Aus der Medizinischen Klinik und Poliklinik IV,

Klinik der Ludwig-Maximilians-Universität München

Vostand: Prof. Dr. Martin Reincke

Expression and putative role of 14-3-3 proteins in corticotroph tumours

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Sicheng Tang

aus
Hubei, V.R. China

Jahr
2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. Marily Theodoropoulou
Mitberichterstatter:	PD Dr. Walter Rachinger
	Prof. Dr. Harald J. Schneider
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	28.07.2022

Table of contents

ZUSAMMENFASSUNG (DEUTSCH)

Das Cushing-Syndrom wird durch eine ACTH-Hypersekretion aus kortikotrophen Tumoren verursacht, die zu Hyperkortisolismus führt. Die genetische Grundlage des Cushing-Syndroms ist ein einzelner somatischer Mutations-Hotspot im *USP8*-Gen. *USP8*-Mutationen finden sich in etwa der Hälfte der Tumoren des Cushing-Syndroms (kortikotrophe Tumoren), wo sie die ACTH-Synthese verstärken. Der Mutations-Hotspot von *USP8* befindet sich in einer Region, die an 14-3-3-Adaptorproteine bindet. Diese regulieren die katalytische Aktivität des Gens indem sie seine Autoinhibition erleichtern. Die 14-3-3-Bindung ist in USP8-mutierten Tumoren gestört, wodurch diese daraufhin eine höhere Deubiquitinase-Aktivität aufweisen. 14-3-3-Proteine sind an vielen Signalwegen beteiligt und bei mehreren menschlichen Krebsarten dereguliert. Ihre Expression und Rolle in kortikotrophen Tumoren wurde jedoch noch nicht untersucht.

Ziel der vorliegenden Arbeit war es, die Expression von USP8-interagierenden 14-3-3-Proteinen in menschlichen kortikotrophen Tumoren zu untersuchen und ihre Rolle für die Funktion und die Lebensfähigkeit von kortikotrophen Tumoren in einem in vitro murinen kortikotrophen Tumorzellmodell zu untersuchen.

Alle 14-3-3-Proteine wurden mit unterschiedlichen Expressionsniveaus nachgewiesen, wobei 14-3-3ε in kortikotrophen Tumoren die höchste Expression im Vergleich zu normaler Hypophyse aufwies. In der Cushing-Patientenkohorte korrelierte eine hohe 14-3-3ε-Expression mit fehlender Invasion, besseren postoperativen Ergebnissen, vollständiger chirurgischer Resektion und postoperativer Remission sowie mit dem USP8-Mutationsstatus. Im Gegensatz dazu war die 14-3-3σ-Expression in kortikotrophen Tumoren im Vergleich zum Normalgewebe signifikant niedriger und es wurden keine signifikanten Korrelationen mit klinischen Parametern gefunden.

Die Überexpression von 14-3-3ε hatte keinen Einfluss auf die Zell-Viabilität der kortikotropen Tumorzellen in vitro, erhöhte aber die Aktivität des menschlichen *POMC*-Promotors. Eine Genanreicherungsanalyse Analyse der Genanreicherung zeigte potenzielle 14-3-3ε-Client-Proteine und -Prozesse auf, die an dieser

stimulierenden *POMC*-Regulierung beteiligt sein könnten. Die Verwendung von niedermolekularen Inhibitoren gegen die wichtigsten Ziele des 14-3-3ε-Signalwegs zeigte, dass MEK bei der 14-3-3ε-induzierten *POMC*-Promotoraktivität eine Rolle spielt.

Zusammenfassend lässt sich sagen, dass die vorliegende Arbeit eine abweichende Expression von 14-3-3ε in kortikotrophen Tumoren nachweist, die möglicherweise Auswirkungen auf die Progression und die Behandlung von Cushing-Tumoren hat.

Abstract (English)

Cushing's disease is caused by ACTH hypersecretion from corticotroph tumours resulting in hypercortisolism. The genetic basis of Cushing's disease is a single somatic mutational hotspot in the *USP8* gene. *USP8* mutations are found in around half of Cushing's disease (corticotroph) tumours, where they potentiate ACTH synthesis. The *USP8* mutational hotspot is within a region that binds to 14-3-3 adaptor proteins, which regulate its catalytic activity by facilitating its autoinhibition. 14-3-3 binding is disrupted in USP8 mutants, which subsequently have higher deubiquitinase activity. 14-3-3 proteins are involved in many signalling pathways and are deregulated in several human cancers. However their expression and role in corticotroph tumours has not been examined.

The aim of the present thesis was to investigate the expression of USP8-interacting 14-3-3 proteins in human corticotroph tumours and examine their role on corticotroph tumour function and cell viability in an *in vitro* murine corticotroph tumour cell model. All 14-3-3 proteins were detected at different expression levels, with 14-3-3ε showing the highest expression in corticotroph tumours compared to the normal pituitary. In the Cushing's patient cohort, high 14-3-3ε expression correlated with lack of invasion, better postoperative outcome, total surgical resection and postoperative remission, as well as, with *USP8* mutational status. In contrast, 14-3-3σ expression was significantly lower in corticotroph tumours compared to the normal tissue and no significant correlations with clinical parameters were found.

14-3-3ε overexpression did not affect corticotroph tumour cell viability *in vitro*, but it increased human *POMC* promoter activity. Gene enrichment analysis indicated potential 14-3-3ε client proteins and processes that may be involved in this stimulatory *POMC* regulation. Using small molecule inhibitors against the main 14-3-3ε signaling targets, revealed a role for MEK on 14-3-3ε-induced *POMC* promoter activity.

In conclusion, the present thesis demonstrates aberrant expression of 14-3-3ε in corticotroph tumours, which may affects Cushing's disease tumour progression and management.

List of Figures

List of Tables

List of Abbreviations

11β-HSD2	11-β-hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropin
AIP	Aryl hydrocarbon receptor-interacting protein
AKT	AK strain transforming
AMPK	AMP-activated protein kinase
AP1	Activator protein 1
ASK1	Apoptosis signal-regulating kinase 1
AVP	Arginine vasopressin
AVPR1B	Arginine vasopressin receptor 1b
BAD	Bcl2 associated agonist of cell death
BAX	Bcl2 associated X protein
Bcl	B-cell lymphoma
BIPSS	Bilateral inferior petrosal sinus sampling
BMI	Body mass index
BMP4	Bone morphogenetic protein 4
BRG1	Brahma-related gene 1
CABLES1	CDK5 and ABL1 enzyme substrate 1
cAMP	Cyclic adenosine monophosphate
CD	Cushing's disease
CDK	Cyclin-dependent kinases
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CRE	cAMP responsive element
CRH	Corticotrophin-releasing hormone
CRHR1	Corticotrophin-releasing hormone receptor 1
D2R	Dopamine receptor D2
E-box	Enhancer box
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FGFR4	Fibroblast growth factor receptor 4
FKHRL1	Forkhead transcription factor
FoxO	Forkhead BoxO
FSH	Follicle stimulating hormone
GC	Glucocorticoid
GH	Growth hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSK3β	Glycogen synthase kinase 3β
HDAC2	Histone deacetylase 2
HPA	Hypothalamic–pituitary–adrenal axis
HSP90	Heat shock protein 90
IGF-I	Insulin-like growth factor-I

IL	Interleukin
IP3	Inositol trisphosphate
IRS	Insulin receptor substrate
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LIF	Leukemia-inhibitory factor
LPH	Lipotropic hormone
MAPK	Mitogen activated protein kinase
MEK	MAP kinase extracellular signal-regulated kinase
MEKK	MEK kinase
MEN	Multiple endocrine neoplasia
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
MSH	Melanocortin stimulating hormones
mTOR	Mammalian target of rapamycin
NeuroD1	Neurogenic differentiation 1
nGRE	Negative glucocorticoid response element
Nurr1	Nur-related factor 1
NurRE	Nur response element
PC	Prohormone convertase
PDK1	Phosphoinositide-dependent kinase 1
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
Pitx	Paired-like homeodomain transcription factor
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POMC	Proopiomelanocortin
PRAS40	Proline-rich Akt substrate of 40kD
PRKCD	Protein kinase C delta
PVN	Paraventricular nucleus
RAF	Rapidly accelerated fibrosarcoma
SCN	Suprachiasmatic nucleus
Shh	Sonic hedgehog
SRC	Steroid receptor coactivator
SSTR	Somatostatin receptors
TNFα	Tumor necrosis factor alpha
TP53	Tumor suppressor 53
Tpit	Pituitary-restricted transcription factor
TR4	Testicular orphan receptor 4
TSC1/2	Tuberous sclerosis complex 1/2
TSH	Thyroid stimulating hormone
UFC	Urinary-free cortisol
USP8	Ubiquitin specific protease 8

1. Introduction

1.1. The pituitary gland

Pituitary gland is an oval body located in the sella turcica of the sphenoid bone on the ventral side of the lower thalamus [1]. It consists of the adenohypophysis and neurohypophysis that have different histological origins and functions.

The neurohypophysis, or posterior lobe, originates from the neuroectoderm and contains axonal projections from hypothalamic neurons. The posterior pituitary does not produce hormones, but stores the neurohypophyseal hormones vasopressin and oxytocin produced in the supraoptic and paraventricular nucleus of the hypothalamus and secreted through nerve fibers.

The adenohypophysis is composed of the anterior and intermediate lobes that derive from the oral ectoderm and include hormone producing endocrine cells. The anterior lobe contains five types of endocrine cells that secrete a specific trophic hormone: somatotrophs producing growth hormone (GH), corticotrophs synthesizing adrenocorticotropin (ACTH), lactrotrophs producing prolactin, thyrotrophs producing thyroid stimulating hormone (TSH) and gonadotrophs producing the gonadotropic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). Non-endocrine cells include the folliculostellate cells, which constitute 5-10 % of all pituitary cells and secrete factors involved in paracrine and juxtacrine signalling [2]. The intermediate lobe contains melanotroph cells that synthesize alpha melanocyte stimulating hormone. The intermediate lobe is rudimentary in humans.

1.2. ACTH

ACTH is mainly produced in the corticotroph cells that constitute 10-20% of endocrine cells in the anterior lobe under the trophic action of hypothalamic stimuli such as corticotrophin-releasing hormone (CRH) [3]. ACTH acts mainly on the adrenals where it stimulates glucocorticoid synthesis in response to stress and environmental changes [4]. In turn it is inhibited by glucocorticoids in a negative feedback loop [5]. (Figure 1.1).

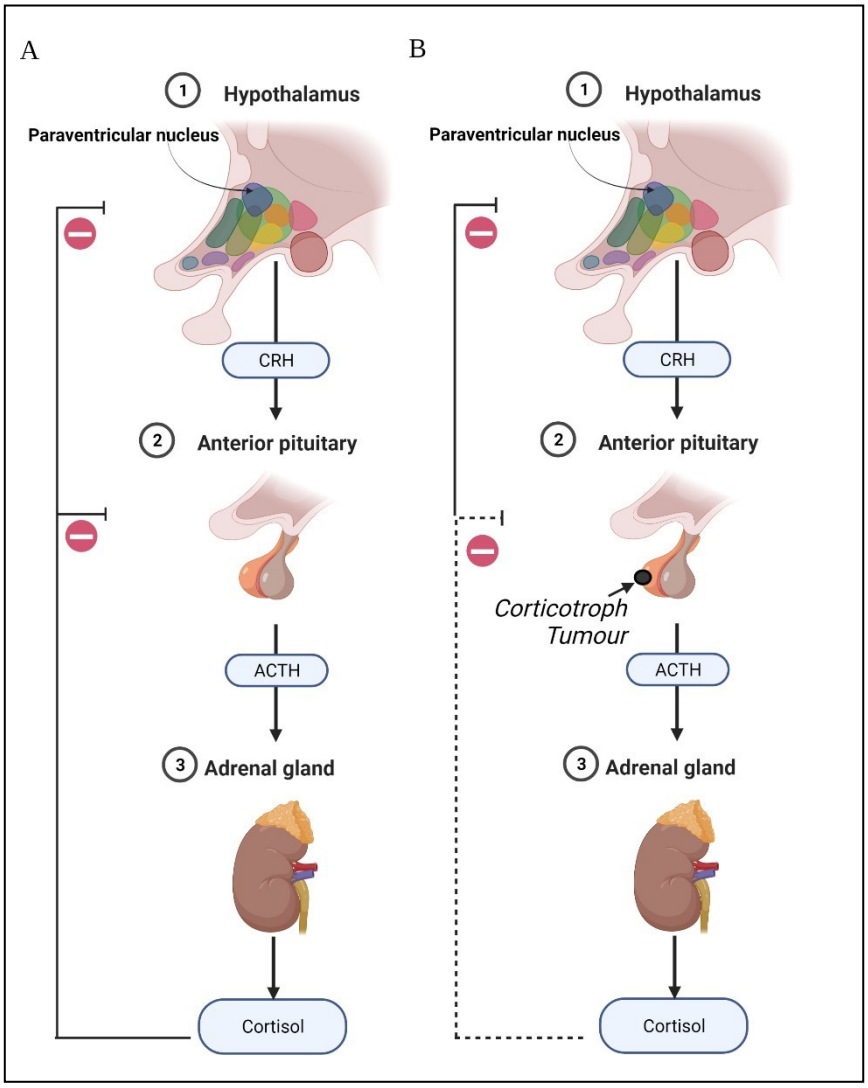


Figure 1.1. Schematic presentation of the hypothalamic pituitary adrenal (HPA) axis under physiological condition and in Cushing's disease. CRH is released from the paraventricular nucleus of the hypothalamus into the anterior pituitary where it stimulates ACTH synthesis and release. ACTH acts on the adrenal gland to trigger glucocorticoid synthesis. In turn, glucocorticoids inhibit ACTH in a negative feedback loop at hypothalamic and pituitary level (A). This glucocorticoid negative feedback loop is compromised in corticotroph tumours (B). This figure was created using BioRender.

ACTH [ACTH (1–39)] is derived from the prohormone proopiomelanocortin (*POMC*). The specific proteases prohormone convertase (PC) 1/3 and 2 cleave *POMC* into ACTH as well as other peptides that include the alpha-, beta- and gamma- melanocortin stimulating hormones (MSH), beta-lipotropin (or lipotropic hormone, LPH) and beta-endorphin [6]. PC1/3 is expressed in the anterior pituitary and predominantly cleaves *POMC* into ACTH, while PC2 is expressed in the intermediate lobe and cleaves into MSH and beta-endorphin [7, 8].

1.2.1. Regulation of ACTH synthesis

Human *POMC* is an 8 kilobase (kb) long gene located on chromosome 2p23 and consisting of three exons: exon 1 (86bp), exon 2 (152 bp) and exon 3 (833 bp, mainly encodes *POMC* in corticotrophs). It is mainly expressed in the pituitary gland and in the hypothalamus. A proximal promoter and enhancer region (-300bp) and 3 distal enhancer regions differentially regulate the pituitary (-7kb) or hypothalamic (-12kb and -10kb) *POMC* expression [6]. A -480 bp proximal promoter drives pituitary *POMC* transcription [9-13] (Figure 1.2). Primary regulatory elements for the pituitary specific transcription include the pituitary homeobox transcription factor Pitx1 (paired-like homeodomain transcription factor 1) and the T-box transcription factor Tpit (pituitary-restricted transcription factor).

Pitx1 belongs to the bicoid-related Pitx gene family that also includes Pitx2 and Pitx3 [14]. Pitx1 and Pitx2 are necessary for early pituitary development throughout the oral ectoderm [15, 16]. Pitx1 activates the expression of the major anterior pituitary hormones in synergy with other pituitary specific transcription factors and its inactivation was found to reduce gonadotroph and thyrotroph cell populations [17, 18]. Tpit (Tbx19) acts in cooperation with Pitx1 and is essential for *POMC* transcription [19, 20]. Tpit is pivotal for corticotroph differentiation and indeed *TBX19* mutations have been detected in patients with isolated ACTH deficiency [21, 22].

Another important, for corticotroph-specific expression, element is located upstream to the Pitx1/Tpit binding sites and is recognized by the basic helix-loop-helix transcription factor NeuroD1 (neurogenic differentiation 1) [23, 24]. NeuroD1 inactivation leads to delayed expression of *POMC* in anterior pituitary corticotrophs [25].

POMC transcription is under hypothalamic trophic and GC negative feedback regulation.

1.2.2. Hypothalamic regulation

ACTH synthesis and secretion is stimulated by the hypothalamic peptides CRH and vasopressin (or arginine vasopressin, AVP).

CRH is released from the paraventricular nucleus of the hypothalamus and reaches the

corticotroph cells where it triggers ACTH synthesis and release upon binding to CRH receptor 1 (CRHR1) [26, 27]. CRHR1 is a G (stimulatory) protein coupled receptor that upon ligand binding stimulates adenylate cyclase to increase cyclic adenosine monophosphate (cAMP) production and subsequent protein kinase A (PKA) activation and calcium dependent channel resulting in calcium accumulation [28, 29]. Although POMC promoter has a cAMP responsive element (CRE) and responds to canonical CRH-cAMP-PKA-CREB signalling cascade [30]. CRH mediates its effect through the Nur family of transcription factors that include Nur77 (also known as nerve growth factor I-B, NGFI-B), Nur-related factor 1 (Nurr1) and NOR-1 [31-33]. The *POMC* promoter has a NBRE site where Nur77 binds as monomer and a unique Nur response element (NurRE) that recognizes Nur77 dimers and is the primary CRH regulatory site [33]. CRH induces the translocation of Nur transcription factors to the nucleus and dimer binding downstream to PKA and MAPK [34, 35]. CRH induced cAMP/PKA facilitates the recruitment of steroid receptor coactivator (SRC)/p160 coactivators to Nur77 dimers and subsequently the synergy between Tpit/Pitx and Nur77 on the *POMC* promoter [36].

AVP is synthesized in the hypothalamic paraventricular nuclear neurons and supraoptic nuclei and stored in the posterior pituitary [37]. It stimulates ACTH release by binding to arginine vasopressin receptor 1b (AVPR1B), a Gq protein coupled receptor that is mainly expressed in corticotroph cells and stimulates *POMC* expression and ACTH secretion through the phospholipase C (PLC)/protein kinase C (PKC) pathway, which generates inositol trisphosphate (IP3) and increases intracellular calcium [38-40].

CRH is the primary trigger of ACTH secretion and both CRH and AVP stimulate ACTH release by activating calcium channels and increasing intracellular calcium concentrations [41-43].

Other factors stimulating ACTH synthesis are interleukin 2 (IL-2) and 6 (IL-6) and leukemia-inhibitory factor (LIF) that act in autocrine/paracrine manner in response to inflammation [44-46]. LIF acts via STAT3 direct or indirect stimulating *POMC* transcription and ACTH release [47, 48].

1.2.3. Glucocorticoid negative feedback

GC inhibit ACTH synthesis indirectly by downregulating *CRH* transcription in the PVN and directly at corticotroph cell level [49]. Glucocorticoids bind to GR that belongs to the nuclear receptor superfamily and may enhance or repress gene transcription by binding to GC response element (GRE) or negative glucocorticoid response elements (nGRE) [50]. The *POMC* promoter has an nGRE that bind GR in dimer-monomer complex [51, 52]. In addition, GC repress *POMC* transcription by antagonizing Nur77 at NurRE [53-55].

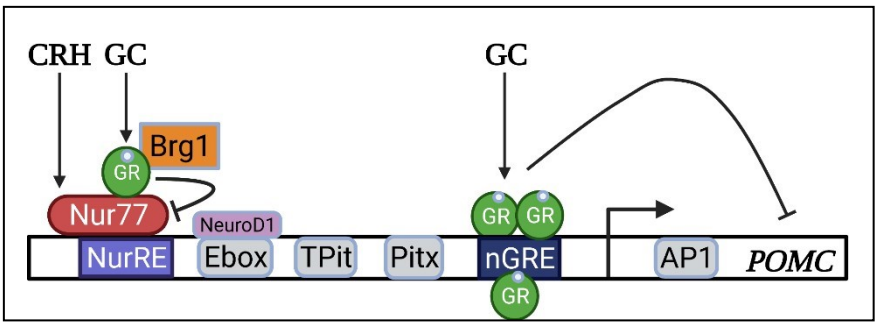


Figure 1.2. Schematic presentation of the human *POMC* promoter. *POMC* promoter contains among others Nur, TPit, Pitx, and AP1 (activator protein 1) regulatory elements. NeuroD1 binds on an E-box (enhancer box) element. CRH stimulates *POMC* promoter at the NurRE site via Nur77. GC bound GR represses *POMC* directly by binding as a trimeric dimer-monomer complex on a negative glucocorticoid responsive element (nGRE) and indirectly on the NurRE by blocking Nur77 in a mechanism involving the transcriptional coregulator Brg1. This figure was created using BioRender.

1.3. Cushing's disease

Harvey Cushing first reported a syndrome of endogenous central hypercortisolism in a young female patient (Minnie G) in 1912 and in the early 30's he linked the syndrome to ACTH producing pituitary tumours [56, 57]. Since then, Cushing syndrome names the disorder caused by endogenous or exogenous (iatrogenic) increase of cortisol and has a number of aetiologies [58, 59]. Endogenous Cushing syndrome may be ACTH-dependent due to corticotroph tumours (Cushing's disease) or in rare cases extrapituitary neuroendocrine tumours (ectopic) or ACTH-independent (primary

hypercortisolism) caused by benign adrenal adenomas or adrenocortical carcinomas [60].

1.3.1. Epidemiology

Cushing's disease (CD) is the most common cause of endogenous Cushing syndrome accounting for ~70% of endogenous hyperocrtisolism cases [61]. Cushing's disease is rare, with annual incidence 1.2-2.4 new cases per million [62, 63].The age at diagnosis is usually at 40-60 years and the disease is more prevalent in females, where it is three times more common than in males [64, 65].

1.3.2. Symptoms

Patients with Cushing's disease present with hot and reddish facial skin, abdominal striae and characteristic full moon face, buffalo back, abdominal fat, but relatively thin limbs. This uneven fat distribution may be due to the varying cortisol sensitivity of the different adipose tissue depots [66]. Metabolic syndrome is the most common presentation of the disease and includes visceral obesity, hyperglycaemia, insulin resistance, dyslipidemia and hypertension due to glucocorticoid excess [67]. Because of the damage to β-cells in pancreatic and reduced insulin sensitivity in the bones, the prevalence of impaired glucose tolerance and diabetes is 21-64% and 20-47% respectively in the patients with Cushing's disease [68]. The effects of excess cortisol on the structure and function of adipose tissue which also affected the secretion of glucose, further promoting insulin resistance and metabolic syndrome [69].

The prevalence of dyslipidemia in Cushing's disease patients ranges from 38% to 71%, mainly manifested by hypercholesterolemia and persistently elevated low density lipoprotein (LDL) cholesterol levels, which further contributes to the high risk of cardiovascular disease in Cushing's disease patients [68, 70].

High cortisol may lead to thickening of blood vessel walls and formation of atherosclerotic plaques in Cushing's disease patients, which further promotes arterial atherosclerosis, which is present in 1/3 of Cushing's disease patients [71]. Most patients with Cushing's disease have hypertensive [72], which are associated with the increase

of vasoconstriction, catecholamine sensitivity, and activation of the renin-angiotensin system [73, 74]. Chronic hypertension in patients with Cushing's disease can eventually lead to congestive heart failure. Excess cortisol also stimulates the synthesis of multiple coagulation factors by liver, such as fibrinogen, factor IX, and von Willebrand factor, and also induces the synthesis of plasma plasminogen activator inhibitors [75]. This suggests that the hypercoagulable state in CD patients which is prone to thromboembolism and increases the risk of cardiovascular disease [76].Cardiovascular disease, like diabetes and insulin resistance, is irreversible in patients with Cushing's disease [77].

Excess cortisol inhibits pituitary gonadotropin, which is more common in women and manifests as decreased libido, low menstruation, amenorrhea, breast overflow, infertility, etc. It manifests as hypogonadism, a sharp drop in testosterone and atrophy of the testicles in men. Spontaneous ACTH excess stimulates an increase in adrenocorticotropic hormones, which can be seen in women as hirsutism, with a significant hair growth on the back and face， and an enlarged laryngeal knot. Acne can occur in men (mostly on the face) [78]. Excess glucocorticoids mainly act on the bone trabeculae, inhibiting bone formation and increasing bone resorption, leading to osteoporosis and bone fractures. Studies have shown that about 40% of patients with Cushing's disease have osteoporosis, 76% have bone fractures. The most common place are vertebral fractures. Spinal compression fractures can affect the patient's height, and cause ribs or pelvis pathological fractures [79, 80]. Bone damage is partially reversible and is associated with the degree of cortisol levels and duration. In children with Cushing's disease, growth retardation often occurs and the damage of height is reversible if with treatment promptly [81].

Chronic high cortisol can compromise immune mechanisms and increase the chance of infection [82, 83]. Fungal infections are especially more common. The type of infection and the degree of risk are related to cortisol levels [84]. The common complication of the infection is sepsis, which will increase the possibility of death in Cushing's disease [85].

Dysregulation of cortisol often leads to psychiatric disorders, neurocognitive impairment, and reduced quality of life in Cushing's disease patients. Mild mental disorders manifest themselves as anxiety, emotional instability, sleep disorders, and in severe cases, depression, mania or even suicide. Depression and anxiety are diagnosed in 51%-84% of Cushing's disease patients [68]. Depression is the most common of these, with 50-80% of patients suffering from it [86]. Studies have shown that patients with Cushing's disease still have psychiatric symptoms after surgery, which may be related to the chronic glucocorticoid overload that stimulates the central nervous system [87]. In terms of neurocognitive impairment, short-term memory and cognitive impairment are the most common and irreversible manifestations, which may be related to partial brain volume damage and brain atrophy in patients [88]. Impairments in language learning and spatial information have also been reported [89]. These lead to a lower quality of life for the patient [90].

1.3.3. Diagnosis of Cushing's disease

The diagnosis of Cushing's disease primarily relies on laboratory tests and radiologic examinations [91]. The most common laboratory tests include 24-hour urinary-free cortisol (UFC), late-night salivary cortisol (or midnight serum cortisol), and low-dose dexamethasone suppression test. Secondly, after confirming the Cushing's syndrome diagnosis, it needs to be determined if it is ACTH-dependent or ACTH-independent. And thirdly, after determining ACTH-dependent cause, magnetic resonance imaging (MRI) and other tests such as bilateral inferior petrosal sinus sampling (BIPSS) are used to determine if the source of ACTH is a pituitary or ectopic tumour [91].

24-hour urinary free cortisol (UFC) represents unbound cortisol in the urine filtered through the glomeruli [60]. If the UFC values are more than 4 times higher than the upper limit of normal, then one should consider Cushing's syndrome [64]. However, this test may be influenced by sex, age, body-mass index (BMI) and should not be considered in patients with renal impairment [92].

Late-night salivary cortisol is able to detect the loss of the circadian nadir of cortisol secretion characteristic of Cushing's sydnrome [93]. It has high accuracy and the

current recommendations call for collection of two independent samples to smoothen out day-to-day variability and the impact of factors like stress [94].

Overnight low-dose (1mg) dexamethasone suppression test (LDDST) is based on the GC negative feedback loop on the HPA axis. Lack of cortisol suppression after dexamethasone indicates the presence of Cushing's syndrome. Patients with Cushing's syndrome have higher cortisol levels, which cannot be supressed by LDDST. The recommended cut off is 1.8µg/dl, which achieves high sensitivity rates and specificity rates of 80% [62]. Cortisol values may vary depending on the assay used [62].

Once the diagnosis of Cushing's syndrome is confirmed, determining of plasma ACTH can differentiate ACTH-dependent causes (pituitary adenoma or ectopic tumour) or ACTH-independent (adrenal tumour) [60]. Morning plasma ACTH values in ACTH-dependent patients are usually higher than 20pg/ml. ACTH levels tend to be higher in ectopic ACTH-secreting than in pituitary. If the plasma ACTH value is less than 5pg/ml, it is mostly ACTH-independent [95]. And if the value is between 5-20pg/ml, CRH stimulation test is required for further determination. If plasma ACTH level is significantly elevated after CRH stimulation, the patient is more likely ACTH-dependent [60]. High-dose (8mg) dexamethasone suppression test can be used to distinguish between pituitary and ectopic origin, since patients with corticotroph tumours retain GC negative feedback response with reduced plasma or urinary cortisol levels [96]. MRI can be used for detecting pituitary tumours. MRI has a higher diagnosis rate which is more helpful to neurosurgeons for further surgical treatment [97]. If the tumour is too small to be seen by MRI positron emission tomography (PET) can be performed [92] (Figure 1.3).

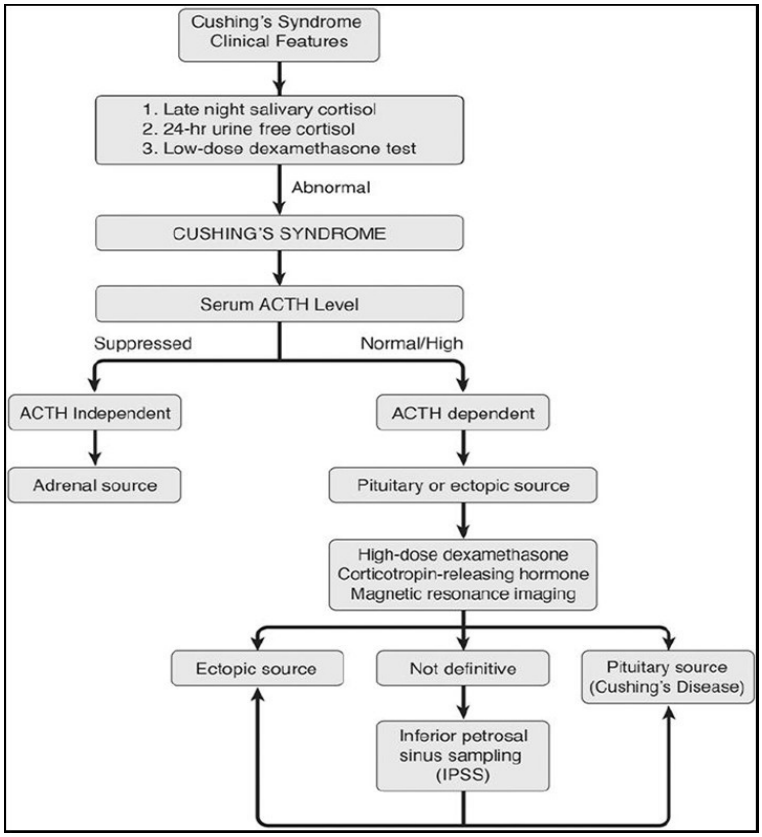


Figure 1.3. Diagnosis of Cushing's disease. Patients who have clinical suspicion of Cushing's syndrome need to do laboratory tests such as late-night salivary cortisol, 24-hour urine free cortisol and low-dose dexamethasone suppression testing. Elevated results of these tests could confirm Cushing's syndrome. Measurement of plasma ACTH can differentiate ACTH-dependent causes (pituitary adenoma or ectopic tumour) or ACTH-independent (adrenal tumour). CRH stimulation to elevate the serum ACTH levels could enhance the possibility of ACTH-dependent. Cortisol suppression after high-dose of dexamethasone suppression test and magnetic resonance imaging (MRI) can be used for detecting pituitary adenomas (Cushing's disease). Inferior petrosal sinus sampling could be used to determine if hypercortisolism is from a pituitary adenoma (CD) for the patients who is ACTH-dependent with negative pituitary MRI. Adapted from Lonser, R. R, et al. Journal of neurosurgery, 2017 [98].

1.4. Treatment of Cushing's disease

For Cushing's disease patients, the first choice of treatment is transsphenoidal pituitary surgery [99]. Repeat transsphenoidal pituitary surgery, radiation therapy, bilateral adrenalectomy and pharmacological therapy constitute additional treatment options.

Patients whose tumour cannot be resected or with recurrent hypercortisolism after operation or who are not candidates for surgery may benefit from pharmacological treatment [100]. The aim of the pharmacological therapy is to lower the excess cortisol

production and associated comorbidities. Currently approved pharmaceuticals are divided into tumour-targeted aimed at decreasing ACTH release and peripheral-targeted that include steroidogenesis inhibitors and glucocorticoids receptor antagonists.

1.4.1. Tumour-targeted

Tumour targeted pharmaceuticals include dopamine agonists and somatostatin analogues.

Dopamine receptor D2 (D2R) are expressed in corticotroph tumours and agonists such as cabergoline achieve UFC normalization and lower ACTH secretion in up to ~40% of Cushing's disease patients [101, 102]. However long-term response to cabergoline was only reported in 30% of patients and some of them showed no sustained control of hypercortisolism and escape phenomenon after 2-5 years treatment was observed in 25% of patients [103, 104]. Nevertheless, cabergoline could be indicated for the management of Cushing's disease during pregnancy [105].

Corticotroph tumours also express somatostatin receptors (SSTR5) and predominantly SSTR5 and respond to the SSTR5 ligand pasireotide by decreasing ACTH secretion [106, 107]. Pasireotide treatment for 12-24 months normalized UFC levels in ~25% of patients with Cushing's disease and showed improvement of clinical signs [108-110]. The long-acting release form (pasireotide-LAR) showed efficacy in around 40% of patients in terms of 24-hour UFC normalization [111]. Pasireotide is the only tumour targeted treatment approved for Cushing's disease. However, a common side effect is hyperglycaemia which needs to be monitored and managed during the treatment [109, 112, 113].

A meta-analysis demonstrated that less than half patients with Cushing's disease respond in term of biochemical normalization to pasireotide or cabergoline (41% and 35.7% respectively) [114]. Potential alternative tumour targeting therapeutics under investigation include retinoic acid, the heat shock protein 90 (HSP90) inhibitor silibinin, the epidermal growth factor receptor (EGFR) inhibitor gefitinib and the cell cycle inhibitor roscovitine (reviewed in [115]).

1.4.2. Periphery-targeted

Steroidogenesis inhibitors include ketoconazole, metyrapone, etomidate and mitotane [116]. Ketoconazole is used as a second-line treatment (after failed surgery or radiotherapy) resulted in normalization or reduction of UFC >50% in 75% of patients [117, 118]. Metyrapone effectively reduced cortisol levels in more than 80% of patients in combination other steroidogenesis inhibitors and more than 50% achieved biochemical eucortisolemia with metyrapone monotherapy [119]. However, its use is limited by side effects such as hirsutism, acne, hypokalemia and hypertension [120]. Etomidate is used under surveillance to control life threatening hypercortisolism in critically ill patients, while the high toxicity of mitotane limits its use to patients with adrenocortical carcinoma [121]. In March 2020, a new oral steroidogenesis inhibitor, osilodrostat, was approved by the FDA for the treatment of Cushing's disease patients who have failed or are unable to undergo surgery [122]. Phase II and III studies showed osilodrostat to have effective rate (UFC or serum cortisol normalization) of 66.4-91.7% and high safety profile, but the long-term observation on pituitary tumour in Cushing's disease patients is still needed [123, 124].

The GR antagonist mifepristone is mainly aimed at alleviating clinical symptoms [125]. It improved clinical symptoms and comorbidities, especially glucose metabolism, weight loss and blood pressure reduction in 38.1-75% of Cushing's disease patients [126].

These periphery-targeted pharmaceuticals result in ~50-80% cortisol normalization [114]. However, they have considerable side effects and they do not target the source of the disease, which is the corticotroph tumour [127].

1.5. Pathogenesis of corticotroph tumours

Corticotroph tumours are monoclonal neoplasms that occur sporadically. Loss of the negative glucocorticoid feedback is a hallmark of Cushing's disease [62]. The *NR3C1* gene that encodes for the glucocorticoid receptor is mutated in ~6% of corticotroph tumours, so genetically defective GR may be responsible for partial resistance to

negative glucocorticoid feedback in only a small subset of cases [128-130]. GR expression is not altered in corticotroph tumours, which show abundant expression of 11-β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) that converts cortisol to cortisone [128, 131]. The GR chaperon HSP90 is overexpressed in corticotroph tumours and its inhibition with a C-terminal inhibitor sensitizes corticotroph tumour cells to glucocorticoids [132].

Ligand bound GR suppresses *POMC* transcription as part of a transcriptional complex that features, among others, Brahma-related gene 1 (BRG1) and histone deacetylase 2 (HDAC2) [133]. Corticotroph tumours show loss of BRG1 and HDAC2 and this may contribute to GC resistance. Another GR regulator is the nuclear testicular orphan receptor 4 (TR4) that blocks GR binding to target promoter and which was shown to be overexpressed in corticotroph tumours [134].

1.5.1. Mechanisms of corticotroph tumorigenesis

Cell cycle deregulations

Corticotroph tumours show aberrant expression of proteins controlling cell cycle like cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors of the Cip/Kip family (p21, p27, and p57) and INK4 family (p16, p18, and p19) [135]. Cyclin E (encoded by the *CCNE1* gene), which activates the CDK2, is overexpressed in corticotroph tumours, while p27/Kip1 (encoded by cyclin-dependent kinase inhibitor 1B, *CDKN1B*) that inhibits the cyclin E/CDK2 complex is reduced at protein level [136, 137]. CDK2 complexed with cyclin E phosphorylates p27/Kip1, marking it for proteasomal degradation [138]. Genetically engineered mice overexpressing cyclin E and insufficient for p27 showed increased frequency, size and proliferation index of pituitary tumours [139]. Corticotroph tumours show loss of the tumour suppressor, transcriptional coregulator BRG1; BRG1 suppresses *CCNE1* transcription and this may account for the high cyclin E levels and subsequent loss of the cell cycle inhibitor p27/Kip1 observed in corticotroph tumours [133]. Another factor that may contribute to p27/Kip1 loss in corticotroph tumours is the downregulation of CABLES1 (CDK5 and ABL1 enzyme substrate 1), a tumour suppressor that stabilizes the members of the

Cip/Kip family of CDK inhibitors and causes CDK inactivation and cell cycle arrest [140]. CABLES1 is mainly downregulated at posttranscriptional level [141].

Growth factors & cytokines

Epidermal growth factor (EGF) acts in a trophic manner similar to CRH to stimulate ACTH synthesis [142]. EGF was found to trigger POMC expression and bromodeoxyuridine (BrdU) incorporation in dispersed corticotroph cells [143]. EGF receptor (EGFR) is highly expressed in corticotroph tumours, which may account for the low p27/Kip1 levels in these tumours [144].

Another growth factor receptor overexpressed in corticotroph tumours is fibroblast-growth factor receptor 4 (FGFR4). Studies showed that the FGFR4-Gly (388) allele polymorphism associates with higher frequency of postoperative recurrence and persistence in patients with Cushing's disease [145, 146].

Contrary to EGFR and FGFR4 overexpression mentioned above, corticotroph tumours show downregulation of bone morphogenetic protein 4 (BMP4), which is a member of the transforming growth factor-beta superfamily. BMP4 inhibits proliferation, *POMC* transcription and ACTH synthesis *in vitro* and *in vivo* [147]. A study suggested that BMP4 might mediate the antiproliferative and antisecretory effects of somatostatin analogues in corticotroph tumours [148].

Another factor involved in corticotroph pathophysiology is the developmental factor sonic hedgehog (Shh), whose expression in the adult pituitary is restricted to the corticotroph cells. Gli1 downstream to Shh activates CRHR1 transcription, enhancing the stimulatory action of CRH on ACTH secretion, while at the same time Shh pathway restricts corticotroph cell proliferation [149]. Shh is downregulated/lost in corticotroph tumours [150].

The pituitary gland also expresses cytokines and their receptors, which regulate hormone synthesis in an autocrine-paracrine manner [151]. IL2 and IL6 are expressed in corticotroph tumours where they stimulate ACTH production [44, 46]. Similarly, the LIF, a pleiotropic immune cytokine that belongs to the IL-6 superfamily, cooperates with CRH to enhance *POMC* transcription and ACTH synthesis [152]. Its

overexpression in transgenic mice induces high levels of ACTH and cortisol, Cushingoid features and reduced sensitivity to dexamethasone [153].

MicroRNAs

MicroRNAs (miRNAs) are 22 nucleotides long RNAs that regulate gene expression post-transcriptionally or repress their translation [154]. Corticotroph tumours show downregulation in miR-let7a, -15a, -16, -21, -141, -143, -145 and -150 and upregulation in miR-26a, -30, -122 and -493 compared to the normal pituitary, but no associations were found with tumour size or remission after surgery, except miR-141 that correlated with higher remission rate [155]. Corticotroph tumours have upregulated miR-493, which targets E2F1 and correlates with tumour aggressiveness [156]. MiR-26a, which targets protein kinase C delta (PRKCD), promotes corticotroph tumour cell viability by regulating cyclin E [157].

1.5.2. Genetics of Cushing's disease

Corticotroph tumours are rarely seen in genetic syndromes such as multiple endocrine neoplasia (MEN) 1, MEN2 and MEN4 (reviewed in [158]). Patients with DICER1 syndrome may present with ACTH-producing pituitary blastoma [159]. Patients with germline mutations in other genes such as *PRKAR1A* (Carney complex), aryl hydrocarbon receptor-interacting protein (*AIP*) and tuberous sclerosis complex 1/2 (*TSC1/2*) only rarely present with corticotroph tumours [160-162]. A study on 146 paediatric and 35 adult Cushing's disease patients detected germline pathogenic missense variants in CABLES1 in four female patients (2 adult and 2 paediatric) presenting with large tumour and higher Ki-67 proliferation index [141].

The majority of corticotroph tumours are sporadic. Their monoclonality established in the early 90's indicates the existence of single somatic genetic events that transform the corticotroph cell and trigger monoclonal tumour expansion [163]. Mutations in oncogenes and tumour suppressor genes frequent in other cancers are rare in corticotroph tumours (reviewed in [164]). Somatic mutations were found in sporadic corticotroph tumours in:

USP8

Whole exome sequencing identified a single mutational hotpot in the ubiquitin specific protease 8 (*USP8*) gene in ~40% of corticotroph tumours [165-168].

USP8 is a deubiquitinase that cleaves the ubiquitin from target proteins such as EGFR rescuing them from lysosomal degradation [165, 169]. Overexpressing USP8 mutants enhances EGFR-induced ACTH synthesis with minimal effect on corticotroph cell proliferation [165]. The two primary signalling pathways downstream to activated EGFR are MEK/ERK and PI3K/Akt; in corticotroph tumour cells, USP8 specifically potentiates the EGF-induced ERK pathway to regulate ACTH synthesis [165].

The *USP8* mutational hotspot is located in exon 14 in a region that contains a motif that binds to 14-3-3 proteins that inhibit its activity [170]. The mutations found in corticotroph tumours, which include p.Ser718Pro, p.Ser718Cys, p.Pro720Arg and p.Ser718del, disrupt the 14-3-3 binding motif RSYSSP and 14-3-3 protein binding, allowing the site to be accessed by proteolytic enzymes that cleave the mutant USP8 to a highly catalytically active C-terminal 40-KD fragment [165]. Furthermore, the USP8 protein has an autoinhibitory region that binds to the ubiquitin-binding pocket of its deubiquitinase domain; as this autoinhibitory interaction is facilitated by association with 14-3-3 proteins, disruption of 14-3-3 binding to USP8 mutants confers them a higher catalytic activity [171, 172].

Patients with *USP8* mutant tumours are predominantly female have smaller, noninvasive tumours, but higher postoperative cortisol levels, worse postoperative prognosis and higher likelihood of tumour recurrence at an earlier time [164, 165, 173]. *USP8* mutant tumours have higher expression levels of somatostatin receptor 5 (SSTR5) suggesting favourable response to pasireotide [167].

USP48

Whole exome sequencing in *USP8*-wild type corticotroph tumours discovered a second mutational hotspot in the *USP48* gene in about 10% of cases [174, 175]. The hotspot concentrates on the conserved Met415 and result in exchange of Met415Ile or Met415Val. USP48 is also a deubiquitinase and the mutant forms have a higher deubiquitinating activity. Overexpressing USP48 mutants has minimal effect on basal *POMC* promoter activity, but potentiates CRH-induced ACTH synthesis [175].

Other

GNAS is rarely mutated in corticotroph tumours [176]. *CDKN1B* mutations are very rare in corticotroph tumours [177].The *BRAF* V600E mutation was reported in 7% of corticotroph tumours in an Asiatic cohort, but it is rarely found in Caucasian patients with Cushing's disease [175].*TP53* is the most frequently mutated tumour suppressor in human cancers, but mutations were considered to be rare in corticotroph tumours (reviewed in [178]). Recent studies, however, concentrating on large and aggressive corticotroph tumours, reported *TP53* mutations in up to 30% of cases [175, 179]. In addition, aggressive corticotroph tumours were also found to harbour *ATRX* mutations [180].

1.6. 14-3-3 Proteins

14-3-3 proteins (gene: *YWHAx*) are a highly conserved protein family with subunit mass 28–33 kDa, present in almost all eukaryotic cells and tissues. In mammals, there are seven proteins: β, γ, ε, ζ, η, σ, and τ [181]. 14-3-3 proteins were originally found to be abundantly expressed in the brain, as well as testis, liver and heart. In eukaryotic cells, they are primarily located in the cytoplasm, but they can also be detected in the plasma membrane and in intracellular organelles such as the nucleus and Golgi apparatus [182, 183]. They consist of nine α-helices and two ligand-binding grooves and function as homodimers or heterodimers [184].

14-3-3 proteins have around 200 different client proteins and control diverse cellular processes such as signal transduction, cell cycle, apoptosis, transcriptional regulation, cell adhesion, chromosome maintenance, protein localization, and metabolic pathways [181, 185] (Figure 1.4). They bind as dimers to phosphorylated serine/threonine residues located within specific sequences of client proteins (canonical 14-3-3 binding motif: RXXpS/TXP, where R is arginine and P is proline), which causes conformational changes in the client protein that may alter its activity, or act as adapter bringing two client proteins in close proximity [186]. 14-3-3 binding may mask recognition sites of the target protein, such as, nuclear localization signals or binding sites for

phosphatases/proteases thereby altering the target protein's intracellular localization or posttranslational modifications respectively [187].

In the case of USP8, binding of 14-3-3 proteins ε, γ, or ζ to the RSXS^{680}XP sequence of mouse Usp8 (or Ubpy) causes conformational changes that keep the deubiquitinase in inactive form [170, 171]. In addition, 14-3-3σ (also known as stratifin, SFN) binds to USP8 to stabilize it [188].

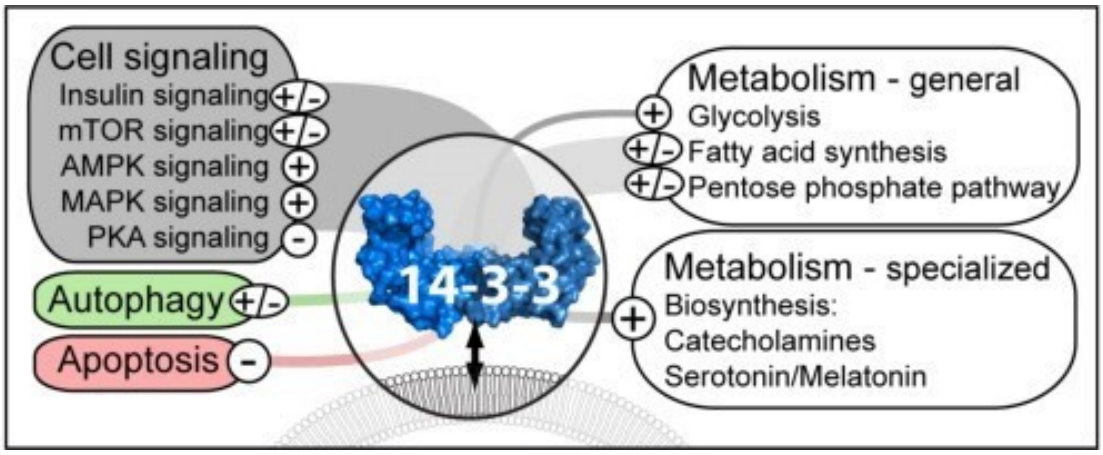


Figure 1.4. 14-3-3 proteins are involved in several signalling pathways: 14-3-3 proteins integrate with client proteins like enzymes and protein kinases to modulate cellular and promote tumorigenesis through insulin-, MAPK-, mTOR- and AMP dependent kinase (AMPK)-signalling pathways, as well as apoptosis and autophagy. Image from Kleppe, R, et al. Seminars in cell & developmental biology, 2011 [220].

1.6.1. 14-3-3 and cancer

14-3-3 proteins are associated with many tumour pathologies. Most 14-3-3 isoforms are upregulated in cancer (Table 1.1) [189]. For example, 14-3-3ζ is upregulated in breast cancer where it activates survival pathways and correlates with worse tumour prognosis [190, 191]. High expression of 14-3-3β in NIH3T3 cells results in the tumour formation in nude mice which indicating it has potential to involve in the cell proliferation and oncogenic transformation [192]. An exception is 14-3-3σ, which was shown to regulate p53 upon DNA damage thereby suppressing tumour growth [193]. 14-3-3σ expression is decreased in many cancers such as breast, lung, liver, ovarian and bladder, and its loss has been suggested to contribute to metastasis and poor tumour

prognosis [194-196].

Table 1.1. 14-3-3 proteins are deregulated in cancers.

14-3-3 Isoform	Cancer Type	Expression
14-3-3ζ	Breast, lung, pancreas, esophageal, head and neck, oral, colon, chronic myeloid leukemia, ovarian	↑
14-3-3σ	Lung, breast, esophageal, chronic myeloid leukemia, uterine, ovarian, skin	↓
	Liver, pancreatic,ductal	↑
14-3-3β	Lung, astrocytoma, glioma, colorectal, gastric squamous, liver	↑
14-3-3ε	Renal, liver, squamous, breast, gastric	↑
14-3-3γ	Liver, breast, lung	↑
14-3-3η	Liver, prostate, squamous, glioma	↑
14-3-3τ/θ	Breast, lung, glioma, prostate	↑

Reproduced from Fan, X., et al., Int J Mol Sci, 2019 [189].

1.6.2. 14-3-3 in MAPK signalling

Cell proliferation is initiated by binding of growth factors to cell surface tyrosine kinase receptors and subsequent activation of Ras (Rat sarcoma) and Raf-1 (Rapidly accelerated fibrosarcoma-1, also known as c-Raf) that trigger the subsequent MEK (MAP kinase extracellular signal-regulated kinase)/MAPK (mitogen activated protein kinase; also known as ERK, Extracellular signal-Regulated Kinase) signalling cascade. The structure of Raf1 includes two phosphorylation-dependent 14-3-3 binding sites, which bind at different sites with different effects [197]. In inactivated cells, Raf-1 is predominantly present in the cytosol and kept in an inactive conformation with 14-3-3 being bound to both C- terminal (catalytic) and N-terminal (regulatory). Upon stimulation, Ras-GTP associates with Raf-1, which translocates to the plasma membrane; during that phase 14-3-3 remains attached to Raf-1 C-terminal, but dissociates from the Raf-1 N-terminal, leaving Raf-1 in an activated state [185]. In addition, 14-3-3 facilitate the heterodimerization of Raf-1 and B-Raf which drives the catalytic activation of Raf-1 [198, 199]. Sequestering 14-3-3 proteins displaces Raf-1 and reduces its activity [200]. In addition there is evidence that 14-3-3 regulate MEK

kinases (MEKK) with 14-3-3ζ and 14-3-3ε interacting with MEKK1 [201].

FOBISIN101 is a radiation-activated small molecule, which blocks the interaction between 14-3-3 and targets like Raf-1 and proline-rich Akt substrate of 40 kD (PRAS40) resulting in inhibition of cell proliferation in lung cancer cells [202]. UTKO1 blocks the activation of Rac1 and EGF by blocking the binding of 14-3-3ζ to Tiam1, thereby preventing the migration and metastasis of tumour cells in human oesophageal tumour EC17 cells [203, 204].

1.6.3. 14-3-3 in PI3K signalling

The phosphoinositide 3-kinase (PI3K) survival pathway initiates downstream to activated insulin/insulin-like growth factor-I (IGF-I) receptors with the generation of inositol lipids by PI3K, which anchor protein kinase AKT (Ak strain transforming; also known as protein kinase B, PKB) to the cell membrane facilitating its activation by 3-Phosphoinositide-dependent kinase 1 (PDK1) [205]. Akt substrates include Forkhead Box O (FoxO; also known as Forkhead transcription factor, FKHR), TSC1/2 (proteins are also known as hamartin and tuberin), PRAS40 and glycogen synthase kinase 3β (GSK3β). Altogether the Akt downstream pathway inhibits apoptosis and facilitates cell metabolism, growth and survival.

There is evidence that 14-3-3 proteins facilitate the activated PI3K pathway. 14-3-3 proteins bind and stabilize the ligand-bound phosphorylated IGF-I receptor as well as its insulin receptor substrate (IRS) docking proteins that connect the activated receptor to downstream signalling components [206]. In addition, 14-3-3ζ was shown to bind the p85 regulatory subunit of PI3K and activate AKT [191]. Furthermore, Akt-phosphorylates the FoxO transcription factors causing their shuttling from the nucleus to the cytosol and this process requires 14-3-3 [207]. Blocking 14-3-3 binding to phosphorylated FOXO3A allowed FOXO3A to enter the nucleus and induce apoptosis in leukaemia cells [208]. In addition, 14-3-3 proteins sequester Akt-phosphorylated TSC2 and PRAS40 thereby blocking their association with mTOR and facilitating mTOR activation [209, 210].

1.6.4. 14-3-3 and the regulation of cell cycle and apoptosis

14-3-3ε inhibits cell cycle at G2 by interacting with CDC25 [211].14-3-3 proteins inhibit apoptosis by binding to proapoptotic proteins phosphorylated by Akt such as Forkhead1 (FKHRL1, also known as FoxO3A) and BAD (Bcl2 associated agonist of cell death); in the case of FKHRL1, 14-3-3 binding retains this transcription factor in the cytoplasm [212]. In the case of BAD, 14-3-3ζ binds the phosphorylated protein preventing its association with the prosurvival Bcl-X_L/Bcl-2 (B-cell lymphoma) in mitochondria [213, 214]. 14-3-3 proteins also bind apoptosis signal-regulating kinase 1 (ASK1) that is induced by death promoting factors such as tumour necrosis factor alpha (TNFα) and Fas (Fas cell surface death receptor) [215].14-3-3ε is anti-apoptotic by inhibiting BAD and Bcl2 associated X protein (BAX) [216]. Disruption of 14-3-3 binding to BAD with the synthetic peptide R18 (difopein) inhibited glioma cell growth and apoptosis [217]. Contrary to the anti-apoptotic action described above, 14-3-3σ binds to DNA damage-induced p53 and facilitates cell cycle arrest [218, 219].

1.7. Aim of the study

The *USP8* mutational hotspot found in almost half of corticotroph tumours affects USP8 binding to 14-3-3 proteins, bringing attention to a potential role of these proteins on corticotroph tumorigenesis. There was no information on the expression and function of 14-3-3 proteins in corticotroph tumours. Therefore the aim of the study was to explore the expression of USP8-interacting 14-3-3 isoforms and investigate their putative role on the pathogenesis of Cushing's disease.

The first goal of the study was to investigate the expression of 14-3-3γ, ε, ζ, σ in normal pituitary versus corticotroph tumours and potential correlation with clinical parameters and *USP8* mutational status.

The second goal was to elucidate the effect of 14-3-3ε on *POMC* promoter activity and cell viability. Furthermore it addressed the signaling processes mediating the stimulatory action on *POMC*.

2. Materials and Methods

2.1. Materials

2.1.1. Laboratory equipment

Table 2.1. Laboratory equipment

Item	Model	Manufacturer
Analytical Balance	770	KERN
Autoclave	VX-75	Systec
Automated Cell Counter	TC20	Bio-Rad
Biological safety cabinet	Safe 2020 Class II	Thermo Scientific
CO2 incubator	BBD 6220	Thermo Scientific
Electronic Rotary Microtome	HM340E	Thermo Scientific
Electrophoresis Apparatus	200/2.0 power supply	Bio-Rad
Embedding Workstation	HistoStar	Thermo Scientific
Fluorescence microscopy	DM 2500	Leica
Ice machine	QM20AC	Manitowoc
Microplate Reader	iMark	Bio-Rad
Microplate Reader/Fluorescence	FLUOstar Omega	BMG LABTECH
Microscope, inverted	TMS	Nikon
Microscope, bright field	DMC2900	Leica
Microwaves	iQ100	Siemens
MiniCentrifuge	SPROUT Plus	Heathrow Scientific
Multimode Plate Reader	VICTOR X4	PerkinElmer
NanoDrop Spectrophotometer	ND-1000	PeqLab
Non-refrigerated centrifuge	5424R	Eppendorf
PCR plate-Spinner Centrifuge	230EU	Axygen
Pipettes	Research® plus	Eppendorf
Precision balance	440-45	KERN
Real-time PCR System	Mx3000P	StrataGene
Refrigerated centrifuge	5804	Eppendorf
Rocker-Shaker	MR-12	Biosan
Shaking incubators	3031	GFL
Thermal cycler	T100	Bio-Rad
Thermomixer	F1.5	Eppendorf
Vortex mixer	Vortex-Genie 2	Scientific Industries
Water bath	TSGP20	Thermo Scientific

2.1.2. Chemicals and Reagents

Table 2.2. Chemicals and Reagents

Chemicals and Reagents	Manufacturer
Beetle-Juice Luciferase Assay Firefly	P.J.K. GmbH
Bovine Serum Albumin	Roth
Cell Proliferation Reagent WST-1 (Roche)	Sigma
Chloroform	Merck KGaA
Citric acid	Roth
Clarity Western ECL Substrate	Bio-Rad
3,3′-Diaminobenzidine (DAB)	Sigma
Dulbecco's modified Eagle medium	Invitrogen,Gibco™
Ethanol 100%	Sigma
Ethanol 75%	Sigma
FBS Qualified HI	Invitrogen
Gaussia-Juice Luciferase Assay	P.J.K. GmbH
Glycine	Carl Roth
GoTaq G2 MasterMix Polymerase HotStart	Promega
Hydrogen peroxide solution	Sigma
Isopropanol	Sigma-Aldrich
LB-Agar	Carl Roth
LB-Medium	Carl Roth
LE Agarose	Biozym
Methanol	Sigma
Milk powder	Roth
NucleoBond Xtra Midi Kit	Macherey-Nagel
Passive Lysis 5X Buffer	Promega
Pen Strep	Invitrogen
PureLink RNA Mini Kit	Thermo Fisher
QuantiTect Reverse Transcription Kit	Qiagen
Roti®-Histokitt II	Roth
SDS pellets	Roth
Serum from goat	Sigma
Sodium chloride	Sigma
SuperFect Transfection Regeant. 4x1,2ml	Qiagen
SsoFast EvaGreen, 500 x 20µl	Bio-Rad
TEMED	Roth
Toluidine Blue	Sigma
Trizma@base powder	Sigma
Trizma hydrochloride	Sigma
Trizol Reagent 100ml 2x	Thermofisher
Trypan Blue, 0,4%, 1ml	Invitrogen
Trypsin-EDTA, 0,05% Phenolrot	Invitrogen
Tween 20	Sigma Aldrich
Vectastain Elite ABC-Peroxidase Kit	Vector laboratories
Xylol	Fisher

2.1.3. PCR Primers

Table 2.3. List of PCR Primers

Primers for Real-Time PCR (human 14-3-3 targets)		
Target	Sequence (5'-3')	Product size(bp)
14-3-3σ (*YWHAS*)	Forward Primer: CGCTGTTCTTGCTCCAAAGG Reverse Primer: ATGACCAGTGGTTAGGTGCG	113
14-3-3ε (*YWHAE*)	Forward Primer: TAACACTGGCGAGTCCAAGG Reverse Primer: AGCCTCCTTCCTGTCGTTTC	97
14-3-3γ (*YWHAG*)	Forward Primer: TGAAGAACGTGACAGAGCTGAAT Reverse Primer: CGCACGACCATCTCAATCT	172
14-3-3ζ (*YWHAZ*)	Forward Primer: CGAGATCCAGGGACAGAGTC Reverse Primer: AGCTCATTTTTATCCATGACTGG	131
GAPDH	Forward Primer: TGCACCACCAACTGCTTAGC Reverse Primer: ACAGTCTTCTGGGTGGCAGTG	110

2.1.4. Plasmid and Antibodies

Table 2.4. Expression vectors and reporter constructs

Vector	Source
Human *POMC* promoter luciferase reporter	Panomics
pcDNA3.1/HisC 14-3-3ε	Addgene
pcDNA3.1/HisC 14-3-3ε K49E	Addgene
pcDNA3.1/HisC	Invitrogen
pMAX GFP	Lonza

HisC, C-terminal tag; GFP, green fluorescence protein

Table 2.5. Antibodies

Primary antibodies	Application	Dilution	Supplier (catalog #)
14-3-3ε (clone N1C3), rabbit polyclonal	IHC	1:1000	GeneTex (GTX109090)
6X His tag, mouse monoclonal	WB	1:1000	Abcam (ab18184)
α-Tubulin, mouse monoclonal	WB	1:10000	Sigma (T6074-100UL)
Phosphorylated p44/42MAPK-	WB	1:1000	Cell Signaling (9101S)
p44/42 MAPK, rabbit polyclonal	WB	1:1000	Cell Signaling(9102S)
Secondary antibodies	**Application**	**Dilution**	**Supplier (catalog #)**
anti-mouse IgG, HRP-linked	WB	1:2000	Cell Signaling (7076S)
anti-rabbit Ig, HRP-linked			Cell Signaling (7074S)
anti-rabbit IgG (H+L)	IHC	1:300	Vector Labs (BA-1000)

IHC, immunohistochemistry; WB, western blot; HRP, horseradish peroxidase; IgG, immunoglobulin G

2.1.5. Patients and corticotroph tumours

Twenty-seven human corticotroph tumours were obtained after transsphenoidal surgery from different neurosurgery centers (LMU Munich-Klinikum Großhadern, Erlangen, Hamburg and Tubingen). Patients were diagnosed following the current guidelines [221]. In brief, diagnosis includes clinical signs and biochemical tests. The biochemical tests include lack of response to 24h-UFC 1 mg overnight dexamethasone suppression test, increased levels of 24 hours UFC and midnight salivary cortisol. The pituitary origin of diagnosis was based on the baseline ACTH >20 pg/Ml and ›50% suppression of serum cortisol to high dose 8 mg overnight dexamethasone suppression testing and CRH stimulation test (100 µg human CRH i.v.).

Tumours were classified according to the Hardy's Classification of pituitary adenoma and Knosp's grading [222, 223]. Hardy Grade I (<10mm diameter) and Grade II (>10mm diameter) are enclosed within the sella. The tumour of Grade III located outside the sella and Grade IV are infiltrated sphenoid and cavernous sinuses and compressed optic nerve. The Knosp's grading of grading 0, 1 and 2 are distinguished from each other by a medial carotid line, the intercarotid line and a lateral of internal carotid artery. Grade 3 means tumour extend beyond internal carotid artery and into the cavernous sinus compartment. Grade 4 corresponds to the total encasement of the intracavernous carotid artery. Clinical information was available from 26 patients and is shown in Table2.6.

For the transportation to the laboratory, tumours were collected in 15ml falcon with DMEM supplemented with 10%FBS and 10^5 U/L penicillin/streptomycin and 500 µg/L partricin. After washing with HDB buffer for three to four times until the blood, fibers and debris were removed, tumours were snap-frozen with dry ice and stored in -80°C. Three human pituitary glands were acquired from autopsies no more than 12h after sudden death from subjects with no evidence of endocrine disease.

Table 2.6. Clinical information of tumour specimens

No.	Clinical diagnosis	Age at diagnosis	Gender	Tumour grade (Knosp)	Tumour grade (Hardy)	Invasive
001	Nelson	65	F	4	III	yes
002	Cushing's disease	15	M	1	II	no
005	Cushing's disease	55	F	4	III	yes
013	Cushing's disease	56	M	3	IV	yes
040	Cushing's disease	31	F	0	I	no
042	Nelson	74	M	4	IV	yes
043	Cushing's disease	49	F	3	III	yes
046	Cushing's disease	29	F	1	I	no
054	Cushing's disease	55	F	1	I	no
055	Cushing's disease	43	M	0	I	no
077	Cushing's disease	29	M	1	I	no
078	Cushing's disease	22	F	2	II	no
085	Cushing's disease	56	F	1	II	no
089	Cushing's disease	27	F	0	I	no
091	Cushing's disease	39	F	0	II	no
117	Cushing's disease	53	F	1	I	no
174	Cushing's disease	55	M	3	III	yes
191	Cushing's disease	20	F	0	II	no
265	Cushing's disease	37	M	1	III	yes
297	Cushing's disease	19	F	1	II	no
309	Cushing's disease	45	F	1	II	no
346	Cushing's disease	57	F	4	IV	ye
350	Cushing's disease	60	F	1	II	no
365	Nelson	54	F	2	II	yes
370	Cushing's disease	81	F	4	III	yes
371	Cushing's disease	60	F	4	IV	yes

2.2. Methods

2.2.1. Cell culture

The murine pituitary corticotroph tumour cell line AtT-20 (American Type Culture Collection (ATCC®) Manassas, VA, USA) were grown in 75cm^2 flasks cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 10^5 U/mL penicillin/streptomycin. Cells were incubated in 37°C, 95% air, 5% CO_2 with humidity of 70%-80%. Cells were used for 10 passages.

2.2.2. Transformation and Plasmid purification

One Shot TOP10 chemically competent bacteria were used as follows: after adding 2µl vector, cells were gently mixed and left for 25 minutes on ice. Cells were heat-shocked at 42°C for 40 seconds and then were left on ice for 2 minutes. 250µl of LB medium without antibiotic was added into the vial and put in shaking incubator at 37°C, 150rpm speed. After 1 hour, 50µl transformed bacteria were spread on a pre-warmed agar plate supplemented with 0.1% ampicillin and incubated at 37°C overnight. The following day, selected colonies were picked and put into sterile conical flask containing 200ml LB medium (supplemented with 0.1% ampicillin), which was left shaking at 37°C, 150rpm speed overnight. The day after, plasmids were isolated using the NucleoBond Xtra Midi kit as follows: after centrifugation the pellet was resuspended in 12ml RES Buffer and lysed with12ml LYS Buffer, mixing and inverting several times. NEU buffer (12ml) was added and mixed thoroughly until the lysate was colourless and left for 5 minutes before loading onto the NucleoBond Xtra Column that has been equilibrated with 12ml Equilibration Buffer. Plasmids were eluted with 5ml Elution buffer in new falcon tubes. After adding 3.5ml isopropanol and vortexing, samples were centrifuged in 4°C, at 15000rpm for 15 minutes. After discarding the supernatant, 2ml 70% ethanol was added to the pellet and centrifuged at room temperature, at 15000 rpm for 15 minutes. Then the ethanol was discarded, the pellet was left to dry in a heated block, and plasmid was fully dissolved in 300µl of TE buffer. The plasmid concentration and purity was determined with the NanoDrop spectrophotometer. All plasmids were stored

in 4°C.

2.2.3. Transfection

AtT20 cells were seed in 48-well plate at a density of 5×10^4 cells/well. After one day, the cells have attached and the confluence reaches 70%-80%, then transfected with plasmids using SuperFect reagent according to the manufacturer's instruction. The pMAX GFP plasmid was included as normalization in each well to monitor transfection efficacy. After 3hours, medium was changed to 2%FBS DMEM and cells were incubated overnight and then treated with compounds for 6 hours. For lysis, the plates were put on ice and washed with ice-cold PBS. Then 75µl passive lysis buffer was added in each well and the plate was shacked on the rocker shaker for 15 minutes, before being put in -80°C overnight to allow for full cell disruption.

2.2.4. Luciferase assay

Cell lysates were collected in 1.5ml Eppendorf tubes and centrifuged at 4°C, 15000 rpm for 10 minutes. For the luciferase assay, 20ul of each supernatant was added in a 96-well luminometer plate and measured in Victor x4 multilabel plate reader. For the GFP assay, 20ul of each supernatant was added in a 96-well black plate and measured in POLARstar Omega (Excitation 485nm, Emission 520nm). Each condition was in triplicates. Three wells of distilled water was included in each plate as blank. Data were calculated as the ratio of (Luciferase-blnk) to (GFP activity-blnk).

2.2.5. RNA extraction

One ml of Trizol was added per 50-100mg of pituitary tissue and homogenized using ULTRA-TURRAX. Between different samples, the disruptor was washed several times with ethanol and distilled water. After homogenizing, RNA was extracted following the TRIzol™ manufacturer's instructions. In brief, lysates were incubated with 1ml Trizol reagent for 5 minutes at room temperature. Then 0.2ml chloroform was added and samples were vigorously shaken for 15 seconds to completely mix and left at room temperature for 3 minutes before being centrifuged at for 15 minutes, 12000rpm at 4°C.

Centrifugation separated each sample in three phases: a low phase, a middle phase that contains DNA and a colourless upper phase that contains the RNA. The upper phase was carefully collected into a new RNase-free tube, where an equal volume of 70% ethanol was added. After thoroughly inverting and vortexing, RNA was isolated using the PureLink RNA Mini Kit as follows: 700µl of sample was added into a Spin Cartridge, centrifuged for 30 seconds, 12000rpm at room temperature and flow through was collected into a Collection Tube and discarded. 700µl Wash Buffer I was added into the Spin Cartridge and centrifuged followed by 500µl Wash Buffer II with ethanol. The steps of centrifuge and discard were repeated once and the membrane was left to dry moderately. RNA was eluted with 30µl RNase-Free Water in a Recovery Tube, incubated for 1 minute at room temperature and centrifuged for 2 minutes, ≥12000rpm, at room temperature. The Recovery Tubes were then kept on ice. RNA concentration and purity was determined using the NanoDrop spectrophotometer and samples were stored in -80°C.

2.2.6. Quantitative reverse transcription polymerase chain reaction (qPCR)

RNA (0.5-1 µg) was reversed transcribed according using the QuantiTect Reverse Transcription Kit as follows: genomic DNA was eliminated using 2µl gDNA Wipeout buffer. Samples were vortexed and centrifuged for few seconds before being incubated in a thermal cycler at 42°C for 2 minutes, after which they were immediately put on ice. The resulting RNA (14µL) was added to the reverse-transcription master mix that contained: 1µl Quantiscript® reverse transcriptase, 4µl RT buffer and 1µl RT primer Mix. Samples were vortexed and centrifuged before being incubated in a thermal cycler at 42°C for 15 minutes and at 95°C for 3 minutes (to inactivate the quantiscript reverse transcriptase). cDNA samples were stored in -20°C.

qPCR was run in Stratagene Mx3000P Multiplex Quantitative PCR machine using 6µl SsoFast EvaGreen master mix, 2µl of cDNA, 1µl of amplification primer-Forward and 1µl of amplification primer-Reverse in each well. The cycling conditions were: 1 cycle denaturation 95°C for 3 minutes, 40 cycles with denaturation at 95°C for 10 seconds and annealing with extension at 60 °C for 10 seconds, 1 cycle dissociation curve with

95°C for 1 minute, 58°C for 30 seconds and 95°C, 30 seconds.

2.2.7. Immunohistochemistry

Fifty-four corticotroph tumours were obtained as formalin fixed paraffin embedded slides (3μm thickness). Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol solution. Antigen retrieval was performed in 10 mM, pH 6.0 citric acid monohydrate using the microwave method, in which slides are heated for 4 minutes at 750W and cooled for 1 minute at room temperature in a process that is repeated twice. Sections were put at room temperature for 20 minutes after the final heating to cool down. After washing for 3 times in distilled water and 1 time in TBS, sections were blocked in goat serum (serum from animal in which the secondary antibody is raised) at room temperature for 30 minutes and then incubated with a rabbit polyclonal antibody against 14-3-3ε used at 1:1000 dilution in TBS buffer overnight at 4°C. The day after slides were washed in TBS for 3 times (5 minutes each) before being incubated with an anti-rabbit secondary antibody used at dilution 1:300 in TBS buffer for 30 minutes at room temperature and avidin-biotin complex for 30 minutes that was prepared in Tris buffer >30 minutes in advance according to the manufacturer's protocol (VECTASTAIN® ABC Kit, Vector Laboratories). Immunoreactivity was visualized after incubating with 1mg/ml diaminobenzidine (DAB) supplemented with 0.01% hydrogen peroxide for 45 seconds in dark. Sections were washed several times in TBS and counterstained in 1% toluidine blue for 20 minutes. Then they were dehydrated in ascending concentration of ethanol and mounted using Roti-Histokit.

Sections were examined under bright field microscope (Leica) and 14-3-3ε immunoreactivity was scored according to H-score [224] which classified as 0(no staining), 1(weak staining), 2(moderate staining), or 3(strong staining) and calculated as: [(percentage of no stained cells×0)+ (percentage of weak staining cells×1) + (percentage of moderate staining cells×2) + (percentage of strong staining cells×3)] / 300 (hypothetical maximum for 100% of the cells being strongly stained), with values varying from 0 (no immunoreactivity) to 1 (maximum immunoreactivity).

2.2.8. WST-1 cell viability assay

AtT20 cells transfected with 14-3-3ε constructs or pcDNA3 empty vector were seeded at a density of 8×10^3 cells/well in 96-well plate. After leaving them overnight to attach, medium was changed to 2% FBS DMEM. Cell viability was determined 24 hours after transfection as follows: 10µl of WST-1 reagent (Roche) were added into each well and the plate was incubated protected from light at 37°C for 0.5-4 hours. Three wells containing only medium (without cells) were used as blank. Absorption was measured using a microplate reader (BioRad) 450 nm with reference at 655 nm.

2.2.9. Protein extraction

Transfected AtT20 cells were washed with ice cold PBS 24 hours after transfection and lysed in 100µl RIPA buffer supplemented with protease and phosphatase inhibitors. Cell lysates were left to shake for 30 minutes and centrifuged for 15 min, 15000rpm at 4 °C. The supernatants were collected on ice, vortexed gently and protein concentration was determined after adding 100µl protein assay dye reagent (BioRad) using the Bradford assay. A series of BSA consecutive dilutions (starting from 25µg/ml) was used to prepare the standard curve. Each sample was measured in triplicates in a transparent 96 well plate, and absorbance was measured using a microplate reader at 595 nm. Protein concentration was calculated using the BSA standard curve.

2.2.10. Western Blot

Laemmli buffer was added according to each samples concentration. Protein was denaturated by heating at 95°C for 5 minutes and 20µl samples were loaded in precast gels and run at constant voltage with Tris-glycine running buffer. Protein was transferred at constant ampere (0.24A) for 2hour to polyvinylidene difluoride (PVDF) membranes which have been activated with methanol. Membranes were blocked in 5 % skimmed milk for 30 minutes at room temperature and incubated with primary antibody (diluted in 5% skimmed milk or 5% BSA, according to each antibody datasheet recommendations) at 4°C under gentle shaking after washed with TBS with 0.1% Tween20. After overnight incubation, membranes were washed and incubated with

corresponding secondary antibody in 5% milk for 1 hour at room temperature. Signal was developmed with Clarity™ Western ECL Substrate Kit or SuperSignal ™ West Femto Maximum Sensitivity Substrate.

2.2.11. 14-3-3ε related gene enrichment analysis

To obtain a list of 14-3-3ε binding proteins the STRING Protein-Protein Interaction Networks Functional Enrichment Analysis (https://string-db.org/) was used, with search words for protein name "YWHAE" (approved human gene nomenclature for 14-3-3ε) and organism "Homo sapiens". The following settings were used: meaning of network edges ("evidence"), minimum required interaction score ["Low confidence (0.150)"], active interaction sources ("experiments") and max number of interactors to show ("no more than 50 interactors" in 1st shell). GEPIA2 was used to get the top 100 genes correlated with YWHAE on the datasets of all TCGA tumour and normal tissues with setting "Similar Gene Detection". In addition, R packages were utilized to administer GO (Gene ontology) of the selected genes. False Discovery Rate (FDR) <0.05 was considered statistically significant.

2.2.12. Statistical analysis

SPSS 25.0 software (IBM), GraphPad Prism 8 software (GraphPad Inc) and R language software [R-3.6.3, 64-bit] were used. Two-tailed Student's t test was used for unpaired data, Mann-Whitney U test for non-parametric variables. In addition One-way ANOVA, Pearson and Spearman's rank correlation coefficient for continuous variables correlation were utilized. A $P<0.05$ was considered statistically significant.

3. Results

3.1. Expression of 14-3-3 proteins in corticotroph tumours

The expression of the 14-3-3 proteins γ, ε, and ζ and σ, described to associate with USP8 [170, 188] were investigated with quantitative qPCR on a cohort of 27 human corticotroph tumours and 3 normal pituitaries. In the normal pituitary gland, 14-3-3γ and ε were the highest expressed 14-3-3 protein, while ζ and σ had very low expression levels (Fig. 3.1).

14-3-3ε transcript levels were significantly higher in corticotroph tumours compared to the normal pituitaries (fold change 3.6, P=0.017) (Fig. 3.1). No differences were found in 14-3-3γ and ζ expression between corticotroph tumours and normal pituitaries (P=0.727 and 0.285 respectively). In contrast 14-3-3σ levels were decreased in corticotroph tumours (P=0.0025).

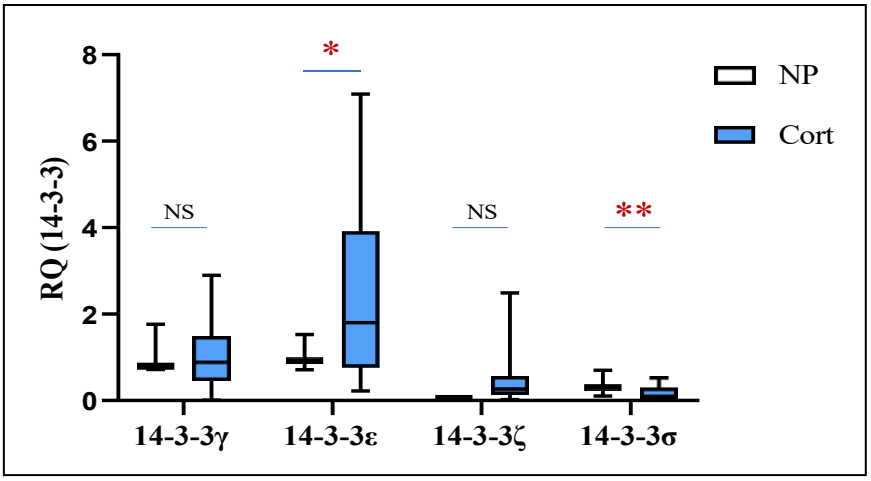


Figure 3.1. Expression of 14-3-3γ, ε, ζ and σ in human normal pituitaries and corticotroph tumours. Real time qPCR data from 27 human corticotroph tumours (Cort) and 3 normal pituitaries (NP). Data are relative quantification using the ΔΔCt calculated from tumour and normal pituitaries ΔCt values normalized to *GAPDH*. *P < 0.05, **P < 0.01 (Mann-Whitney U-Test).

3.2. Correlation of 14-3-3ε expression with clinical features

14-3-3ε transcript levels were analysed in the context of clinical data available from 26 cases (19 females and 7 males; mean age at diagnosis 46±18 years; Table 3.1). 14-3-3ε

expression did not significantly correlate with age at diagnosis (P=0.279), gender (male vs. female, P=0.277), body mass index (BMI, P=0.700) and tumour size (microadenoma vs. macroadenoma, P=0.274). Similarly no differences in 14-3-3ε expression were found in Cushing's disease tumours and those from corticotroph tumour progression after bilateral adrenalectomy (Nelson's syndrome) (P=0.233).

14-3-3ε expression levels correlated positively with total tumour resection after pituitary surgery and with postoperative remission (P=0.017 and P=0.019 respectively). There was no difference in intratumoral 14-3-3 ε expression in the six patients that received radiation postoperatively (P=0.145). Noninvasive tumours had 2.5x higher 14-3-3ε transcript levels compared to the invasive ones (P=0.007). No correlation was found within each Hardy or Knosp grade subgroup (P=0.213 and 0.439 respectively). However, grouping Hardy's I-II and III-IV revealed significantly higher 14-3-3ε expression in Hardy's I-II (2.88±2.08 vs. 1.31±0.96, P=0.019). Similarly, grouping Knosp 0-2 and 3-4 showed higher 14-3-3ε expression in the non-invasive Knosp 0-2 (2.74±2.10 vs. 1.41±0.96, P=0.044).

No correlation was found with basal and postoperative plasma ACTH, serum cortisol, and 24 hour-UFC values (Table 3.2).

Regarding the tumour mutational status, 14-3-3ε expression was significantly higher in *USP8* mutant tumours compared to wild type (3.58±2.04 vs 1.46±1.39, P=0.004) and lower in the 3 *TP53* mutant tumours (1.23±0.72 vs. 2.91±2.01, P=0.026; Table 3.1).

Table 3.1. 14-3-3ε Patient cohort and Clinical features

	Total number	n (%)	14-3-3ε (mean±SD)	*P*
Sex (Females)	26			0.277
Female, n(%)		19 (73.1%)	2.53±2.10	
Male, n(%)		7 (26.9%)	1.59±1.29	
Age at diagnosis (years), mean±SD	25	45.72±17.9		0.279*
BMI (kg/m2), mean±SD	24	30±7.2		0.700*
USP8 status	26			0.004
wild type, n(%)		16 (61.5%)	1.46±1.39	
mutant, n(%)		10 (38.5%)	3.58±2.04	
TP53 status	21			0.026
wild type, n(%)		18 (85.7%)	2.91±2.01	
mutant, n(%)		3 (14.3%)	1.23±0.72	
Type, n (%)	26			0.233
Cushing, n(%)		23 (88.5%)	2.44±1.98	
Nelson, n(%)		3 (11.5%)	1.00±0.94	
Tumour size (mm), median (IQR)	26	15 (12.3)		0.274
Microadenoma, n (%)		6 (23.1%)	3.05±2.01	
Macroadenoma, n (%)		20 (76.9%)	2.05±1.90	
Total resection after OP	24			0.017
Yes, n(%)		15 (62.5%)	2.86±2.19	
No, n(%)		9 (37.5%)	1.15±0.99	
Postoperative remission	25			0.019
Yes, n(%)		17 (68%)	2.80±2.12	
No, n(%)		8 (32%)	1.19±1.05	
Received postOP radiation, n (%)	24			0.145
Yes, n(%)		6 (25%)	1.31±1.21	
No, n(%)		18 (75%)	2.69±2.11	
Hardy grade	26			0.213#
Ⅰ, n(%)		7 (26.9%)	3.25±1.91	
Ⅱ, n(%)		9 (34.6%)	2.60±2.40	
Ⅲ, n(%)		6 (23.1%)	1.39±1.16	
Ⅳ, n(%)		4 (15.4%)	1.19±0.89	
Knosp grade	26			0.439#
0, n(%)		5 (19.2%)	3.15±2.50	
1, n(%)		10 (38.5%)	2.67±2.15	
2, n(%)		2 (7.7%)	2.05±2.59	
3, n(%)		3 (11.5%)	2.16±1.49	
4, n(%)		6 (23.1%)	1.03±0.51	
Tumour invasion	26			0.007
Invasion, n(%)		11 (42.3%)	1.21±1.01	
No invasion, n(%)		15 (57.7%)	3.06±2.10	
Survival	24			0.498
Yes, n(%)		22 (91.7%)	2.42±2.06	
No, n(%)		2 (8.3%)	1.40±0.93	

14-3-3ε values are ΔΔCt expression presented as mean (±SD, standard deviation). P values were calculated by *t*-test, Pearson (*) or One-Way ANOVA (#).BMI, body mass index; PostOP, postoperative.

Table 3.2. 14-3-3ε and hormonal values

	median (IQR)	#	*P*
PreOP basal plasma ACTH (pg/mL)	95 (141.3)	24	0.373
PostOP basal plasma ACTH (pg/mL)	10.5 (52.9)	24	0.231
PreOP basal serum cortisol (μg/dl)	96 (196.4)	19	0.175
PostOP serum cortisol (μg/dl)	11 (27.8)	23	0.276
PreOP UFC (μg/24h)	406(587.4)	13	0.090
PostOP UFC (μg/24h)	38 (237)	6	0.623

P values were calculated with Spearman's rank correlation. Data are expressed as median (IQR, interquartile range). PreOP, preoperative; PostOP, postoperative.

3.3. Correlation of 14-3-3σ expression with clinical features

14-3-3σ transcript levels were analysed in the context of clinical data available from 25 cases (18 females and 7 males; mean age at diagnosis 45±18 years; Table 3.3). 14-3-3σ expression did not significantly correlate with age at diagnosis (P=0.693), gender (male vs. female, P=0.745), body mass index (BMI, P=0.827) and tumour size (microadenoma vs. macroadenoma, P=0.827). Similarly no differences in 14-3-3σ expression were found in Cushing's disease tumours and those from corticotroph tumour progression after bilateral adrenalectomy (Nelson's syndrome) (P=0.733).

14-3-3σ expression levels did not correlate with total tumour resection after pituitary surgery and postoperative remission (P=0.577 and P=0.989 respectively) and were not different in the five patients that received radiation postoperatively (P=0.917).

No correlation was found with either Hardy's, Knosp grade or tumour invasion (P=0.942, 0.523 and 0.635 respectively). Similarly, no correlation was found with basal and postoperative plasma ACTH, serum cortisol, and 24 hour-UFC values (Table 3.4).

Regarding the tumour mutational status, 14-3-3σ expression was significantly higher in *TP53* wild type tumours compared to mutant (0.148±0.128 vs 0.013±0.016, P=0.042; Table 3.3).There was no difference of 14-3-3σ expression between *USP8* wild-type and mutant tumours (P=0.439).

Table 3.3. 14-3-3σ Patient cohort and clinical features

	Total number	n (%)	14-3-3σ (mean±SD)	*P*
Sex (Females)	25			0.745
Female, n(%)		18 (72%)	0.159±0.154	
Male, n(%)		7 (28%)	0.137±0.144	
Age at diagnosis (years), mean±SD	24	44.92±17.8		0.693*
BMI (kg/m2), mean±SD	23	29.76±7.31		0.827*
USP8 status	25			0.439
wild type, n(%)		15 (60%)	0.172±0.165	
mutant, n(%)		10 (40%)	0.124±0.122	
TP53 status	20			0.042##
wild type, n(%)		18 (90%)	0.148±0.128	
mutant, n(%)		2 (10%)	0.013±0.016	
Type, n (%)	25			0.733##
Cushing, n(%)		23 (92%)	0.143±0.132	
Nelson, n(%)		2 (8%)	0.267±0.344	
Tumour size (mm), median (IQR)	25	15 (13.4)		0.827
Microadenoma, n (%)		6 (24%)	0.165±0.159	
Macroadenoma, n (%)		19 (76%)	0.149±0.149	
Total resection after OP	23			0.577
Yes, n(%)		15 (65.2%)	0.177±0.140	
No, n(%)		8 (34.8%)	0.139±0.174	
Postoperative remission	24			0.989
Yes, n(%)		17 (70.8%)	0.159±0.140	
No, n(%)		7 (29.2%)	0.158±0.178	
Received postOP radiation, n (%)	23			0.917
Yes, n(%)		5 (21.7%)	0.165±0.220	
No, n(%)		18 (78.3%)	0.157±0.135	
Hardy grade	25			0.942#
Ⅰ, n(%)		7 (28%)	0.180±0.151	
Ⅱ, n(%)		9 (36%)	0.152±0.178	
Ⅲ, n(%)		5 (20%)	0.141±0.158	
Ⅳ, n(%)		4 (16%)	0.123±0.100	
Knosp grade	25			0.523#
0, n(%)		5 (20%)	0.143±0.150	
1, n(%)		10 (40%)	0.172±0.149	
2, n(%)		2 (8%)	0.271±0.338	
3, n(%)		3 (12%)	0.175±0.127	
4, n(%)		5 (20%)	0.062±0.066	
Tumour invasion	25			0.635
Invasion, n(%)		10 (40%)	0.171±0.170	
No invasion, n(%)		15 (60%)	0.141±0.137	
Survival	23			na
Yes, n(%)		22 (95.7%)	0.172±0.148	
No, n(%)		1 (4.3%)		

14-3-3σ values are ΔΔCt expression presented as mean (±SD, standard deviation). *Pearson correlation, # One-way ANOVA, ## Mann-Whitney test. na, not applicable.

Table 3.4. 14-3-3σ and hormonal values

	median (IQR)	#	*P*
PreOP basal plasma ACTH (pg/mL)	93.5 (89.5)	23	0,638
PostOP basal plasma ACTH (pg/mL)	10 (14.8)	23	0,261
PreOP basal serum cortisol (µg/dl)	96 (196.4)	19	0,689
PostOP serum cortisol (µg/dl)	11 (27.8)	23	0,511
PreOP UFC (µg/24h)	406(587.4)	13	0,642
PostOP UFC (µg/24h)	38.4 (237)	6	0,872

P values were calculated with Spearman's rank correlation. Data are expressed as median (IQR, interquartile range). PreOP, preoperative; PostOP, postoperative.

3.4. 14-3-3 expression and *USP8* mutational status

USP8 functionally crosstalk with 14-3-3 proteins. To investigate a possible association between 14-3-3 expression and USP8 mutational status, the study cohort of 27 corticotroph tumours was screened for *USP8* mutations in the hotspot region of exon 14. Ten corticotroph tumours were found to be *USP8* mutant (2 p.S718P, 2 p.P720R, 1 p.P720Q and 5 p.Ser718del) and 16 wild type. One specimen was not informative.

No differences were found in the expression of 14-3-3γ, ζ and σ between *USP8*wt and mutant tumours (P=0.462, 0.328 and 0.356 respectively; Fig. 3.2). In contrast, 14-3-3ε expression levels were higher in *USP8*mut corticotroph tumours compared to wild type (Fig. 3.2, P=0.012).

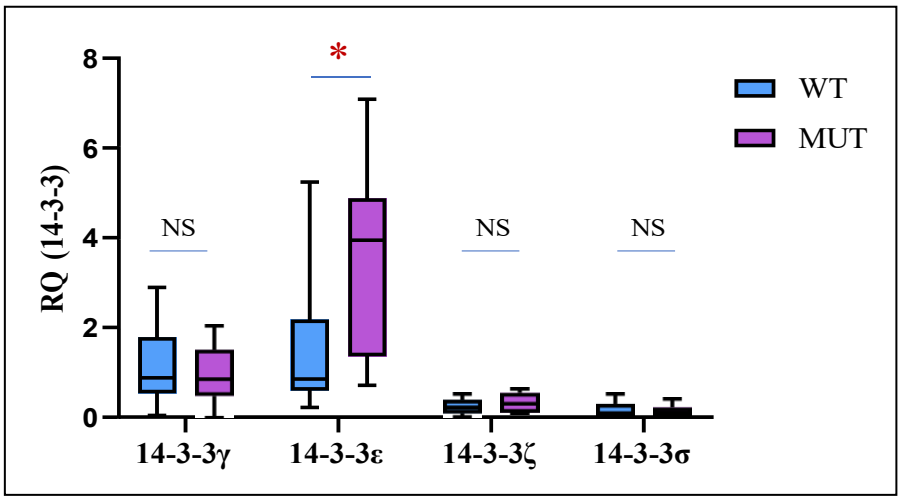


Figure 3.2. Expression of 14-3-3γ, ε, ζ and σ in *USP8* wild type versus mutant corticotroph tumours. Data are ΔΔCt normalized to *GAPDH*. WT: *USP8* wild type; Mut: *USP8* mutant; NS: not significant. *P < 0.01 (Mann-Whitney U-Test and Unpaired *t* test).

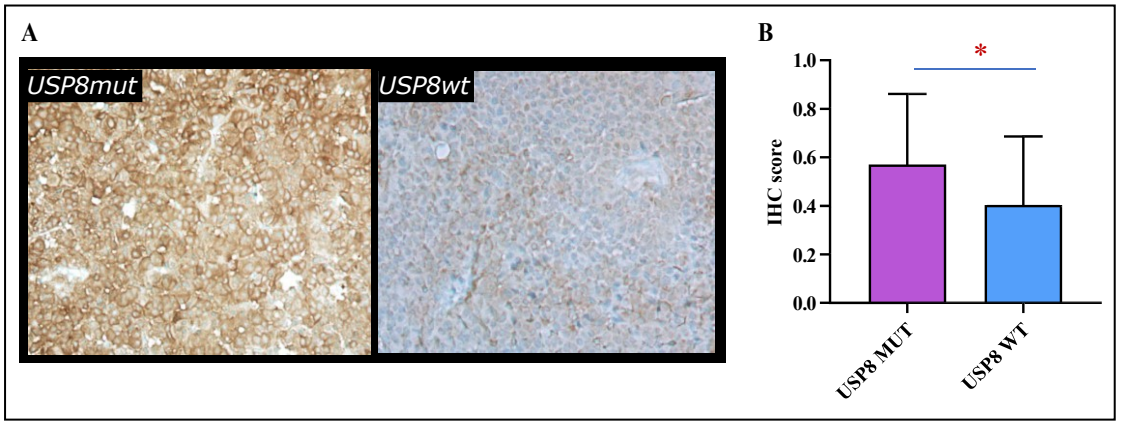


Figure 3.3. 14-3-3ε immunoreactivity in *USP8* mutant versus wild type corticotroph tumours. A. Representative immunostainings (DAB, brown colour) in *USP8*mut and wild type corticotroph tumours. Sections were counterstained with toluidine blue. B. Graph showing mean 14-3-3ε histoscore (H score) from 27 *USP8*mut versus 22 *USP8*wt. Data are mean±SD. *P < 0.05 (Unpaired *t* test).

Immunohistochemistry for 14-3-3ε was performed on an additional cohort of 53 formalin fixed paraffin embedded corticotroph tumours (23 *USP8*wt, 30 *USP8*mut). Cytoplasmic 14-3-3ε immunoreactivity was detected in 49 out of 53 cases and was higher in *USP8*mut compared to *USP8*wt (H score 0.57 versus 0.40, P=0.049; Fig. 3.3).

3.5. Effect of 14-3-3ε on human *POMC* promoter activity

The data presented herein show overexpression of 14-3-3ε in corticotroph tumours compared to the normal pituitary that correlates with more severe pathology of Cushing's disease, suggesting a role on corticotroph pathophysiology.

An *in vitro* corticotroph tumour cell model (AtT-20) was used to analyse the impact of 14-3-3ε on *POMC*. 14-3-3ε overexpression significantly increased human *POMC* promoter activity compared to mock control (P=0.022; Fig. 3.4). This effect was not observed using a mutant 14-3-3ε^{K49E} construct that is unable to bind to its client proteins (P=0.234), indicating the presence of a 14-3-3 signalling hub in *POMC* regulation.

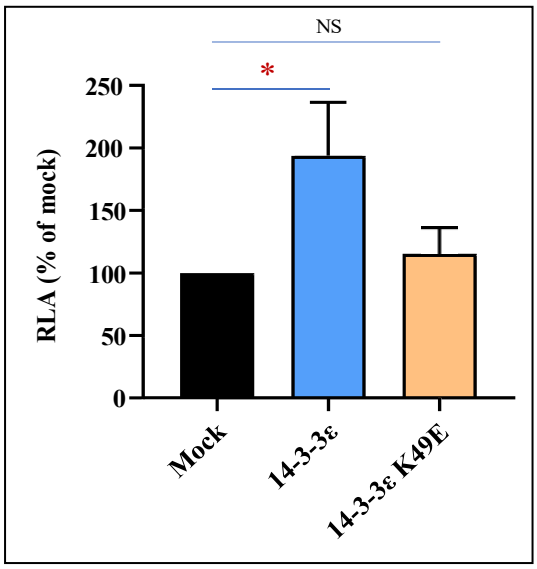


Figure 3.4. Effect of 14-3-3ε overexpression on human *POMC* promoter activity. AtT-20 cells were transfected with the human POMC promoter luciferase reporter and 14-3-3ε/14-3-3ε^{K49E} overexpressing vectors for 24 hours. The 14-3-3ε^{K49E} mutant is incapable of protein binding. The empty pcDNA3 vector (mock) was used as control. Data are means ± standard deviation of three independent experiments measured as luciferase to GFP ratio and presented as percentage of mock. **P*<0.05 (Welch's *t*-test). NS, not significant. RLA, relative luciferase activity.

3.6. 14-3-3ε enrichment analysis

To identify putative molecular mechanisms mediating 14-3-3ε effects in corticotroph tumour cells, the STRING and GEPIA2 tools were utilized. The STRING clustering tool revealed in addition to USP8, RAF, RICTOR and HDAC proteins as putative 14-3-3ε clients (Fig.3.5A). GO enrichment analysis showed the majority of the potential 14-3-3ε partners belong to "MAP kinase kinase kinase" in addition to "protein binding" pathways (Fig.3.5B).

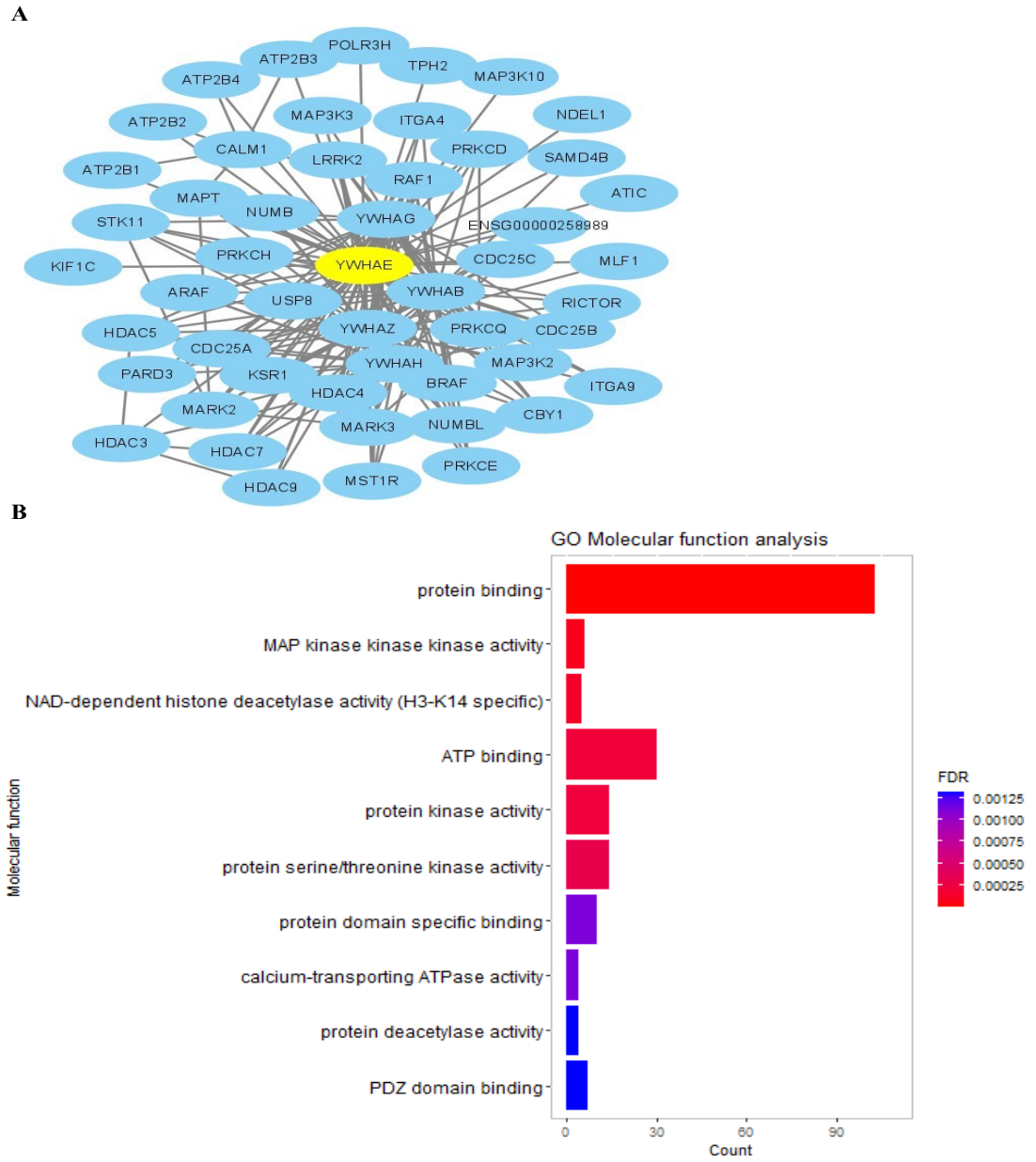


Figure 3.5. 14-3-3ε related gene enrichment analysis. A. STRING clustering showing 14-3-3ε ('YWHAE' in the figure) protein interacting proteins. B. Bar plots coloured by enrichment scores showing the top 10 terms. FDR<0.05 was considered statistically significant. GO, gene ontology.

3.7. 14-3-3ε induces *POMC* promoter activity via the MEK pathway

The enrichment analysis indicated that 14-3-3ε affects pathways associated with MEK signalling and protein serine/threonine kinase activity. To identify the mechanisms mediating the stimulatory effect of 14-3-3ε on human *POMC* promoter, inhibitors against major serine/threonine kinases were utilized in 14-3-3ε overexpressing AtT-20 cells. 14-3-3ε overexpression was able to significantly induce *POMC* promoter activity

in the presence of the prototype non-selective serine/threonine kinase inhibitor staurosporin (PKC>>PKG, S6K, CaMKII, and others; source: Selleckchem) (Fig. 3.6). In contrast, treatment with the selective MAP kinase kinase (MEK1/2) inhibitor ARRY-162 (also known as MEK162 or binimetinib) completely abolished the stimulatory action of 14-3-3ε.

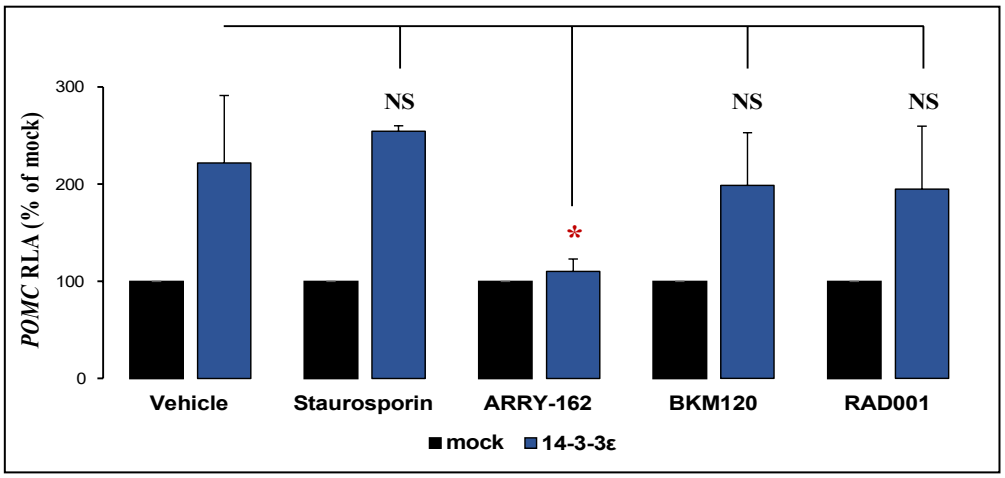


Figure 3.6. Effect of small molecule inhibitors on 14-3-3ε stimulatory action on human *POMC* promoter activity. AtT-20 cells were transfected with the human *POMC* promoter luciferase reporter and 14-3-3ε overexpressing vector (or empty pcDNA3 vector, mock) for 24 hours before being treated with the indicated inhibitors (staurosporin 1μM, ARRY-162 1μM, BKM120 0.1μM, and RAD001 0.1μM) in serum free DMEM for 6 hours. All compounds were dissolved in DMSO (Vehicle). Data are means ± standard deviation of three independent experiments measured as luciferase to GFP ratio and presented as percentage of each mock. *P<0.05 (*t*-test). NS, not significant. RLA, relative luciferase activity.

Use of selective PI3K inhibitor (BKM120, also known as buparlisib) and mTOR inhibitor RAD001 (also known as everolimus) had no effect, indicating a specific role of the MEK pathway in mediating 14-3-3ε action on *POMC*.

Western blot on 14-3-3ε overexpressing cell lysates showed increased MAPK phosphorylation at Thr202/Tyr204, a site phosphorylated by MEK1, compared to mock transfected control (Fig 3.7A). In these cells, ARRY-162 treatment abolished the stimulatory action of 14-3-3ε (p=0,017; Fig. 3.7B), confirming the notion that in corticotroph tumour cells 14-3-3ε induces MEK activity to stimulate *POMC*.

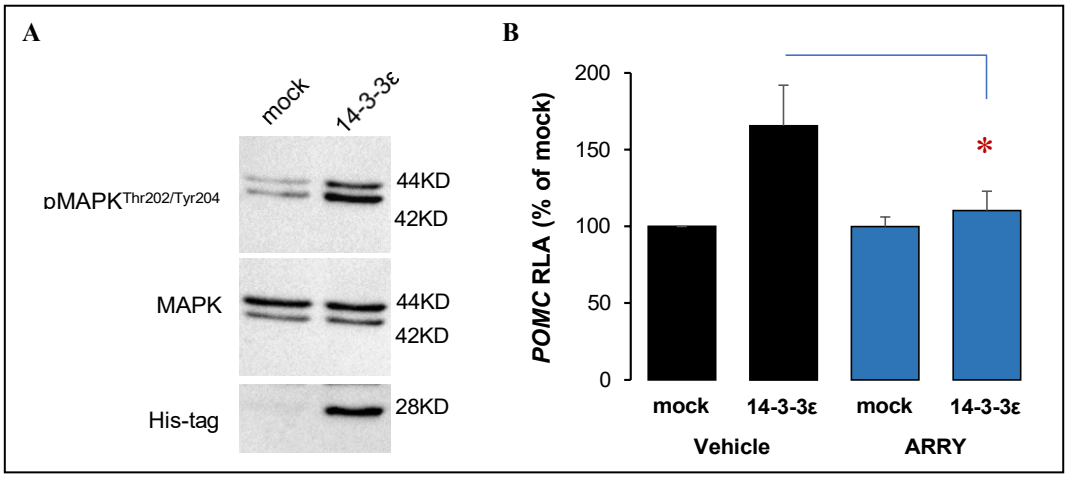


Figure 3.7. 14-3-3ε stimulates *POMC* promoter activity via MAPK. A. Western blot for phosphorylated pMAPK-Thr202/Tyr204 using cell lysates of AtT-20 overexpressing 14-3-3ε or mock empty pcDNA3 vector. Membranes were blotted for total MAPK and His-tag (for 14-3-3ε overexpression) after sequential stripping with Tris pH2.0 buffer. B. Effect of the MEK inhibitor ARRY-162 on 14-3-3ε action on human POMC promoter activity. AtT-20 cells overexpressing 14-3-3 or empty pcDNA3 plasmid (mock) were treated with vehicle (DMSO) or ARRY-162 (1μM) in serum free DMEM for 6 hours. Data are means ± standard deviation of four independent experiments measured as luciferase to GFP ratio and presented as percentage of each mock control. **P*<0.05 to mock vehicle treated control (unpaired *t*-test). RLA, relative luciferase activity.

3.8. 14-3-3ε on corticotroph tumour cell viability

14-3-3ε overexpression did not affect AtT-20 cell viability (P=0.893, Fig. 3.8).

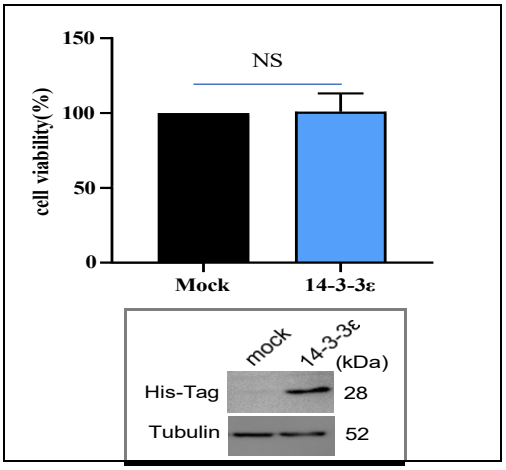


Figure 3.8: Effect of overexpressing of 14-3-3ε on cell viability. AtT-20 cells were transfected with 14-3-3ε or mock empty pcDNA3 vector for 24 hours before being assayed with the WST-1 colorimetric assay. Data are mean ± standard deviation of three independent experiments presented as percentage of mock. NS: not significant (unpaired *t*-test). Western blot for His-Tag confirmed the successful 14-3-3ε overexpression.

4. Discussion

4.1. 14-3-3ε is highly expressed in corticotroph tumours

My study examined the expression in corticotroph tumours of 14-3-3 proteins that interact with USP8. The results show higher expression of 14-3-3ε in these tumours compared to the normal pituitary and a predominance for epsilon over the other 14-3-3 proteins.

The present study shows that in corticotroph tumours 14-3-3ε expression is higher compared to the normal pituitary. Other intracranial tumours, such as meningiomas and astrocytomas, also show 14-3-3ε overexpression, which correlates positively with tumour grade [225, 226]. In corticotroph tumours, no correlation was observed between 14-3-3ε expression and each tumour grade (Hardy's and Knosp) possibly due to the relatively low number of cases in each subgroup. Nevertheless, high 14-3-3ε expression correlated with lack of invasion and better postoperative outcome. In fact, grouping together the no/low invasive groups (Hardy's grade I-II, Knosp 0-2) showed higher 14-3-3ε expression compared to the grades representing invasive tumours (Hardy's III-IV and Knosp 3-4). Tumour grade reflects postoperative outcome [227]. Indeed, total surgical resection and postoperative remission were more frequent in tumours with higher 14-3-3ε expression. However, no correlation was found between 14-3-3ε expression and biochemical parameters, which may be due to the retrospective and multicentre nature of the study. Indeed in the study cohort presented herein, hormonal values were not available for all cases and diagnostic protocols differed from centre to centre.

14-3-3ε is frequently deregulated in cancer, where it was reported to act as oncogene or tumour suppressor depending on the tumour type [189]. In gastric cancer and hepatocellular carcinoma, high 14-3-3ε expression correlates with higher risk of metastasis and reduced progression-free survival [228-230]. In addition, 14-3-3ε is overexpressed in prostate cancer, where it is implicated in inducing tumour survival by inactivating FOXO [231]. In contrast, in laryngeal squamous cell carcinoma and breast cancer high 14-3-3ε expression correlated with lower stage and better prognosis [232,

233]. Finally, 14-3-3ε expression was shown to be downregulated in small cell lung cancers, where it acts as a tumour suppressor [234]. In corticotroph tumours, the high 14-3-3ε expression correlated with non-invasive tumours and a more favourable postoperative outcome.

4.2. 14-3-3σ is downregulated in corticotroph tumours

Corticotroph tumours had reduced of 14-3-3σ expression compared to the normal pituitary tissue. Although there have been reports of upregulation in example pancreatic and gastric cancer [235, 236], the majority of studies reported 14-3-3 downregulation in cancers like prostate, lung, breast, liver and colorectal cancer [237-241].

In some cancers such as breast and endometrial cancer, 14-3-3σ correlated with tumour invasion, tumour stage and clinical outcome, while in others no correlation was found [242-246]. In the present study no correlation between corticotroph tumour 14-3-3σ expression and clinical parameters such as tumour invasion, total tumour resection and postoperative remission was found.

14-3-3σ acts as a tumour suppressor that causes cell cycle arrest [187, 218, 247]. It is induced by p53 and in turn it improves its stability and transcriptional activity [193, 218, 248]. This may explain the significantly lower 14-3-3σ levels in the few *TP53* mutant corticotroph tumours included in the present study.

14-3-3σ interacts with numerous proteins and controls crucial for tumour survival processes [249, 250] (reviewed in [187]). Its interaction with CDK2 [247], is of potential significance for corticotroph pathophysiology, since 14-3-3σ downregulation in corticotroph tumours may release CDK2 to drive cell cycle progression. Unfortunately, the lack of expression in the available corticotroph cell model AtT-20 did not allow testing the impact of 14-3-3σ downregulation on corticotroph cell cycle.

4.3. 14-3-3 and USP8 mutational status

From the two 14-3-3 proteins deregulated in corticotroph tumours compared to normal pituitary, only 14-3-3ε, but not 14-3-3σ, correlated with the USP8 mutational status.

The higher expression of 14-3-3ε in *USP8* mutant tumours may explain the correlation with invasion, since these tumours are more frequently noninvasive [165, 166, 173].
The reason for the higher 14-3-3ε levels in *USP8* mutant tumours is at present unclear. Still it is noteworthy that from the USP8 binding proteins, only 14-3-3ε was found to be deregulated in corticotroph tumours (compared to the normal pituitary) and its expression to correlate with the USP8 mutational status. Since the USP8 mutations affect the 14-3-3 binding site, 14-3-3ε cannot inhibit the deubiquitinase activity of USP8 mutants as it does with the wild-type protein [171]. It is possible that this loss of autoinhibitory regulation promotes the upregulation of 14-3-3 possibly as a compensatory mechanism in a yet to be elucidated mechanism.

4.4. 14-3-3ε regulate *POMC* in corticotroph tumour cells

The findings of relatively high 14-3-3ε expression in corticotroph tumours suggests a potential role in corticotroph function and indeed, 14-3-3ε overexpression *in vitro* increased human *POMC* promoter activity.
14-3-3ε proteins are involved in diverse signalling cascades [181]. The most relevant in Cushing's disease 14-3-3 client is USP8. However, since 14-3-3ε inhibits USP8 deubiquitinase activity [171], this interaction is unlikely to mediate its stimulatory action on human *POMC*.
To explore alternative possible mechanisms of 14-3-3ε action on *POMC*, enrichment analysis was utilized and revealed association with proteins involved in MEK kinase activity and protein serine/threonine kinase activity. Using inhibitors against the main serine/threonine kinases showed that 14-3-3ε stimulates *POMC* via MEK, but not via PKC, PI3K or mTOR. 14-3-3ε binding proteins include RAF1 (also known as CRAF), ARAF and BRAF proteins. BRAF overexpression was shown to induce *POMC* promoter activity and transcription in AtT-20 corticotroph tumour cells [174]. 14-3-3 dimers bind and activate BRAF in complex with its target MEK1 [251, 252], indicating a potential mechanism of 14-3-3ε action.

In the present study, use of the MEK inhibitor ARRY-162 abolished the stimulatory action of 14-3-3ε overexpression on *POMC* promoter, but had no effect on basal promoter activity at 1μM concentration after 6 hours of treatment. A previous study has shown that 48-hours treatment with very high (up to 80μM) concentrations of ARRY-162 (also known as binimetinib) inhibited ACTH synthesis in both murine AtT-20 cells and in human corticotroph tumours in primary cell culture after 48 hours [253]. This effect however may have been due to the strong suppression of cell viability and high rate of apoptosis observed at this time point.

There is increasing evidence for a central role of MEK signalling pathway on *POMC* regulation. CRH utilizes the MEK-target MAPK to stimulate *POMC* transcription [36, 254]. In addition, EGF induces *POMC* promoter activity through MAPK [255]. Finally, USP8 mutants were shown to potentiate EGFR signalling on ACTH synthesis via MAPK, but not AKT, activation [165]. Altogether these findings suggest that targeting the MEK pathway may be promising for the management of ACTH hypersecretion in Cushing's disease.

4.5. 14-3-3ε does not affect corticotroph tumour cell viability

In the present study, 14-3-3ε overexpression in corticotroph tumour cells *in vitro* did not affect cell viability. The effect of 14-3-3 on cell proliferation is not clear. 14-3-3ε was found to block cell cycle progression to mitosis [211, 256]. 14-3-3ε overexpression decreased proliferation in a small cell lung cancer [234] and gastric cancer [257] cell lines. In contrast, 14-3-3ε was found to induce proliferation of breast cancer cells in vitro [258]. It was also found to maintain the survival of cardiac cells and cardiomyocytes [259]. In fact, it was shown to block apoptosis by inhibiting the proapoptotic proteins BAD and BAX [216].

The role of 14-3-3 proteins on cell cycle regulation is complex and may depend on the tumour subtype [260]. The evidence provided by the present study indicates that 14-3-3ε may not play a role in corticotroph tumour cell proliferation, but rather in corticotroph function.

5. Conclusion

The present work shows deregulated expression of 14-3-3ε and σ in corticotroph tumours compared to the human normal pituitary. High 14-3-3ε expression was linked to lower tumour grade and postoperative remission, indicating a more favourable disease outcome for Cushing's disease patients. Interestingly, this study revealed a correlation between 14-3-3ε, but not σ, and positive *USP8* mutational status, suggesting a bidirectional regulatory loop between 14-3-3ε and its client. The *in vitro* investigation in 14-3-3ε overexpressing corticotroph tumour cells did not show any significant impact on cell viability, but revealed a stimulatory action on *POMC* that was mediated by the MEK signalling pathway. Altogether these observations suggest a role for 14-3-3ε, possibly as a signalling hub connecting diverse regulatory pathways, in corticotroph cell physiology.

References

1. Larkin, S. and O. Ansorge, Development and microscopic anatomy of the pituitary gland. 2017.
2. Devnath, S. and K. Inoue, An insight to pituitary folliculo-stellate cells. J Neuroendocrinol, 2008. 20(6): p. 687-91.
3. Melmed, S., The pituitary. 2010: Academic press.
4. Herman, J.P., et al., Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Compr Physiol, 2016. 6(2): p. 603-21.
5. Keller-Wood, M.E. and M.F. Dallman, Corticosteroid inhibition of ACTH secretion. Endocr Rev, 1984. 5(1): p. 1-24.
6. Chretien, M. and M. Mbikay, 60 YEARS OF POMC: From the prohormone theory to pro-opiomelanocortin and to proprotein convertases (PCSK1 to PCSK9). J Mol Endocrinol, 2016. 56(4): p. T49-62.
7. Benjannet, S., et al., PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. Proc Natl Acad Sci U S A, 1991. 88(9): p. 3564-8.
8. Day, R., et al., Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. Mol Endocrinol, 1992. 6(3): p. 485-97.
9. Tremblay, Y., et al., Pituitary-specific expression and glucocorticoid regulation of a proopiomelanocortin fusion gene in transgenic mice. Proc Natl Acad Sci U S A, 1988. 85(23): p. 8890-4.
10. Lavoie, P.L., et al., Developmental dependence on NurRE and EboxNeuro for expression of pituitary proopiomelanocortin. Mol Endocrinol, 2008. 22(7): p. 1647-57.
11. Hammer, G.D., V. Fairchild-Huntress, and M.J. Low, Pituitary-specific and hormonally regulated gene expression directed by the rat proopiomelanocortin promoter in transgenic mice. Mol Endocrinol, 1990. 4(11): p. 1689-97.
12. Liu, B., et al., Identification of DNA elements cooperatively activating proopiomelanocortin gene expression in the pituitary glands of transgenic mice. Mol Cell Biol, 1992. 12(9): p. 3978-90.
13. Rubinstein, M., et al., Rat and mouse proopiomelanocortin gene sequences target tissue-specific expression to the pituitary gland but not to the hypothalamus of transgenic mice. Neuroendocrinology, 1993. 58(4): p. 373-80.
14. Gage, P.J., H. Suh, and S.A. Camper, The bicoid-related Pitx gene family in development. Mamm Genome, 1999. 10(2): p. 197-200.
15. Lanctôt, C., B. Lamolet, and J.J.D. Drouin, The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. 1997. 124(14): p. 2807-2817.
16. Suh, H., et al., Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. 2002.
17. Kelberman, D., et al., Genetic regulation of pituitary gland development in human and mouse. Endocr Rev, 2009. 30(7): p. 790-829.
18. Tremblay, J.J., C. Lanctot, and J. Drouin, The pan-pituitary activator of

transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. Mol Endocrinol, 1998. 12(3): p. 428-41.
19. Lamolet, B., et al., A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. Cell, 2001. 104(6): p. 849-59.
20. Langlais, D., et al., A pituitary-specific enhancer of the POMC gene with preferential activity in corticotrope cells. Mol Endocrinol, 2011. 25(2): p. 348-59.
21. Pulichino, A.M., et al., Tpit determines alternate fates during pituitary cell differentiation. Genes Dev, 2003. 17(6): p. 738-47.
22. Pulichino, A.M., et al., Human and mouse TPIT gene mutations cause early onset pituitary ACTH deficiency. Genes Dev, 2003. 17(6): p. 711-6.
23. Therrien, M. and J. Drouin, Cell-specific helix-loop-helix factor required for pituitary expression of the pro-opiomelanocortin gene. Mol Cell Biol, 1993. 13(4): p. 2342-53.
24. Poulin, G., B. Turgeon, and J. Drouin, NeuroD1/beta2 contributes to cell-specific transcription of the proopiomelanocortin gene. Mol Cell Biol, 1997. 17(11): p. 6673-82.
25. Poulin, G., et al., Specific protein-protein interaction between basic helix-loop-helix transcription factors and homeoproteins of the Pitx family. Mol Cell Biol, 2000. 20(13): p. 4826-37.
26. Gysling, K., et al., Corticotropin-releasing hormone and urocortin: redundant or distinctive functions? Brain Res Brain Res Rev, 2004. 47(1-3): p. 116-25.
27. Hauger, R.L., et al., Corticotropin releasing factor (CRF) receptor signaling in the central nervous system: new molecular targets. CNS Neurol Disord Drug Targets, 2006. 5(4): p. 453-79.
28. Labrie, F., et al., Corticotropin-releasing factor stimulates accumulation of adenosine 3', 5'-monophosphate in rat pituitary corticotrophs. Science, 1982. 216(4549): p. 1007-8.
29. Gutknecht, E., et al., Molecular mechanisms of corticotropin-releasing factor receptor-induced calcium signaling. Mol Pharmacol, 2009. 75(3): p. 648-57.
30. Boutillier, A.L., et al., Transcriptional activation of the proopiomelanocortin gene by cyclic AMP-responsive element binding protein. Pituitary, 1998. 1(1): p. 33-43.
31. Law, S.W., et al., Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol, 1992. 6(12): p. 2129-35.
32. Ohkura, N., et al., Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. Biochem Biophys Res Commun, 1994. 205(3): p. 1959-65.
33. Philips, A., et al., Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. Mol Cell Biol, 1997. 17(10): p. 5946-51.
34. Maira, M., et al., Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. 1999.

19(11): p. 7549-7557.
35. Kovalovsky, D.n., et al., Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. 2002. 16(7): p. 1638-1651.
36. Maira, M., et al., The T-box factor Tpit recruits SRC/p160 co-activators and mediates hormone action. J Biol Chem, 2003. 278(47): p. 46523-32.
37. Wotjak, C.T., et al., Release of vasopressin from supraoptic neurons within the median eminence in vivo. A combined microdialysis and push-pull perfusion study in the rat. 1996. 726(1-2): p. 237-241.
38. Tanoue, A., et al., The vasopressin V1b receptor critically regulates hypothalamic-pituitary-adrenal axis activity under both stress and resting conditions. J Clin Invest, 2004. 113(2): p. 302-9.
39. Thibonnier, M., et al., Molecular pharmacology and modeling of vasopressin receptors. Prog Brain Res, 2002. 139: p. 179-96.
40. Aguilera, G., S. Volpi, and C. Rabadan-Diehl, Transcriptional and post-transcriptional mechanisms regulating the rat pituitary vasopressin V1b receptor gene. J Mol Endocrinol, 2003. 30(2): p. 99-108.
41. Duncan, P.J., et al., Large conductance Ca(2)(+)-activated K(+) (BK) channels promote secretagogue-induced transition from spiking to bursting in murine anterior pituitary corticotrophs. J Physiol, 2015. 593(5): p. 1197-211.
42. Mollard, P., et al., Electrical properties of cultured human adrenocorticotropin-secreting adenoma cells: effects of high K+, corticotropin-releasing factor, and angiotensin II. Endocrinology, 1987. 121(1): p. 395-405.
43. Takano, K., et al., Corticotropin-releasing hormone excites adrenocorticotropin-secreting human pituitary adenoma cells by activating a nonselective cation current. J Clin Invest, 1996. 98(9): p. 2033-41.
44. Arzt, E., et al., Interleukin-2 and interleukin-2 receptor expression in human corticotrophic adenoma and murine pituitary cell cultures. J Clin Invest, 1992. 90(5): p. 1944-51.
45. Auernhammer, C.J., V. Chesnokova, and S. Melmed, Leukemia inhibitory factor modulates interleukin-1beta-induced activation of the hypothalamo-pituitary-adrenal axis. Endocrinology, 1998. 139(5): p. 2201-8.
46. Pereda, M.P., et al., Interleukin-6 is inhibited by glucocorticoids and stimulates ACTH secretion and POMC expression in human corticotroph pituitary adenomas. Exp Clin Endocrinol Diabetes, 2000. 108(3): p. 202-7.
47. Bousquet, C., M.C. Zatelli, and S. Melmed, Direct regulation of pituitary proopiomelanocortin by STAT3 provides a novel mechanism for immuno-neuroendocrine interfacing. J Clin Invest, 2000. 106(11): p. 1417-25.
48. Ray, D.W., S.G. Ren, and S. Melmed, Leukemia inhibitory factor (LIF) stimulates proopiomelanocortin (POMC) expression in a corticotroph cell line. Role of STAT pathway. J Clin Invest, 1996. 97(8): p. 1852-9.
49. Evans, A.N., et al., Regulation of hypothalamic corticotropin-releasing hormone transcription by elevated glucocorticoids. Mol Endocrinol, 2013. **27**(11): p. 1796-807.

50. Mangelsdorf, D.J., et al., The nuclear receptor superfamily: the second decade. Cell, 1995. **83**(6): p. 835-9.
51. Drouin, J., et al., Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. EMBO J, 1993. **12**(1): p. 145-56.
52. Riegel, A.T., et al., Proopiomelanocortin gene promoter elements required for constitutive and glucocorticoid-repressed transcription. Mol Endocrinol, 1991. **5**(12): p. 1973-82.
53. Drouin, J., et al., Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. Mol Cell Biol, 1989. **9**(12): p. 5305-14.
54. Jenks, B.G., Regulation of proopiomelanocortin gene expression: an overview of the signaling cascades, transcription factors, and responsive elements involved. Ann N Y Acad Sci, 2009. **1163**: p. 17-30.
55. Philips, A., et al., Antagonism between Nur77 and glucocorticoid receptor for control of transcription. Mol Cell Biol, 1997. **17**(10): p. 5952-9.
56. Cushing, H., The Pituitary Body and Its Disorders, Philadelphia, J. B. 1912, Lippincott Company.
57. Cushing, H., The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). 1932. Obes Res, 1994. **2**(5): p. 486-508.
58. Carroll, T.B. and J.W. Findling, Cushing's syndrome of nonpituitary causes. Curr Opin Endocrinol Diabetes Obes, 2009. **16**(4): p. 308-15.
59. Raff, H., S.T. Sharma, and L.K. Nieman, Physiological basis for the etiology, diagnosis, and treatment of adrenal disorders: Cushing's syndrome, adrenal insufficiency, and congenital adrenal hyperplasia. Compr Physiol, 2014. **4**(2): p. 739-69.
60. Raff, H. and J.W. Findling, A physiologic approach to diagnosis of the Cushing syndrome. Ann Intern Med, 2003. **138**(12): p. 980-91.
61. Tritos, N.A. and B.M. Biller, Cushing's disease. Handb Clin Neurol, 2014. **124**: p. 221-34.
62. Nieman, L.K., et al., The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab, 2008. **93**(5): p. 1526-40.
63. Ragnarsson, O., et al., The incidence of Cushing's disease: a nationwide Swedish study. Pituitary, 2019. **22**(2): p. 179-186.
64. Pivonello, R., et al., Cushing's Syndrome. Endocrinol Metab Clin North Am, 2008. **37**(1): p. 135-49, ix.
65. Steffensen, C., et al., Epidemiology of Cushing's syndrome. Neuroendocrinology, 2010. **92 Suppl 1**: p. 1-5.
66. Felig, P. and L.A. Frohman, Endocrinology and Metabolism. 2001: McGraw-Hill.
67. Valassi, E., et al., The European Registry on Cushing's syndrome: 2-year experience. Baseline demographic and clinical characteristics. Eur J Endocrinol, 2011. **165**(3): p. 383-92.
68. Feelders, R.A., et al., The burden of Cushing's disease: clinical and health-

related quality of life aspects. Eur J Endocrinol, 2012. **167**(3): p. 311-26.
69. Pivonello, R., et al., Pathophysiology of diabetes mellitus in Cushing's syndrome. Neuroendocrinology, 2010. **92 Suppl 1**: p. 77-81.
70. Arnaldi, G., et al., Pathophysiology of dyslipidemia in Cushing's syndrome. Neuroendocrinology, 2010. **92 Suppl 1**: p. 86-90.
71. Faggiano, A., et al., Cardiovascular risk factors and common carotid artery caliber and stiffness in patients with Cushing's disease during active disease and 1 year after disease remission. J Clin Endocrinol Metab, 2003. **88**(6): p. 2527-33.
72. Newell-Price, J., et al., The diagnosis and differential diagnosis of Cushing's syndrome and pseudo-Cushing's states. Endocr Rev, 1998. **19**(5): p. 647-72.
73. Cicala, M.V. and F. Mantero, Hypertension in Cushing's syndrome: from pathogenesis to treatment. Neuroendocrinology, 2010. **92 Suppl 1**: p. 44-9.
74. van der Pas, R., et al., Cushing's disease and hypertension: in vivo and in vitro study of the role of the renin-angiotensin-aldosterone system and effects of medical therapy. Eur J Endocrinol, 2014. **170**(2): p. 181-91.
75. Van Zaane, B., et al., Hypercoagulable state in Cushing's syndrome: a systematic review. J Clin Endocrinol Metab, 2009. **94**(8): p. 2743-50.
76. Faggiano, A., et al., Sulfur amino acids in Cushing's disease: insight in homocysteine and taurine levels in patients with active and cured disease. J Clin Endocrinol Metab, 2005. **90**(12): p. 6616-22.
77. Colao, A., et al., Persistence of increased cardiovascular risk in patients with Cushing's disease after five years of successful cure. J Clin Endocrinol Metab, 1999. **84**(8): p. 2664-72.
78. Luton, J.P., et al., Reversible gonadotropin deficiency in male Cushing's disease. J Clin Endocrinol Metab, 1977. **45**(3): p. 488-95.
79. Ohmori, N., et al., Osteoporosis is more prevalent in adrenal than in pituitary Cushing's syndrome. Endocr J, 2003. **50**(1): p. 1-7.
80. Pivonello, R., et al., Complications of Cushing's syndrome: state of the art. Lancet Diabetes Endocrinol, 2016. **4**(7): p. 611-29.
81. Di Somma, C., et al., Effect of 2 years of cortisol normalization on the impaired bone mass and turnover in adolescent and adult patients with Cushing's disease: a prospective study. Clin Endocrinol (Oxf), 2003. **58**(3): p. 302-8.
82. Auphan, N., et al., Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science, 1995. **270**(5234): p. 286-90.
83. Cohn, L.A., Glucocorticosteroids as immunosuppressive agents. Semin Vet Med Surg Small Anim, 1997. **12**(3): p. 150-6.
84. Fareau, G.G. and R. Vassilopoulou-Sellin, Hypercortisolemia and infection. Infect Dis Clin North Am, 2007. **21**(3): p. 639-57, viii.
85. Bakker, R.C., et al., Cushing's syndrome complicated by multiple opportunistic infections. J Endocrinol Invest, 1998. **21**(5): p. 329-33.
86. Sonino, N. and G.A. Fava, Psychiatric disorders associated with Cushing's syndrome. Epidemiology, pathophysiology and treatment. CNS Drugs, 2001.

15(5): p. 361-73.
87. Espinosa-de-Los-Monteros, A.L., et al., Persistence of Cushing's disease symptoms and comorbidities after surgical cure: a long-term, integral evaluation. Endocr Pract, 2013. **19**(2): p. 252-8.
88. Bourdeau, I., et al., Loss of brain volume in endogenous Cushing's syndrome and its reversibility after correction of hypercortisolism. J Clin Endocrinol Metab, 2002. **87**(5): p. 1949-54.
89. Starkman, M.N., Neuropsychiatric findings in Cushing syndrome and exogenous glucocorticoid administration. Endocrinol Metab Clin North Am, 2013. **42**(3): p. 477-88.
90. Lindsay, J.R., et al., Long-term impaired quality of life in Cushing's syndrome despite initial improvement after surgical remission. J Clin Endocrinol Metab, 2006. **91**(2): p. 447-53.
91. Arnaldi, G., et al., Diagnosis and complications of Cushing's syndrome: a consensus statement. J Clin Endocrinol Metab, 2003. **88**(12): p. 5593-602.
92. Fleseriu, M., et al., Consensus on diagnosis and management of Cushing's disease: a guideline update. Lancet Diabetes Endocrinol, 2021. **9**(12): p. 847-875.
93. Raff, H., Cushing's syndrome: diagnosis and surveillance using salivary cortisol. Pituitary, 2012. **15**(1): p. 64-70.
94. Petersenn, S., Biochemical diagnosis of Cushing's disease: Screening and confirmatory testing. Best Pract Res Clin Endocrinol Metab, 2021. **35**(1): p. 101519.
95. Newell-Price, J., et al., Cushing's syndrome. Lancet, 2006. **367**(9522): p. 1605-17.
96. Liddle, G.W., Tests of pituitary-adrenal suppressibility in the diagnosis of Cushing's syndrome. J Clin Endocrinol Metab, 1960. **20**: p. 1539-60.
97. Nieman, L.K. and I. Ilias, Evaluation and treatment of Cushing's syndrome. Am J Med, 2005. **118**(12): p. 1340-6.
98. Lonser, R.R., L. Nieman, and E.H. Oldfield, Cushing's disease: pathobiology, diagnosis, and management. J Neurosurg, 2017. **126**(2): p. 404-417.
99. Nieman, L.K., et al., Treatment of Cushing's Syndrome: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab, 2015. **100**(8): p. 2807-31.
100. Albani, A. and M. Theodoropoulou, Persistent Cushing's Disease after Transsphenoidal Surgery: Challenges and Solutions. Exp Clin Endocrinol Diabetes, 2021. **129**(3): p. 208-215.
101. Ferriere, A., et al., Cabergoline for Cushing's disease: a large retrospective multicenter study. Eur J Endocrinol, 2017. **176**(3): p. 305-314.
102. Stefaneanu, L., et al., Dopamine D2 receptor gene expression in human adenohypophysial adenomas. Endocrine, 2001. **14**(3): p. 329-36.
103. Cuevas-Ramos, D. and M. Fleseriu, Treatment of Cushing's disease: a mechanistic update. J Endocrinol, 2014. **223**(2): p. R19-39.
104. Godbout, A., et al., Cabergoline monotherapy in the long-term treatment of Cushing's disease. Eur J Endocrinol, 2010. **163**(5): p. 709-16.

105. Nakhleh, A., et al., Cabergoline treatment for recurrent Cushing's disease during pregnancy. Hormones (Athens), 2016. **15**(3): p. 453-458.
106. Hofland, L.J., et al., The multi-ligand somatostatin analogue SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor type 5. Eur J Endocrinol, 2005. **152**(4): p. 645-54.
107. Batista, D.L., et al., The effects of SOM230 on cell proliferation and adrenocorticotropin secretion in human corticotroph pituitary adenomas. J Clin Endocrinol Metab, 2006. **91**(11): p. 4482-8.
108. Boscaro, M., et al., Treatment of pituitary-dependent Cushing's disease with the multireceptor ligand somatostatin analog pasireotide (SOM230): a multicenter, phase II trial. J Clin Endocrinol Metab, 2009. **94**(1): p. 115-22.
109. Schopohl, J., et al., Pasireotide can induce sustained decreases in urinary cortisol and provide clinical benefit in patients with Cushing's disease: results from an open-ended, open-label extension trial. Pituitary, 2015. **18**(5): p. 604-12.
110. Pivonello, R., et al., Pasireotide treatment significantly improves clinical signs and symptoms in patients with Cushing's disease: results from a Phase III study. Clin Endocrinol (Oxf), 2014. **81**(3): p. 408-17.
111. Boscaro, M., et al., Extended treatment of Cushing's disease with pasireotide: results from a 2-year, Phase II study. Pituitary, 2014. **17**(4): p. 320-6.
112. Petersenn, S., et al., Long-term treatment of Cushing's disease with pasireotide: 5-year results from an open-label extension study of a Phase III trial. Endocrine, 2017. **57**(1): p. 156-165.
113. Lacroix, A., et al., Efficacy and safety of once-monthly pasireotide in Cushing's disease: a 12 month clinical trial. Lancet Diabetes Endocrinol, 2018. **6**(1): p. 17-26.
114. Broersen, L.H.A., et al., Effectiveness of medical treatment for Cushing's syndrome: a systematic review and meta-analysis. Pituitary, 2018. **21**(6): p. 631-641.
115. Theodoropoulou, M. and M. Reincke, Tumor-Directed Therapeutic Targets in Cushing Disease. J Clin Endocrinol Metab, 2019. **104**(3): p. 925-933.
116. Tritos, N.A. and B.M.K. Biller, Medical Therapy for Cushing's Syndrome in the Twenty-first Century. Endocrinol Metab Clin North Am, 2018. **47**(2): p. 427-440.
117. Feelders, R.A., L.J. Hofland, and W.W. de Herder, Medical treatment of Cushing's syndrome: adrenal-blocking drugs and ketaconazole. Neuroendocrinology, 2010. **92 Suppl 1**: p. 111-5.
118. Castinetti, F., et al., Ketoconazole in Cushing's disease: is it worth a try? J Clin Endocrinol Metab, 2014. **99**(5): p. 1623-30.
119. Daniel, E., et al., Effectiveness of Metyrapone in Treating Cushing's Syndrome: A Retrospective Multicenter Study in 195 Patients. J Clin Endocrinol Metab, 2015. **100**(11): p. 4146-54.
120. Ceccato, F., et al., Metyrapone treatment in Cushing's syndrome: a real-life study. Endocrine, 2018. **62**(3): p. 701-711.

121. Rubinstein, G., et al., Therapeutic options after surgical failure in Cushing's disease: A critical review. Best Pract Res Clin Endocrinol Metab, 2019. **33**(2): p. 101270.
122. Duggan, S., Osilodrostat: First Approval. Drugs, 2020. **80**(5): p. 495-500.
123. Fleseriu, M., et al., Osilodrostat, a potent oral 11beta-hydroxylase inhibitor: 22-week, prospective, Phase II study in Cushing's disease. Pituitary, 2016. **19**(2): p. 138-48.
124. Pivonello, R., et al., Efficacy and safety of osilodrostat in patients with Cushing's disease (LINC 3): a multicentre phase III study with a double-blind, randomised withdrawal phase. Lancet Diabetes Endocrinol, 2020. **8**(9): p. 748-761.
125. Johanssen, S. and B. Allolio, Mifepristone (RU 486) in Cushing's syndrome. Eur J Endocrinol, 2007. **157**(5): p. 561-9.
126. Pivonello, R., et al., Medical Treatment of Cushing's Disease: An Overview of the Current and Recent Clinical Trials. Front Endocrinol (Lausanne), 2020. **11**: p. 648.
127. Ciato, D., et al., Currently used and investigational drugs for Cushing s disease. Expert Opin Investig Drugs, 2017. **26**(1): p. 75-84.
128. Dahia, P.L., et al., Expression of glucocorticoid receptor gene isoforms in corticotropin-secreting tumors. J Clin Endocrinol Metab, 1997. **82**(4): p. 1088-93.
129. Theodoropoulou, M., Glucocorticoid Receptors Are Making a Comeback in Corticotroph Tumorigenesis. Endocrinology, 2022. **163**(2).
130. Sbiera, S., et al., The New Genetic Landscape of Cushing's Disease: Deubiquitinases in the Spotlight. Cancers (Basel), 2019. **11**(11).
131. Korbonits, M., et al., Expression of 11 beta-hydroxysteroid dehydrogenase isoenzymes in the human pituitary: induction of the type 2 enzyme in corticotropinomas and other pituitary tumors. J Clin Endocrinol Metab, 2001. **86**(6): p. 2728-33.
132. Riebold, M., et al., A C-terminal HSP90 inhibitor restores glucocorticoid sensitivity and relieves a mouse allograft model of Cushing disease. Nat Med, 2015. **21**(3): p. 276-80.
133. Bilodeau, S., et al., Role of Brg1 and HDAC2 in GR trans-repression of the pituitary POMC gene and misexpression in Cushing disease. Genes Dev, 2006. **20**(20): p. 2871-86.
134. Du, L., et al., Evidence for orphan nuclear receptor TR4 in the etiology of Cushing disease. Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8555-60.
135. Araki, T. and N.A. Liu, Cell Cycle Regulators and Lineage-Specific Therapeutic Targets for Cushing Disease. Front Endocrinol (Lausanne), 2018. **9**: p. 444.
136. Jordan, S., et al., Cyclin D and cyclin E expression in normal and adenomatous pituitary. Eur J Endocrinol, 2000. **143**(1): p. R1-6.
137. Lidhar, K., et al., Low expression of the cell cycle inhibitor p27Kip1 in normal corticotroph cells, corticotroph tumors, and malignant pituitary tumors. J Clin Endocrinol Metab, 1999. **84**(10): p. 3823-30.

138. Sheaff, R.J., et al., Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev, 1997. **11**(11): p. 1464-78.
139. Roussel-Gervais, A., et al., Cooperation between cyclin E and p27(Kip1) in pituitary tumorigenesis. Mol Endocrinol, 2010. **24**(9): p. 1835-45.
140. Roussel-Gervais, A., et al., The Cables1 Gene in Glucocorticoid Regulation of Pituitary Corticotrope Growth and Cushing Disease. J Clin Endocrinol Metab, 2016. **101**(2): p. 513-22.
141. Hernandez-Ramirez, L.C., et al., Loss-of-function mutations in the CABLES1 gene are a novel cause of Cushing's disease. Endocr Relat Cancer, 2017. **24**(8): p. 379-392.
142. Polk, D.H., et al., Epidermal growth factor acts as a corticotropin-releasing factor in chronically catheterized fetal lambs. J Clin Invest, 1987. **79**(3): p. 984-8.
143. Childs, G.V., et al., Epidermal growth factor enhances ACTH secretion and expression of POMC mRNA by corticotropes in mixed and enriched cultures. Mol Cell Neurosci, 1991. **2**(3): p. 235-43.
144. Theodoropoulou, M., et al., Expression of epidermal growth factor receptor in neoplastic pituitary cells: evidence for a role in corticotropinoma cells. J Endocrinol, 2004. **183**(2): p. 385-94.
145. Brito, L.P., et al., Influence of the fibroblast growth factor receptor 4 expression and the G388R functional polymorphism on Cushing's disease outcome. J Clin Endocrinol Metab, 2010. **95**(10): p. E271-9.
146. Nakano-Tateno, T., et al., FGFR4 polymorphic variants modulate phenotypic features of Cushing disease. Mol Endocrinol, 2014. **28**(4): p. 525-33.
147. Giacomini, D., et al., Bone morphogenetic protein-4 inhibits corticotroph tumor cells: involvement in the retinoic acid inhibitory action. Endocrinology, 2006. **147**(1): p. 247-56.
148. Tsukamoto, N., et al., Effects of bone morphogenetic protein (BMP) on adrenocorticotropin production by pituitary corticotrope cells: involvement of up-regulation of BMP receptor signaling by somatostatin analogs. Endocrinology, 2010. **151**(3): p. 1129-41.
149. Vila, G., et al., Sonic hedgehog regulates CRH signal transduction in the adult pituitary. FASEB J, 2005. **19**(2): p. 281-3.
150. Vila, G., et al., Expression and function of sonic hedgehog pathway components in pituitary adenomas: evidence for a direct role in hormone secretion and cell proliferation. J Clin Endocrinol Metab, 2005. **90**(12): p. 6687-94.
151. Arzt, E., et al., Pathophysiological role of the cytokine network in the anterior pituitary gland. Front Neuroendocrinol, 1999. **20**(1): p. 71-95.
152. Bousquet, C., D.W. Ray, and S. Melmed, A common pro-opiomelanocortin-binding element mediates leukemia inhibitory factor and corticotropin-releasing hormone transcriptional synergy. J Biol Chem, 1997. **272**(16): p. 10551-7.
153. Yano, H., et al., Pituitary-directed leukemia inhibitory factor transgene causes Cushing's syndrome: neuro-immune-endocrine modulation of pituitary development. Mol Endocrinol, 1998. **12**(11): p. 1708-20.

154. Bartel, D.P., MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 2004. **116**(2): p. 281-97.
155. Amaral, F.C., et al., MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. J Clin Endocrinol Metab, 2009. **94**(1): p. 320-3.
156. Stilling, G., et al., MicroRNA expression in ACTH-producing pituitary tumors: up-regulation of microRNA-122 and -493 in pituitary carcinomas. Endocrine, 2010. **38**(1): p. 67-75.
157. Gentilin, E., et al., miR-26a plays an important role in cell cycle regulation in ACTH-secreting pituitary adenomas by modulating protein kinase Cdelta. Endocrinology, 2013. **154**(5): p. 1690-700.
158. Denes, J. and M. Korbonits, The clinical aspects of pituitary tumour genetics. Endocrine, 2021. **71**(3): p. 663-674.
159. de Kock, L., et al., Pituitary blastoma: a pathognomonic feature of germ-line DICER1 mutations. Acta Neuropathol, 2014. **128**(1): p. 111-22.
160. Kiefer, F.W., et al., PRKAR1A mutation causing pituitary-dependent Cushing disease in a patient with Carney complex. Eur J Endocrinol, 2017. **177**(2): p. K7-K12.
161. Nandagopal, R., et al., Cushing's syndrome due to a pituitary corticotropinoma in a child with tuberous sclerosis: an association or a coincidence? Clin Endocrinol (Oxf), 2007. **67**(4): p. 639-41.
162. Cazabat, L., et al., Germline AIP mutations in apparently sporadic pituitary adenomas: prevalence in a prospective single-center cohort of 443 patients. J Clin Endocrinol Metab, 2012. **97**(4): p. E663-70.
163. Biller, B.M., et al., Clonal origins of adrenocorticotropin-secreting pituitary tissue in Cushing's disease. J Clin Endocrinol Metab, 1992. **75**(5): p. 1303-9.
164. Albani, A., et al., The USP8 mutational status may predict long-term remission in patients with Cushing's disease. Clin Endocrinol (Oxf), 2018.
165. Reincke, M., et al., Mutations in the deubiquitinase gene USP8 cause Cushing's disease. Nat Genet, 2015. **47**(1): p. 31-8.
166. Ma, Z.Y., et al., Recurrent gain-of-function USP8 mutations in Cushing's disease. Cell Res, 2015. **25**(3): p. 306-17.
167. Hayashi, K., et al., The USP8 mutational status may predict drug susceptibility in corticotroph adenomas of Cushing's disease. Eur J Endocrinol, 2016. **174**(2): p. 213-26.
168. Perez-Rivas, L.G., et al., Somatic USP8 mutations are frequent events in corticotroph tumor progression causing Nelson's tumor. Eur J Endocrinol, 2018. **178**(1): p. 57-63.
169. Mizuno, E., et al., Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. Mol Biol Cell, 2005. **16**(11): p. 5163-74.
170. Mizuno, E., N. Kitamura, and M. Komada, 14-3-3-dependent inhibition of the deubiquitinating activity of UBPY and its cancellation in the M phase. Exp Cell Res, 2007. **313**(16): p. 3624-34.
171. Kakihara, K., et al., Molecular basis of ubiquitin-specific protease 8

autoinhibition by the WW-like domain. Commun Biol, 2021. **4**(1): p. 1272.
172. Centorrino, F., et al., Biophysical and structural insight into the USP8/14-3-3 interaction. FEBS Lett, 2018. **592**(7): p. 1211-1220.
173. Perez-Rivas, L.G., et al., The Gene of the Ubiquitin-Specific Protease 8 Is Frequently Mutated in Adenomas Causing Cushing's Disease. J Clin Endocrinol Metab, 2015. **100**(7): p. E997-1004.
174. Chen, J., et al., Identification of recurrent USP48 and BRAF mutations in Cushing's disease. Nat Commun, 2018. **9**(1): p. 3171.
175. Sbiera, S., et al., Driver mutations in USP8 wild-type Cushing's disease. Neuro Oncol, 2019. **21**(10): p. 1273-1283.
176. Riminucci, M., et al., An R201H activating mutation of the GNAS1 (Gsalpha) gene in a corticotroph pituitary adenoma. Mol Pathol, 2002. **55**(1): p. 58-60.
177. Dahia, P.L., et al., Mutation and expression analysis of the p27/kip1 gene in corticotrophin-secreting tumours. Oncogene, 1998. **16**(1): p. 69-76.
178. Albani, A., et al., Pathogenesis of Cushing Disease: An Update on the Genetics of Corticotropinomas. Endocr Pract, 2018. **24**(10): p. 907-914.
179. Uzilov, A.V., et al., USP8 and TP53 Drivers are Associated with CNV in a Corticotroph Adenoma Cohort Enriched for Aggressive Tumors. J Clin Endocrinol Metab, 2021. **106**(3): p. 826-842.
180. Casar-Borota, O., et al., Corticotroph Aggressive Pituitary Tumors and Carcinomas Frequently Harbor ATRX Mutations. J Clin Endocrinol Metab, 2021. **106**(4): p. 1183-1194.
181. van Heusden, G.P., 14-3-3 proteins: regulators of numerous eukaryotic proteins. IUBMB Life, 2005. **57**(9): p. 623-9.
182. Garcia-Guzman, M., et al., Cell adhesion regulates the interaction between the docking protein p130(Cas) and the 14-3-3 proteins. J Biol Chem, 1999. **274**(9): p. 5762-8.
183. Freed, E., et al., Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. Science, 1994. **265**(5179): p. 1713-6.
184. Yang, X., et al., Structural basis for protein-protein interactions in the 14-3-3 protein family. Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17237-42.
185. Fu, H., R.R. Subramanian, and S.C. Masters, 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol, 2000. **40**: p. 617-47.
186. Pennington, K.L., et al., The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein-protein interactions. Oncogene, 2018. **37**(42): p. 5587-5604.
187. Hermeking, H., The 14-3-3 cancer connection. Nat Rev Cancer, 2003. **3**(12): p. 931-43.
188. Kim, Y., et al., Stratifin regulates stabilization of receptor tyrosine kinases via interaction with ubiquitin-specific protease 8 in lung adenocarcinoma. Oncogene, 2018. **37**(40): p. 5387-5402.
189. Fan, X., et al., 14-3-3 Proteins Are on the Crossroads of Cancer, Aging, and Age-Related Neurodegenerative Disease. Int J Mol Sci, 2019. **20**(14).
190. Neal, C.L., et al., 14-3-3zeta overexpression defines high risk for breast cancer

recurrence and promotes cancer cell survival. Cancer Res, 2009. **69**(8): p. 3425-32.
191. Neal, C.L., et al., Overexpression of 14-3-3zeta in cancer cells activates PI3K via binding the p85 regulatory subunit. Oncogene, 2012. **31**(7): p. 897-906.
192. Takihara, Y., Y. Matsuda, and J. Hara, Role of the beta isoform of 14-3-3 proteins in cellular proliferation and oncogenic transformation. Carcinogenesis, 2000. **21**(11): p. 2073-7.
193. Yang, H.Y., et al., 14-3-3 sigma positively regulates p53 and suppresses tumor growth. Mol Cell Biol, 2003. **23**(20): p. 7096-107.
194. Raungrut, P., et al., Methylation of 14-3-3sigma gene and prognostic significance of 14-3-3sigma expression in non-small cell lung cancer. Oncol Lett, 2017. **14**(5): p. 5257-5264.
195. Ling, C., et al., Loss of the 14-3-3sigma tumor suppressor is a critical event in ErbB2-mediated tumor progression. Cancer Discov, 2012. **2**(1): p. 68-81.
196. Vercoutter-Edouart, A.S., et al., Proteomic analysis reveals that 14-3-3sigma is down-regulated in human breast cancer cells. Cancer Res, 2001. **61**(1): p. 76-80.
197. Wellbrock, C., M. Karasarides, and R. Marais, The RAF proteins take centre stage. Nat Rev Mol Cell Biol, 2004. **5**(11): p. 875-85.
198. Garnett, M.J., et al., Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol Cell, 2005. **20**(6): p. 963-9.
199. Ritt, D.A., et al., Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. Mol Cell Biol, 2010. **30**(3): p. 806-19.
200. Tzivion, G., Z.J. Luo, and J. Avruch, Calyculin A-induced vimentin phosphorylation sequesters 14-3-3 and displaces other 14-3-3 partners in vivo. J Biol Chem, 2000. **275**(38): p. 29772-8.
201. Fanger, G.R., et al., 14-3-3 proteins interact with specific MEK kinases. J Biol Chem, 1998. **273**(6): p. 3476-83.
202. Zhao, J., et al., Discovery and structural characterization of a small molecule 14-3-3 protein-protein interaction inhibitor. Proc Natl Acad Sci U S A, 2011. **108**(39): p. 16212-6.
203. Sawada, M., et al., Synthesis and anti-migrative evaluation of moverastin derivatives. Bioorg Med Chem Lett, 2011. **21**(5): p. 1385-9.
204. Kobayashi, H., et al., Involvement of 14-3-3 proteins in the second epidermal growth factor-induced wave of Rac1 activation in the process of cell migration. J Biol Chem, 2011. **286**(45): p. 39259-68.
205. Vanhaesebroeck, B. and D.R. Alessi, The PI3K-PDK1 connection: more than just a road to PKB. Biochem J, 2000. **346 Pt 3**: p. 561-76.
206. Neukamm, S.S., et al., Phosphorylation of serine 1137/1138 of mouse insulin receptor substrate (IRS) 2 regulates cAMP-dependent binding to 14-3-3 proteins and IRS2 protein degradation. J Biol Chem, 2013. **288**(23): p. 16403-16415.

207. Van Der Heide, L.P., M.F. Hoekman, and M.P. Smidt, The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J, 2004. **380**(Pt 2): p. 297-309.
208. Arrendale, A., et al., Synthesis of a phosphoserine mimetic prodrug with potent 14-3-3 protein inhibitory activity. Chem Biol, 2012. **19**(6): p. 764-71.
209. Cai, S.L., et al., Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol, 2006. **173**(2): p. 279-89.
210. Vander Haar, E., et al., Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol, 2007. **9**(3): p. 316-23.
211. Telles, E., et al., A novel pocket in 14-3-3epsilon is required to mediate specific complex formation with cdc25C and to inhibit cell cycle progression upon activation of checkpoint pathways. Exp Cell Res, 2009. **315**(8): p. 1448-57.
212. Brunet, A., et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, 1999. **96**(6): p. 857-68.
213. Datta, S.R., et al., 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell, 2000. **6**(1): p. 41-51.
214. Sluchanko, N.N., et al., Crystal structure of human 14-3-3zeta complexed with the noncanonical phosphopeptide from proapoptotic BAD. Biochem Biophys Res Commun, 2021. **583**: p. 100-105.
215. Zhang, L., J. Chen, and H. Fu, Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. Proc Natl Acad Sci U S A, 1999. **96**(15): p. 8511-5.
216. Won, J., et al., Cleavage of 14-3-3 protein by caspase-3 facilitates bad interaction with Bcl-x(L) during apoptosis. J Biol Chem, 2003. **278**(21): p. 19347-51.
217. Masters, S.C., et al., Survival-promoting functions of 14-3-3 proteins. Biochem Soc Trans, 2002. **30**(4): p. 360-5.
218. Hermeking, H., et al., 14-3-3sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell, 1997. **1**(1): p. 3-11.
219. Stavridi, E.S., et al., Substitutions that compromise the ionizing radiation-induced association of p53 with 14-3-3 proteins also compromise the ability of p53 to induce cell cycle arrest. Cancer Res, 2001. **61**(19): p. 7030-3.
220. Kleppe, R., et al., The 14-3-3 proteins in regulation of cellular metabolism. Semin Cell Dev Biol, 2011. **22**(7): p. 713-9.
221. Dimopoulou, C., et al., Long-term remission and recurrence rates after first and second transsphenoidal surgery for Cushing's disease: care reality in the Munich Metropolitan Region. Eur J Endocrinol, 2014. **170**(2): p. 283-92.
222. Hardy, J. and M. Somma, Clinical management of pituitary disorders. 1979, Raven Press, New York.
223. Knosp, E., et al., Pituitary adenomas with invasion of the cavernous sinus space: a magnetic resonance imaging classification compared with surgical findings. Neurosurgery, 1993. **33**(4): p. 610-7; discussion 617-8.
224. Detre, S., G. Saclani Jotti, and M. Dowsett, A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in

breast carcinomas. J Clin Pathol, 1995. **48**(9): p. 876-8.
225. Cao, L., et al., Identification of 14-3-3 protein isoforms in human astrocytoma by immunohistochemistry. Neurosci Lett, 2008. **432**(2): p. 94-9.
226. Liu, Y., et al., The expression of seven 14-3-3 isoforms in human meningioma. Brain Res, 2010. **1336**: p. 98-102.
227. Micko, A.S., et al., Invasion of the cavernous sinus space in pituitary adenomas: endoscopic verification and its correlation with an MRI-based classification. J Neurosurg, 2015. **122**(4): p. 803-11.
228. Gong, X., et al., 14-3-3epsilon functions as an oncogene in SGC7901 gastric cancer cells through involvement of cyclin E and p27kip1. Mol Med Rep, 2014. **10**(6): p. 3145-50.
229. Ko, B.S., et al., Overexpression of 14-3-3epsilon predicts tumour metastasis and poor survival in hepatocellular carcinoma. Histopathology, 2011. **58**(5): p. 705-11.
230. Wu, C.Y., et al., Prognostic Significance of 14-3-3epsilon, Aldo-keto Reductase Family 1 B10 and Metallothionein-1 in Hepatocellular Carcinoma. Anticancer Res, 2018. **38**(12): p. 6855-6863.
231. Angeles, A.K., et al., The ERG-Regulated LINC00920 Promotes Prostate Cancer Cell Survival via the 14-3-3epsilon-FOXO Pathway. Mol Cancer Res, 2020. **18**(10): p. 1545-1559.
232. Che, X.H., et al., 14-3-3epsilon contributes to tumour suppression in laryngeal carcinoma by affecting apoptosis and invasion. BMC Cancer, 2010. **10**: p. 306.
233. Mei, J., et al., Characterization of the expression and prognostic value of 14-3-3 isoforms in breast cancer. Aging (Albany NY), 2020. **12**(19): p. 19597-19617.
234. Konishi, H., et al., Identification of frequent G(2) checkpoint impairment and a homozygous deletion of 14-3-3epsilon at 17p13.3 in small cell lung cancers. Cancer Res, 2002. **62**(1): p. 271-6.
235. Li, Y.L., et al., 14-3-3sigma is an independent prognostic biomarker for gastric cancer and is associated with apoptosis and proliferation in gastric cancer. Oncol Lett, 2015. **9**(1): p. 290-294.
236. Li, Z., et al., Role of 14-3-3sigma in poor prognosis and in radiation and drug resistance of human pancreatic cancers. BMC Cancer, 2010. **10**: p. 598.
237. Urano, T., et al., 14-3-3sigma is down-regulated in human prostate cancer. Biochem Biophys Res Commun, 2004. **319**(3): p. 795-800.
238. Osada, H., et al., Frequent and histological type-specific inactivation of 14-3-3sigma in human lung cancers. Oncogene, 2002. **21**(15): p. 2418-24.
239. Ferguson, A.T., et al., High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6049-54.
240. Winter, M., M. Rokavec, and H. Hermeking, 14-3-3sigma Functions as an Intestinal Tumor Suppressor. Cancer Res, 2021. **81**(13): p. 3621-3634.
241. Iwata, N., et al., Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. Oncogene, 2000. **19**(46): p. 5298-302.

242. Boudreau, A., et al., 14-3-3sigma stabilizes a complex of soluble actin and intermediate filament to enable breast tumor invasion. Proc Natl Acad Sci U S A, 2013. **110**(41): p. E3937-44.
243. Wu, Q., et al., The clinicopathological and prognostic impact of 14-3-3 protein isoforms expression in human cholangiocarcinoma by immunohistochemistry. Asian Pac J Cancer Prev, 2012. **13**(4): p. 1253-9.
244. Holm, R., et al., Expression of 14-3-3sigma in cervical squamous cell carcinomas: relationship with clinical outcome. Oncol Rep, 2009. **22**(1): p. 11-5.
245. Wang, Z., et al., The clinicopathological and prognostic impact of 14-3-3 sigma expression on vulvar squamous cell carcinomas. BMC Cancer, 2008. **8**: p. 308.
246. Ito, K., et al., 14-3-3sigma in endometrial cancer--a possible prognostic marker in early-stage cancer. Clin Cancer Res, 2005. **11**(20): p. 7384-91.
247. Laronga, C., et al., Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. J Biol Chem, 2000. **275**(30): p. 23106-12.
248. Chan, T.A., et al., 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature, 1999. **401**(6753): p. 616-20.
249. Phan, L., et al., The cell cycle regulator 14-3-3sigma opposes and reverses cancer metabolic reprogramming. Nat Commun, 2015. **6**: p. 7530.
250. Benzinger, A., et al., Targeted proteomic analysis of 14-3-3 sigma, a p53 effector commonly silenced in cancer. Mol Cell Proteomics, 2005. **4**(6): p. 785-95.
251. Park, E., et al., Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. Nature, 2019. **575**(7783): p. 545-550.
252. Kondo, Y., et al., Cryo-EM structure of a dimeric B-Raf:14-3-3 complex reveals asymmetry in the active sites of B-Raf kinases. Science, 2019. **366**(6461): p. 109-115.
253. Zhang, D., et al., Targeting the ERK pathway for the treatment of Cushing's disease. Oncotarget, 2016. **7**(43): p. 69149-69158.
254. Kovalovsky, D., et al., Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol, 2002. **16**(7): p. 1638-51.
255. Fukuoka, H., et al., EGFR as a therapeutic target for human, canine, and mouse ACTH-secreting pituitary adenomas. J Clin Invest, 2011. **121**(12): p. 4712-21.
256. Dalal, S.N., M.B. Yaffe, and J.A. DeCaprio, 14-3-3 family members act coordinately to regulate mitotic progression. Cell Cycle, 2004. **3**(5): p. 672-7.
257. Leal, M.F., et al., YWHAE silencing induces cell proliferation, invasion and migration through the up-regulation of CDC25B and MYC in gastric cancer cells: new insights about YWHAE role in the tumor development and metastasis process. Oncotarget, 2016. **7**(51): p. 85393-85410.
258. Yang, Y.F., et al., YWHAE promotes proliferation, metastasis, and chemoresistance in breast cancer cells. Kaohsiung J Med Sci, 2019. **35**(7): p. 408-416.
259. Kosaka, Y., et al., 14-3-3epsilon plays a role in cardiac ventricular compaction

by regulating the cardiomyocyte cell cycle. Mol Cell Biol, 2012. **32**(24): p. 5089-102.

260. Hermeking, H. and A. Benzinger, 14-3-3 proteins in cell cycle regulation. Semin Cancer Biol, 2006. **16**(3): p. 183-92.

Acknowledgements

Studying in Munich is a valuable and wonderful experience in my life from 2018 to 2022.

I would like to thank the director of the Department of Medicine IV, Professor Martin Reincke, for giving me the opportunity to do this work in his department.

My heartiest thanks flow to my supervisor, Professor Marily Theodoropoulou, for giving me the opportunity to join her wonderful group and complete my doctoral degree. Thanks to her patience and precious instructions during four years no matter in experiments or in life. She always encourages me when I feel disappointed in doing experiments and cultivates my experimental thinking, and also provides a good platform and freedom for me. What's more, I would like to thank her for giving me the opportunity to attend some academic conferences, so that I can have a more comprehensive study of related research directions and share my study with other scholars.

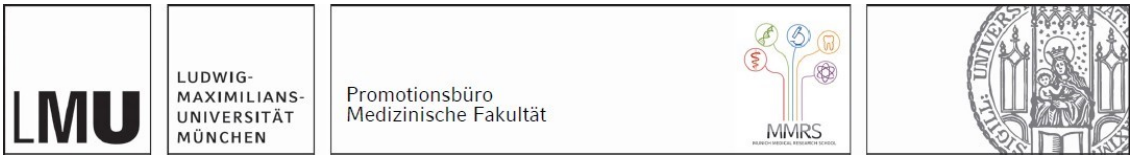
Special thanks to the post doctor Luis G. Perez-Rivas in our group. He really helps me a lot in experimental basics, scientific knowledge and statistical analysis. And also thanks for his useful advice. I would also like to thank Julia Simon and all the people in the lab.

Thanks to my best friends like Mengmeng Song , Qilin Tang, Xudong Zhao ,we really have a very special and happy time in Munich.

Additionally, I would like to thank the China Scholarship Council (CSC) for providing me during the four years of my doctoral study.

At last, I would like to extend my deep gratefulness to my parents who support me all the time whenever in any circumstances or in any time. Even though they are in China, we are talking about life and share my happiness and frustrations through the internet. And they support my study here with endless love and inspiration. And special acknowledgement to my husband Pan Gao, for his care and being here with me. Thanks for his absolute trust, forever support and accompanying when I need to doing experiments at night and feel lonely in abroad during these four years.

Affidavit



Affidavit

Sicheng Tang

Surname, first name

Street

Zip code, town, country

I hereby declare, that the submitted thesis entitled:

Expression and Putative role of 14-3-3 proteins in corticotroph tumours

……

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.
I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 20.01.2022 Sicheng Tang

place,date Signature doctoral candidate