P
1
2
3
4
1
2
3
4
5
6
RALBP1
β-actin
B
ACC
NA
WT
PM
WT
1
2
3
4
S45P
1
RALBP1
β-actin
C
Relative RALBP1 protein levels
NA
WT AA
Mutant AA
ACC
Mutant ACC
D
PNU74654 (μM)
PKF115-584 (μM)
Vehicle
30
100
1
5
10
RALBP1
β-catenin
β-actin
Amount protein (% of control)
β-catenin
RALBP1
Vehicle
PNU74654 (μM)
PKF115-584 (μM)



Supplemental Figure 1. β-catenin status in adrenocortical cells. **(A)** Western blot of H295R and SW13 total protein extracts, cytoplasmic and nuclear fractions probed with anti-β-catenin antibody showing over-expression of β-catenin in H295R cells. Anti-β-actin antibody was used as control for total protein and cytoplasmic extracts and anti-histone H4 for nuclear fraction. **(B)** Immunoprecipitation of TCF4 (IP TCF) in H295R and SW13 cells. TCF4/β-catenin-bound fraction has higher expression in H295R than in SW13 cells. Non-immunoprecipitated fraction (flow through) was analyzed to detect TCF4-unbound β-catenin and β-actin levels.



S

Supplemental Figure 2. mRNA expression levels of *ISM1, RALPB1*, *ENC1*, *PDE2A*, *CDH12* and *CITED2* in H295R and SW13 cell lines relative to normal adrenal glands. The results represent the mean of 2 experiments performed with 2 different RNA extractions per tissue analyzed. Real-time PCR reactions were performed in triplicate.

Supplemental Table 1. Among the initial 1,509 probe sets statistically significant under Microarray analysis, PANTHER recognized 22 biological processes as being expressed differentially in adrenocortical tumors harbouring *CTNNB1* mutations compared to WT AA ($p<0.01$). Multiple probe sets associated with the same gene were counted as 1. The expected count for each class is based on representation of total classified genes in the human genome. Individual genes may belong to multiple classes. Classes highlighted in bold are overrepresented in over-expressed and under-expressed genes. The "Unclassified" category refers to genes which have not been associated with any biological process. Probe sets corresponding to hypothetical protein or transcripts, chromosome open reading frames were excluded from analysis.

Biological process	**Genes with under-expressed probe sets**			**Genes with over-expressed probe sets**		
	# found	# expected	p-value	# found	# expected	p-value
Unclassified	159	53.69	0.0000	94	31.88	0.000
Developmental process	44	24.12	0.0000	25	14.32	0.003
System development	33	16.32	0.0001	19	9.69	0.003
Immune system process	38	21.12	0.0002	15	12.54	0.268
Cell communication	54	35.08	0.0004	32	20.83	0.006
Cell adhesion	23	10.71	0.0004	16	6.36	0.001
Signal transduction	52	33.68	0.0005	30	20	0.011
Ectoderm development	23	11.46	0.0011	14	6.8	0.008
Nervous system development	21	10.11	0.0012	12	6	0.017
Cell motion	17	7.75	0.0021	9	4.6	0.041
Cellular process	68	50.29	0.0021	43	29.86	0.003
Cell-cell adhesion	15	6.42	0.0021	10	3.81	0.005
System process	30	17.81	0.0030	16	10.57	0.060
Immune response	14	6.08	0.0033	3	3.61	0.511
Response to stimulus	25	14.45	0.0049	8	8.58	0.509
Sensory perception	13	5.69	0.0050	7	3.38	0.053
Mesoderm development	22	12.28	0.0056	12	7.29	0.060
Sex determination	2	0.12	0.0067	0	0.07	0.931
Blood circulation	6	1.69	0.0074	1	1	0.735
Lipid metabolic process	17	8.99	0.0090	9	5.34	0.086
Muscle contraction	9	3.6	0.0107	2	2.14	0.639
Cell-cell signaling	19	10.7	0.0109	9	6.35	0.184
Heart development	0	2.93	0.0518	6	1.74	0.008

Supplemental Table 2. Genes related to Wnt/β-catenin signaling. 14 genes related to Wnt/β-catenin signaling were found to be de-regulated in adrenocortical adenomas with *CTNNB1* point mutations compared to *CTNNB1* WT by Microarray.

Probe set	Gene ID	Implication	Fold expression	p-value
204672_s_at	ANKRD6	WNT signaling pathway Negative regulator	1.93	0.008
219889_at	FRAT1	WNT signaling pathway	0.7	0.006
213222_at	PLCB1	WNT signaling pathway non-canonical	2.03	0.05
234040_at	HELLS	WNT signaling pathway	4	0.025
207416_s_at	NFATC3	WNT signaling pathway, Wnt3-independent	0.73	0.043
217656_at	SMARCA4	Chromatin remodelling	0.711	0.022
240297_at	SFRP4	WNT signaling pathway	0.523	0.045
207149_at	CDH12	WNT signaling Cadherin pathway	25.79	0.039
203874_s_at	SMARCA1	Chromatin remodelling	0.8	0.05
213251_at	SMARCA5	Chromatin remodelling	0.7	0.02
231767_at	HOXB4	WNT target gene	1.69	0.041
201341_at	ENC1	WNT target gene	6.04	0.035
201667_at	GJA1 (connexin 43)	WNT target gene	0.243	0.08
220640_at	CSNK1G1	WNT signaling	3.42	0.001

Chapter III. Discussion

Our laboratory focuses on identifying Wnt/β-catenin pathway alterations and understanding the implications/effects of Wnt/β-catenin in adrenocortical tumourigenesis. Our laboratory was the first to find β-catenin mutations in adrenal adenomas (*188*). β-catenin mutations are known to occur in about 20% of all adrenocortical tumours (benign and malignant). Theses mutations lead to tumour growth and progression. In the adrenal gland β-catenin is essential for development (143). At the beginning of this work, the effects of β-catenin mutations in the adrenal tumours had not been characterized and information was speculative. Relevance of the mutations in an adrenal tumour context was first demonstrated by Doghman et al (*218*), who demonstrated that the β-catenin/TCF inhibitor PKF115-584 inhibited growth and increased apoptosis of H295R cells (*218*). We have confirmed that this inhibitor as well as another inhibitor; PNU74654 which does affect H295R growth (results not shown). Recently, overexpression of a stable β-catenin mutant in mice was shown to induce adrenal hyperplasia and promote malignancy (*152*). Given the importance of β-catenin, it is surprising that only a few studies (*123-125, 127, 219, 220*) have been performed to globally find β-catenin target genes in a specific context. We designed a microarray experiment to distinguish gene expression profile of 3 adenomas with wild-type β-catenin and 3 adenomas with β-catenin mutations. Wild-type β-catenin adrenal tissues may have sporadic cytoplasmic and sparse diffuse nuclear staining. In order to determine β-catenin regulated genes, samples used with mutations were selected based on availability and strong widespread nuclear and cytoplasmic staining throughout the tumour.

The microarray analysis lead to the identification of novel target genes that may be regulated by β-catenin specifically in adrenal tissues. Our preliminary analysis showed that most genes known to be regulated by β-catenin various cancers were not aberrantly expressed in tumours with β-catenin mutations suggesting that most genes deregulated by β-catenin in the adrenal gland are tissue specific. In fact only one gene, *ENC1*, wich was previously shown to be regulated by β-catenin/TCF (*107*) was highly overexpressed (over 4-fold) in adenomas with *CTNNB1* point mutations. *ENC1* may contribute to colorectal carcinogenesis by suppressing cell differentiation (*107*) and has been linked to meningioma progression (*221*), but its role in adrenocortical tumourigenesis remains to be determined. *ENC1* was also found to be generally overexpressed in ACC but less than in tumours with β-catenin point mutations. As mentioned earlier, up to 70% of ACC may have aberrant accumulation of β-catenin (Figure 13, page 48); hence it is not surprising that certain genes deregulated in ACC may also be deregulated in adenomas with β-catenin mutations. The different levels of deregulation may be due to the fact that in some tissues aberrant accumulation of β-catenin may be heterogeneous across the tissue whereas the tissues harbouring β-catenin mutations that were studied in our microarray experiment had very high homogeneous nuclear accumulation of β-catenin as determined by IHC (previously (*153*) and results not shown). β-catenin nuclear accumulation is not the only element of gene expression that should be take into account since other co-factors may be required and this may explain why β-catenin activity may lead to expression of genes other than those previously reported in colon cancer. Microarray studies in other cancers (*123, 126*) have shown widespread difference in target genes and our results are in accordance to this variability between tissues.

ACCs and adenomas typically have very distinctive expression profiles(*62*), but ACCs often present β-catenin accumulation regardless of β-catenin mutational status (*164*). In regard to this fact, we searched for genes that were deregulated in our study compared to those found deregulated in ACC. Nine highly deregulated genes *ENC1, PHYHIP, FIBLN1, GPR98, HTR2B, RARRES2, SEMA6A, LRRN3, SLC16A9* in tumours with β-catenin mutations were found previously deregulated in ACC by previous microarray studies (*61, 62, 187*). In fact, the latter six genes have been found to be good diagnostic markers to differentiate benign and malignant adrenal tumours (*222*), and both *ENC1* and *PHYHIP* are found overexpressed in ACC compared to adenomas and normal adrenal glands. Although the functionnal implications of these genes in adrenal cell proliferation/survival is yet unknown, these finding do supports the hypothesis that deregulation of the Wnt/β-catenin pathway may cause malignant growth potential.

It is important to underline, that most genes identified as aberrantly expressed in tumours harbouring *CTNNB1* mutations have not been well characterized and their role in the development of adrencortical tumours is unknown. In addition, most genes were not previously known to be linked to β-catenin. Although, previous studies reported a link betweenβ-catenin and Wnt signaling in adrenocortical steroidogenesis (via the transcription factor SF1) (*151, 162*), no genes involved in adrenal steroidogenesis (Figure 2, page 4) were aberrantly expressed in β-catenin mutant adenomas. β-catenin mutations are found in all types of secreting adrenal tumours as well as non-secreting adenomas and carcinomas. Therefore, this is highly suggestive that β-catenin mutations do not affect the hormonal secretion profile of adrenal tumours.

In this study, we determined gene expression profile of 3 adrenocortical adenomas harbouring 3 point mutations in the *CTNNB1* gene including the S45P, T41A and, S37C mutations. Previous studies showed that depending of the localisation of the mutations, activation of gene expression by β-catenin may vary (*170, 223*) and therefore mutations in the regulatory sites (S45, S33, S37, T41) have been shown to lead to different potential activation of gene expression. Hence variability between the three tissues in the expression of β-catenin target genes was expected and this may have lead to false negative results in regard to β-catenin target genes. This fact may explain why genes typically regulated by β-catenin/TCF were not significantly differently expressed in our study. In fact, the gene *ENC1* was the only highly upregulated gene which was previously proven to be regulated by β-catenin/TCF (*107*). *ENC1* was also shown to be upregulated generally in ACC. As mentioned previously up to 70% of ACC (Figure 13, page 48) may have aberrant β-catenin accumulation, then it is not surprising that certain genes deregulated in ACC may also be deregulated in adenomas with β-catenin mutations. Aberrant accumulation of β-catenin may be heterogeneous but the mutant tissues selected for microarray analysis exhibit very high and homogeneous nuclear accumulation of β-catenin as detected by IHC (results not shown, and as described in Tadjine *et al* (*153*)) and this may explain why genes selected for validation were highly deregulated but in a lesser extent in Wt ACCs.

This study also permitted the identification of genes that had never been characterized in the adrenal gland. For example, although ISTHMIN 1*/ISM1* expression has never been reported in human tissues, it was previously found as regulated by β-catenin/TCF in zebrafish (*224*). *ISM1* as well has *CDH12* are apparently very weakly expressed in the normal adrenal but genes such as *RALBP1* and *PDE2A* are quite readily expressed suggesting that WNT/β-catenin not only activates gene expression of a subset of

genes, but may increase expression of constitutively expressed genes in the adrenal gland directly or indirectly. The low levels of *ISM1* expression in normal adrenal glands and WT adenomas suggest little or no role of ISM1 in the adrenal gland. In Xenopus, ISM1 was found to be a secreted protein apparently involved in development (*225*). In mice ISM1 was recently found to have a tumour suppressor role leading to inhibition of angiogenesis (*226*). If in fact β-catenin does directly lead to adrenal tumour formation, ISM1 may be activated as part of an auto regulatory loop, or hold a yet undetermined function in humans, as secreted proteins are known to potentially have contradictory roles in different contexts.

RALBP1 was another gene of particular interest due to its well characterized role in drug resistance (*227-230*). In adrenocortical carcinomas, drug resistance to typical chemotherapeutic agents is quite high and it is why surgery is the only efficient treatment since the response to chemotherapy is poor (*204*). Multidrug resistance is brought in part by the multi drug resistance gene (MDR1) which encodes for a p-glycoprotein. MDR1 levels are high (*231*) in normal adrenal glands and adrenocortical tumours. Moreover, MDR1 seems to be involved in aldosterone secretion (*232*). Drugs targeting p-glycoprotein activity have shown limited success in a clinical setting (*233*). Thus, *RALBP1* may be a new potential drug target to counteract multidrug resistance in adrenal cancers.

The *CDH12* gene also chosen for further validation due to its high differential expression was found to have a high variable expression in all adenomas with highest levels in those with β-catenin mutations. Originally thought to be a brain specific protein, it has been now found to be linked to tumor progression in non-small-cell lung carcinomas (*234*). CDH12 is part of the cadherins family of proteins, cadherins are calcium-dependent cell-cell adhesion molecules that mediate cell-cell binding to maintain cell:cell structures such as the epithelium. The impact of CDH12 on adrenal pathophysiology remains unknown. In

Wt adrenocortical adenomas, its expression is either downregulated or over expressed suggesting that it is not involved in tumour survival/growth. Other cadherins such as E-cadherin and N-cadherin are known to interact with β-catenin mediating cell junction and differentiation. Whether or not β-catenin and CDH12 interact, warrants further study since our work with inhibitors of β-catenin transcriptional activity did not show clear effect on *CDH12* expression (results not shown).

We also validated expression levels of *PDE2A* in our adrenal samples. *PDE2A* is a phosdiesterase capable of hydrolyzing both cGMP and cAMP (*235*). In adrenal glomerulosa cells, ANP (Atrial natriuretic peptide) activated PDE2A can block aldosterone accumulation by inhibiting cAMP (*236*). ANP is a peptide known to decrease secretarory responses in both the adrenal and pituitary gland (*237*). It is important to underline that in our microarray study we studied secreting-adrenocortical tumours whereas most β-catenin mutants found to date in other laboratories are in non-secreting lesions. Interestingly Wnt signaling has been linked to aldosterone secretion (*151, 152, 162*). In addition, forkskolin, a cAMP stimulator, leads to increased aldosterone levels in H295R cells despite the overexpression of *PDE2A*. In summary, *PDE2A* overexpression is probably not sufficient to affect hormone production without exterior activation, but increased PDE2A could provide an environment to decrease hormone production upon activation.

Most probes downregulated in adrenocortical tissues harbouring β-catenin mutations corresponded to partial transcripts and uncharacterized transcripts. Underexpressed *CITED2* and *PHYHIP* genes were validated by real-time PCR. *CITED2* like β-catenin is essential to adrenal development. The significance of decreased expression of *CITED2* in adrenal tumor is not clear but in colon cancer increasing *CITED2* leads to cell growth and inhibition of cell invasion in RKO cells (*238*). *PHYHIP* has been

characterized as an angiogenesis and neuronal differentiation inhibitor (*239*). *PHYHIP* expression is known to be repressed by the transcriptional repressor AP4 (*240*). Interestingly AP4 interacts with β-catenin and leads to its nuclear translocation (*241*) which supports the possible involvement of β-catenin in regulation of *PHYHIP* levels in a TCF independent fashion.

The main drawback of our project is the limited number of samples studied. However we were limited by the clinical availability of adrenocortical tissues harbouring β-catenin mutations. Due to the limited number of tissues studied, it is possible that subgroups of genes was found by chance in our dataset and are not linked to β-catenin.

Treatments of adrenocortical carcinoma cells H295R which harbour the *CTNNB1* S45P mutation with β-catenin/TCF inhibitors PKF115-584 and PNU74654 led to a decrease of *ISM1*, *PDE2A*, *ENC1,RALBP1* expression which further strengthen the link between β-catenin and the expression of these genes. *CITED2* mRNA expression in cells was increased upon treatment with PKF115-584 but not with PNU74654. It is therefore possible that β-catenin regulates *CITED2* in an alternative fashion. *CITED2* levels can be regulated by FOXO3A (*242*). FOXO competes with TCF for interaction with β-catenin, therefore inhibiting TCF transcriptional activity (*132*) . FOXO overexpression leads to reduced TCF/ β-catenin binding after oxidative stress, which simultaneously increases binding between β-catenin and FOXO (*132*). Furthermore, small interfering RNA-mediated knock down of FOXO reverts loss of β-catenin binding to TCF after cellular oxidative stress(*132*). PKF115-584 may lead to oxidative stress (*243*) in cells and therefore could lead to an increase of β-catenin and FOXO binding causing CITED2 overexpression.

Chapter IV. Conclusions

Through the use of microarray technology we have identified new genes potentially regulated by β-catenin. Our dataset incuded genes with various functions such as cAMP/cGMP regulation, angiogenesis, proliferation and tumour survival. RALBP1 was found at the protein level to not be only overexpressed in mutant tumours but also in direct correlation to tumour status (normal <adenoma<carcinoma). Further work is needed to better understand the role of Ralbp1 in drug resistance of treatment of adrenocortical cancer. Our work led also to the identification of *ISM1,* a newly discovered gene for which its implication in the adrenal gland is undetermined. Further studies using chromatin immunoprecipitation for example should be performed to confirm that ISM1 is a TCF/β-catenin target.

It must also be noted that our results, represent long term effects of β-catenin activation in a clinical perspective and thus most genes discovered to be deregulated in adrenocortical adenomas, with β-catenin mutations, may not be direct targets of β-catenin. We also highly agree that our data is based on a limited amount of tissues causing false positive and negative results and that this data would require confirmation with studies in future samples. *CDH12* for example was upregulated in adenomas with β-catenin mutations but not in the ACC mutant and consequently no changes in *CDH12* expression was detected upon treatment with β-catenin inhibitors in the H295R ACC cell line. However *ISM1*, *PDE2A*, *ENC1, RALBP1* were postviely confirmed by inhibitor treatement, demonstrated probable β-catenin gene targets. Further work using more direct approaches such as chromatin immupopreciptation assays (CHIP), electrophoresis mobility shift assay

(EMSA) or promoter assays will be required to determine and confirm genes directly regulated by β-catenin.

Finally, we believe that this project was a first step in understanding the molecular effects induced by β -catenin mutations in adrenal tumours and strengthened that β-catenin regulated genes are context and cell specific. Studying the whole transcriptome of β-catenin mutant tumours provided numerous avenues for further research and potential new therapeutic targets in the management of patients affected by adrenocortical tumours.

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