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Molecular mechanisms of glucocorticoid resistance in Cushing's disease

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Résumé

La maladie de Cushing est caractérisée par une sécrétion excessive de l'hormone adrénocorticotrope (ACTH) à partir des tumeur corticotropes de l'hypophyse. Un excès d'ACTH entraîne un hypercortisolisme et provoque des symptômes tels que diabète, hypertension, obésité et les maladies cardiovasculaires entraînant un risque accru de mortalité si la maladie n'est pas traitée. Les tumeurs corticotropes sont caractérisées par la perte du rétro-contrôle négatif exercé par les glucocorticoïdes (GCs) sur la proopiomélanocortine (POMC) qui est le précurseur de l'ACTH : c'est la caractéristique majeure de la maladie de Cushing. Les causes de la résistance aux GC dans les adénomes corticotropes sont encore mal connues. Des études récentes ont montré une surexpression du récepteur du facteur de croissance épidermique (EGFR) dans les adénomes corticotropes provoquant une augmentation de l'activité du gène POMC et de la sécrétion d'ACTH. Les principaux objectifs de ce travail étaient de comprendre la relation entre la signalisation dérégulée de EGF et la résistance aux GCs.

Dans le présent travail, nous avons identifié la voie JAK/STAT3 comme la principale voie de signalisation EGFR qui active la transcription du gène POMC. De plus, nous montrons que l'activation de la signalisation EGFR entraîne une résistance du promoteur POMC aux GCs et que l'activation de STAT3 est responsable de cette résistance. STAT3 affecte le mécanisme de transrepression de GR sans affecter le recrutement de GR au promoteur POMC. L'utilisation d'un inhibiteur de STAT3 restaure la répression de la transcription du promoteur POMC par les GCs. Nous avons aussi trouvé que 50% des adénomes corticotropes humains montrent une surexpression de la forme active de STAT3.

Nous avons aussi étudié les mécanismes sous le contrôle des GCs qui régulent la prolifération cellulaire et qui pourraient être dérégulés dans la maladie de Cushing. CABLES1 est un régulateur négatif du cycle cellulaire et son expression est sous le contrôle des GCs. L'expression de CABLES1 est perdue dans 55 % des adénomes hypophysaires corticotropes, mais la cause de cette perte est encore mal comprise. Dans ce travail, nous avons identifié quatre variants faux-sens dans le gène *CABLES1*, deux chez de jeunes adultes (c.532G > A, c.718C > T) et deux chez des enfants (c.935G > A, et c.1388A > G) atteints de la maladie de Cushing. Les quatre variants touchent une

région de la protéine CABLES1 qui est proche du motif de liaison de la kinase-3 dépendante des cyclines (CDK3). Ces variants ont perdu la capacité d'inhiber la croissance de cellules corticotropes tumorales (AtT20). Les quatre variantes sont donc des mutations de perte de fonction.

En résumé, nos travaux révèlent le rôle important de STAT3 dans la résistance aux GC et ainsi, le blocage de l'action de STAT3 peut être une nouvelle stratégie pour le traitement de la maladie de Cushing. Nous avons aussi supporté un rôle de *CABLES1* en tant que nouveau gène prédisposant aux tumeurs hypophysaires.

Mots-clés: POMC, tumeur corticotrope, résistance aux glucocorticoïdes, voie EGFR, STAT3, CABLES1, mutation.

Abstract

Cushing's disease (CD) is characterized by excess secretion of adrenocorticotropic hormone (ACTH) from corticotroph tumors of the pituitary gland. Excessive ACTH leads to hypercortisolism that causes disabling symptoms such as diabetes, hypertension, obesity and cardiovascular disease resulting in an increased risk of mortality if it is not treated. Corticotroph tumors are characterized by the loss of glucocorticoid (GC) feedback repression of the proopiomelanocortin (POMC) that encodes the precursor of ACTH: this is the hallmark of CD. The causes of GC resistance in corticotroph adenomas of CD patients remain unknown. Recent findings showed overexpression of epidermal growth factor receptor (EGFR) in corticotroph adenomas causing increased POMC activity and ACTH secretion. The main objectives of this work were to understand the relationship between deregulated EGF signaling and GC resistance in the tumorigenesis of CD.

In the present work, we identified the JAK/STAT3 pathway as the main EGFR pathway activating transcription of the POMC gene. We found that sustained activation of EGFR signaling or overactivation of STAT3 causes unresponsiveness of the POMC promoter to GCs and that activated STAT3 is responsible for GC resistance. STAT3 affects the transrepression mechanism of GR without affecting GR recruitment to the POMC promoter. The use of STAT3 inhibitor restores the repressive effect of GC on POMC transcription. Importantly, 50% of human corticotroph adenomas showed overexpression of activated STAT3.

We also studied the mechanisms under the control of GCs that regulate cell proliferation and that could be deregulated in CD. CABLES1 is a negative cell cycle regulator, its expression is under the control of GC. CABLES1 expression is lost in 55 % of corticotroph adenomas and the underlying reasons remain unclear. In this work, we identified the presence of four missense variants in CABLES1 gene, two in young adults (c.532G > A, c.718C > T) and two in children (c.935G > A, and c.1388A > G) with CD. The four variants are close to the predicted cyclin-dependent kinase-3 (CDK3)-binding region of the CABLES1 protein. The variants have lost the ability to inhibit growth of corticotropinoma cells (AtT20). The four variants are thus loss of function mutations.

In summary, our work revealed the important role of STAT3 in GC resistance and further indicates that inhibition of STAT3 action may be a novel strategy for CD treatment. We also provided evidence for a role of *CABLES1* as a novel pituitary tumor-predisposing gene.

Keywords: POMC, corticotroph tumor, glucocorticoid resistance, EGFR pathway, STAT3, CABLES1, mutation.

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Acronyms and Abbreviations

A
ACTH: Adrenocortical-stimulating hormone
11βHSD: 11β-hydroxysteroid dehydrogenases
ADH: Anti-diuretic hormone
AL: anterior lobe
aPV: anterior periventricular nucleus
AR: amphiregulin
AVP: Arginine vasopressin
AVPR1b: Vasopressin receptor 1b
B
BTC: betacellulin

C
Cables1: Cdk5 And Abl Enzyme Substrate
CAMKII: Calmodulin kinase II
CD: Cushing's disease
CLIP: Corticotropin-like intermediate lobe peptide
CRH: Corticotropin releasing hormone
CRHR1: CRH receptor 1
CRHR2: CRH receptor 2

D
DNA-PK: DNA-dependent protein kinase

E
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ELK-1: ETS Like-1 protein Elk-1
EPR: epiregulin
ERKs: extracellular-signal-regulated kinases

F
FoxO: Forkhead Box O
FSH: Follicle-stimulating hormone

G
GC: Glucocorticoids
GH: Growth hormone
GHRH: Growth hormone-releasing hormone
GnRH: Gonadotropin-releasing hormone
GR: Glucocorticoid receptor

GREs: Glucocorticoid response elements
GSK3: Glycogen Synthase Kinase 3

H
HAT: histone acetyltransferases
HB-EGF: heparin-binding EGF
HDAC: Histone deacetylase
HMT: histone methyltransferases
HPA axis: Hypothalamic-Pituitary-Adrenal

I
IGF I: Insulin-like growth factors I
IGF II: Insulin-like growth factors II
IL-6: Interleukin-6
IL: Intermediate lobe
ILK: integrin-linked kinase

J
JAK: Janus kinase
JNKs: Jun amino-terminal kinases

L
LH: Luteinizing hormone
LIF: Leukemia inhibitory factor
LIF: Leukemia inhibitory factor
LMW-DSP2: low molecular weight-dual specificity phosphatase 2
LPH: Lipotropins

M
MAPK: Mitogen protein kinase
MCR2: Melanocortin type 2 receptor
MSH: Melanotrophin
mTORC1: Rapamycin Complex 1
mTORC2: mechanistic target of rapamycin complex 2
Mucin-1: MUC-1

N
nGRE: Negative glucocorticoid-responsive element

O
OT: oxytocin

P
PDK1: phosphoinositide-dependent kinase 1 and 2

PIAS: Protein inhibitors of activated STATs
PKA: Protein kinase A
PL: Posterior lobe
POMC: Pro-opiomelanocortin
PRH: Prolactin-releasing hormone
PTPases: Protein tyrosine phosphatases
PTTG: Pituitary tumor transforming gene
PVN: paraventricular nucleus

R
Rb: Retinoblastoma
RHA: RNA helicase A
RSK1: p90 ribosomal S6 kinase 1

S
SBE: STAT binding element
SCN: suprachiasmatic nucleus
SOCS: Suppressors of cytokine signaling
SON: Supraoptic nucleus

T
TF: Transcription factor
TGF-α: Transforming growth factor alpha
TNF-α: tumor necrosis factor-α
TRH: Thyrotropin releasing hormone
TSC2: Tuberous Sclerosis Complex 2
TSH: Thyroid-stimulating hormone
TSS: Transcription Start site

U
USP8: Ubiquitin-specific protease 8

Acknowledgements/Remerciements

CHAPTER 1 – INTRODUCTION

Cells in the human body communicate with each other to regulate processes that are necessary for survival such as metabolism, respiration, reproduction, stress, digestion, sleep, excretion, movement, sensory perception, growth, development. This communication is mediated through hormones that are secreted by the endocrine system. Hormones are small molecules released by glands directly in blood to target and regulate different organs in the body. These glands constitute the endocrine system that works in synergism with the central nervous system. The endocrine system is composed of 8 endocrine glands: Pineal gland, pituitary gland, pancreas, gonads (ovaries, testes), thyroid gland, parathyroid gland and adrenal glands (Figure 1.1). Each gland produces hormones that achieve a specific role in the body, and one hormone can regulate different organs and one organ may be affected by more than one hormone. A deficit or an excess in one hormone can be lethal in same cases. Those small molecules can be classified in 3 categories based on their chemical composition, they can be amine, such as dopamine, derived from single amino acid, they can be peptide, such as insulin, consist of three to 200 amino acid or they can be steroids, such as glucocorticoids, grouped by the receptor to which they bind. The understanding of hormones role and regulation and globally the understanding of endocrine system function has been revolutionized since the discovery of insulin by Frederick Bantinq and Charles Best in 1922. The discovery of insulin gene structure and receptor completely transformed the treatment of diabetes, million lives have been saved all over the world.

Many conditions may increase risk of endocrine system disorder such as genetics and unbalanced lifestyle, leading to serious diseases appearance and cancer formation.

In order to maintain the body's homeostasis, hormone production and secretion must be controlled tightly. For many hormone systems, the hypothalamus is responsible for secreting a releasing hormone that reach the pituitary gland to stimulate it to secrete hormones which in turn are circulated and transported in the blood to their target glands.

This introduction will briefly summarize our knowledge about normal pituitary gland and pituitary tumors.
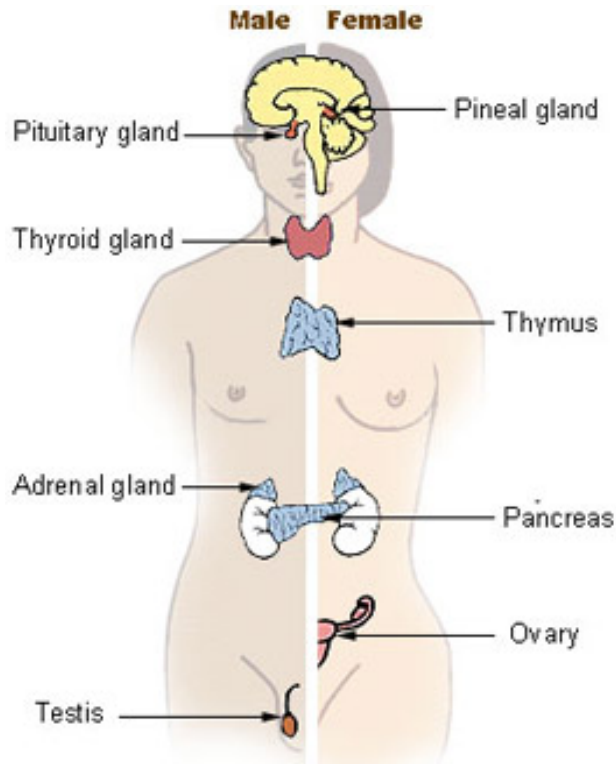
Figure 1.1: Principal endocrine glands

https://training.seer.cancer.gov/anatomy/endocrine/glands

Schematic representation of the location of the eight endocrine glands in the body: the pituitary, pineal gland, thyroid gland thymus, adrenal gland, pancreas, testis and ovary.

1. THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The HPA axis (Hypothalamic-Pituitary-Adrenal) is a major neuroendocrine system. It regulates different biological process in the body such as digestion, sleep, sexuality, mood, immune system, and cardiovascular system. It is composed of three organs: the hypothalamus, pituitary and adrenals. The HPA axis is activated in response to physical or psychological stress. The hypothalamus starts by releasing CRH (the corticotropin releasing hormone). CRH is then transported in the blood to the anterior pituitary where it stimulates ACTH (Adrenocortical-stimulating hormone) secretion. ACTH in turn, activates the adrenals to produce cortisol. Cortisol is the major stress hormone, it controls blood pressure, blood glucose levels and inflammatory responses. But it controls also the HPA axis activity by exerting a negative feedback to inhibit both

hypothalamic CRH and pituitary ACTH release (Figure 1.2) [1]. The HPA axis is severely impaired in diseases such as Isolated ACTH Deficiency, Addison's disease, and Cushing's syndrome.
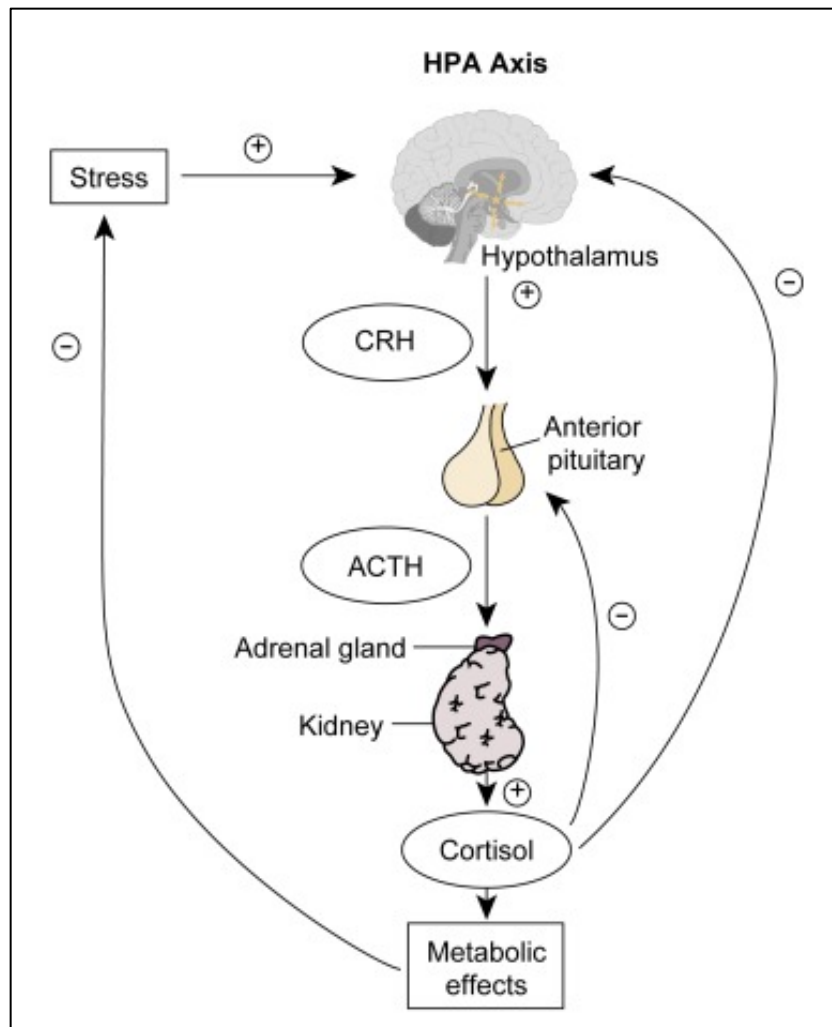
Figure 1.2: Schematic representation of the HPA axis

https://insideouthealthwellness.com/the-hpa-axis-and-the-stress-response

Organization of the HPA axis. Under stress the HPA axis release CRH which is transported to the anterior pituitary to enable access to corticotroph cells. Corticotroph cells then secrete ACTH into the systemic circulation and simulate production and secretion of glucocorticoids from the adrenal cortex. Glucocorticoids then through their receptors have access to targets organs including the hypothalamus and the pituitary and exert negative feedback.

1.1 The hypothalamus

The hypothalamus has a major role in control of homeostasis which is essential for survival.

The hypothalamus is a brain structure located in the ventral position of the midbrain. It contains a number of small functional nuclei of specialised neurones. These nuclei express neurotransmitters and peptide hormones. Anatomically the hypothalamus is divided into seven zones. Each zone is composed of specific nuclei and achieve specific function. For example, in the anterior hypothalamus we find the supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN) and anterior periventricular nucleus (aPV). In the PVN and SCN the magnocellular neurons project to the posterior lobe of the pituitary to induce oxytocin and arginine vasopressin secretion. There are five hypothalamic hormones:

The Corticotropin-releasing hormone (CRH) which stimulates ACTH production and POMC (Pro-opiomelanocortin) gene transcription in corticotrope cells. The Gonadotropin-releasing hormone (GnRH) that induce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. The Growth hormone-releasing hormone (GHRH) that stimulates growth hormone (GH) production from somatotroph cells in the anterior pituitary. The Somatostatin: A peptide inhibitory hormone that inhibit GH and TSH release from the anterior pituitary and the thyrotropin releasing hormone (TRH) that induce the secretion of thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary.

1.2 The pituitary

The pituitary gland is a small and complex gland that has central role in the endocrine system, so it is called "the master gland". It controls the majority of other glands in the body. It is localised in a protective bony enclosure called the sella turcica. The pituitary is linked to the hypothalamus through the pituitary stalk which form a portal system permitting the hypothalamic hormone to reach directly their targets in the pituitary. The adult pituitary is composed of three lobes: The posterior lobe (PL), the intermediate lobe (IL) and the anterior lobe (AL).

1.2.1 The posterior lobe

The posterior lobe is an extension of the hypothalamus. It is composed of axonal projections from the hypothalamus [2, 3]. The posterior lobe stores arginine vasopressin (AVP) and oxytocin (OT) hormones and secretes them upon signal activation.

Oxytocin: Is a peptide hormone and its major role is in female reproduction. It is released in the bloodstream during labor in response to stretching of cervix and uterus. OT is also involved in control of lactation. Also, mice lacking OT receptor showed deficits in social interaction that characterize autism disorders [4] [5].

Arginine vasopressin: Also called antidiuretic hormone (ADH), it is secreted in case of body dehydration promoting reabsorption of water from the tubular fluid in the collecting duct of the kidney, it is suppressed in response to water overload.

1.2.2 The intermediate lobe

The intermediate lobe (IL) derives from the surface ectoderm. It is a homogeneous tissue because it is composed of same population of endocrine cells, the melanotrophs. These cells synthesize and release α-melanotrophin (αMSH) that is produced from the major precursor protein proopiomelanocortin (POMC) [6]. POMC is proteolytically cleaved by proprotein convertases 1 and 2 (PC1 and PC2) into αMSH and CLIP (Corticotropin-like intermediate lobe peptide). αMSH is released after IL stimulation by hypothalamic dopaminergic neurons [7]. It is responsible for hair and skin pigmentation through stimulation of melanin production and release.

The IL is absent in adult humans because it regresses after the 15th week of gestation, but it is present in many species such as mice and rats [8].

All the melanotroph cells express the transcription factor (TF) Tpit (T-box 19 (TBX19). Inactivation of Tpit results in failed differentiation of melanotroph: either they are blocked in the noncycling precursor state, or they differentiate in another cell fate; gonadotrophs [9, 10]. Another transcription factor, PAX7, is also restricted to IL. Pax7 positively controls the melanotrope fate: its inactivation results in loss of melanotrope gene expression and derepression of corticotrope genes. It is thus a switch between melanotrope and corticotrope identity. It exerts this function by acting as a pioneer transcription factor [8].

1.2.3 The anterior lobe

The anterior lobe (AL) has the same developmental origin as the IL, the ectoderm. IL secretes hormones in response to releasing or inhibiting hormones from the hypothalamus. Hormones

secreted by the AL are trophic and they are released directly in the bloodstream to stimulate their target organ (Figure 1.3). The AL is heterogeneous tissue as it contains five different types of endocrine cells: the thyrotrophs, gonadotrophs, somatotrophs, lactotrophs and corticotrophs. Each cell type secretes a specific hormone that is regulated tightly, because inappropriate secretion of these hormones has deleterious consequences.
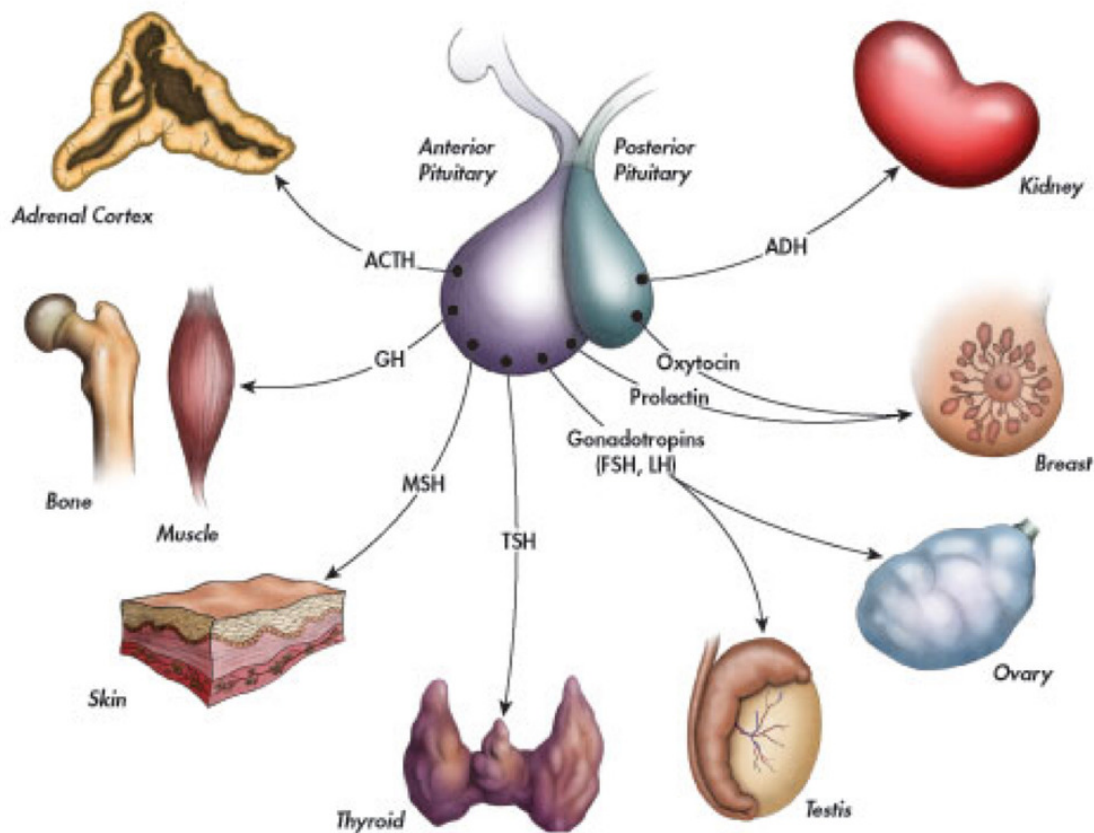
Figure 1.3: Pituitary hormones and their target tissue

https://quizlet.com/580149925/pituitary-hormones-target-tissues-diagram

Anterior pituitary cells and their hormones and target organs: Each pituitary cell secrete one hormone to regulate one organ, but the gonadotroph cells secrete two hormones LH and FSH.

1.2.3.1 Thyrotroph cells

The thyrotroph cells are the least abundant cells in the pituitary, they represent only 2-3% of all AL cells. The thyrotroph cells produce Thyroid Stimulation Hormone (TSH) in response to TRH

released from the paraventricular nucleus of the hypothalamus. TSH is a glycoprotein composed of two subunits, the α GSU (glycoprotein hormone alpha subunit) and βTSH (TSH beta subunit) chain. TSH stimulates the thyroid to produce thyroxine (T4) and triiodothyronine (T3). T4 and T3 exert negative feedback on the pituitary to inhibit TSH secretion in order maintain homeostasis of the pituitary thyroid axis. An increase or decrease in pituitary activity may cause either hyperthyroidism or a hypothyroidism. In the case of hyperthyroidism, only 1% is caused by TSH hypersecretion from the pituitary, the most common cause is autoimmune pathological stimulation of the thyroid gland, for example in Grave's disease. People with hyperthyroidism suffer from increased sensitivity to heat, fatigue, muscle weakness, and increased sweating. In the case of hypothyroidism, the major cause is insufficient TSH secretion by the pituitary, but it is a rare disorder. The most frequent cause is inactivating mutations in the TSH receptor gene [11].

1.2.3.2 Gonadotroph cells

Gonadotroph cells represent about 5 to 10% of AL cells. Gonadotroph cells secrete gonadotropins; Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in response to GnRH. There are three gonadotropic subtypes: One contains only FSH, one contains only LH, and one contains both LH and FSH. FSH and LH are glycoprotein hormones that stimulate the gonads: ovaries, and testis, to produce steroid hormones. In male, LH act on Leydig cells and stimulates the production of testosterone, FSH stimulates spermatogenesis by inducing Sertoli cells proliferation and maintenance of sperm quality. In females, LH and FSH are secreted to elicit the menstrual cycle. Gonadotropin excess cause hypergonadism and it is mainly caused by gonadotroph adenomas. A deficiency of gonadotropin secretion causes hypogonadism and it usually results from functional deficiency of GnRH neurones as observed in Kallmann syndrome. [12]

1.2.3.3 Somatotroph cells

Somatotroph cells are the most abundant cell in the anterior pituitary, they represent about 50% of the total number of AL cells in males. Somatotroph cells secrete growth hormone (GH). GH secretion is positively regulated by the hypothalamic hormone GHRH, and negatively regulated

by somatostatin. GH has an essential role in postnatal growth. It stimulates production of insulin-like growth factors I and II (IGFI and IGFII) from the liver. Increased level of GH and IGF1 lead to inhibition of GHRH secretion and stimulation of somatostatin release in order to inhibit GH secretion. In adults, hypersecretion of GH causes acromegaly and gigantism in children before puberty. GH excess is usually because of somatotroph tumors. GH deficiency cause nanism in children.

1.2.3.4 Lactotroph cells

Lactotroph cells represent 15-20% of all AL endocrine cells but its proportion can increase to 30% when females is are lactating. Lactotroph cells secrete prolactin (PRL).

Its most apparent function is the regulation of lactation, but it has also a role in reproduction, regulation of the immune system and pancreatic development. PRL-inhibiting factor is dopamine and PRL-releasing factors are TRH, OT and galanin. Isolated PRL deficiency has been reported in a few cases. Excess of PRL production is usually caused by prolactinomas and is associated with amenorrhea, galactorrhea and infertility.

1.2.3.5 Corticotroph cells

Corticotroph cells represent 5-10% of all AL endocrine cells. Their major role is to produce the prohormone POMC that is proteolytically cleaved by the proprotein convertase PC1 (Figure 1.4). To yield ACTH and β-lipotropin (βLPH: LPHs stimulate lipolysis in adipose tissue, it can inhibit OT release).

ACTH release from corticotroph cells in under the positive control of CRH and AVP. CRH and AVP bind to their respective receptors CRHR1 and AVPR1b expressed at the membrane of corticotroph cells. ACTH is released in blood and binds to melanocortin type 2 receptors (MCR2) in the adrenal gland where it stimulates synthesis of glucocorticoids (GC). Gc exerts negative feedback on the hypothalamus and pituitary via the glucocorticoid receptor (GR) to inhibit CRH and ACTH production. Gc have important roles in fetal development and in regulating homeostasis.

Isolated adrenocorticotropic hormone deficiency (IAD) is a rare disorder, characterised by very low or absent ACTH production from the corticotroph cells. ACTH deficiency results in deficient glucocorticoid production from the adrenals, leading to hypoglycemia, weakness, and

hypotension. Neonatal IAD is caused by mutations in the TPIT gene. Tpit is a transcription factor essential for terminal differentiation of corticotroph cells. Mice with inactivation of the TPIT gene fail to differentiate pituitary corticotroph and melanotroph [10].

Excess ACTH production cause hypercortisolism as in Cushing's disease that is caused by corticotroph adenomas. These are typically poorly responsive to negative feedback of GC.
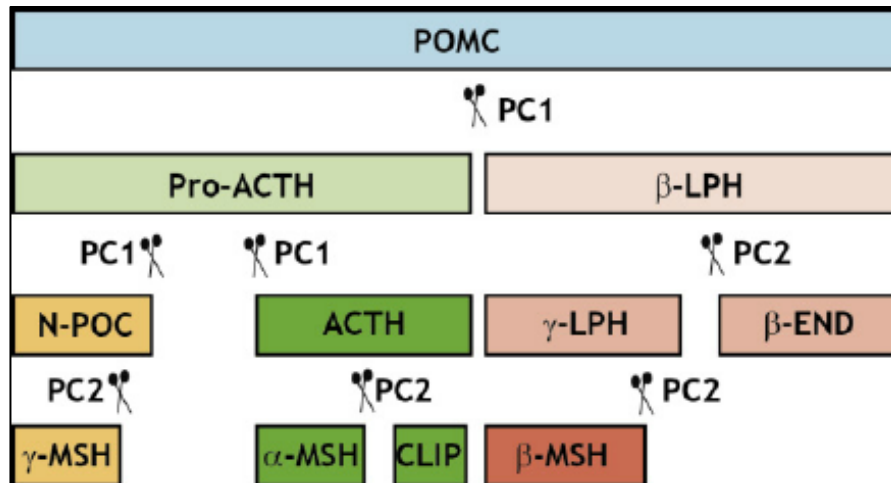
Figure 1.4: Post-translational processing of POMC by the action of proprotein convertase 1 and 2 (PC1 and PC2)

The POMC gene encode a large propeptide that is cleaved to small peptide with the proconvertase 1 and 2 (PC1) and (PC2). The anterior pituitary expresses the PC1, and POMC is processed on adrenocorticotropin (ACTH). The intermediate lobe expresses PC1 and PC2, ACTH is processed into alpha-melanostimuling hormone (α-MSH), adapted from [12].

2. REGULATION OF POMC TRANSCRIPTION

Transcription is a vital process of all cells. The regulation of gene transcription is more complex in eukaryotes than in prokaryotes. The POMC gene transcription is regulated by its promoter that contain different regulatory sequences which can be bind by diverse transcription factors and co-activators. The recruitment of these factors either induce or inhibit the RNA polymerase activity and then gene transcription. The regulation of POMC gene transcription play an important role in the regulation of the HPA axis and its regulation has been studied extensively in the past decades. In this section, I will discuss in more detail the POMC promoter and its regulatory elements.

2.1 POMC promoter

The human POMC gene is 8 kilobases (kb) long composed of: Proximal regulatory sequence promoter with 500 base pairs (bp), three exons and two introns (Figure 1.5A). Exon 1 is not translated and it the conserved less than 50% between spices. Exon 3 encodes the biologically active part of POMC: ACTH, β-LPH, α-MSH, CLIP, and β-endorphin [13].

The POMC promoter contains DNA regulatory elements that mediate either increase or decrease of transcription. In human's POMC promoter these DNA elements exist within the 700 bp 5' to the transcription start site, while in rats these DNA regulatory elements are present within 543 bp of the transcription start site. The rat POMC promoter has been studied extensively. By mutagenesis in AtT20 cells (a model of mouse corticotroph cells in culture), Drouin and Therrien defined three domains of the promoter that have distinct and complementary activities: distal, central and proximal domain (Figure 1.5B) [14]. The proximal and the distal domain alone have low activity, but the central domain in combination with the distal domain have synergetic activity. These domains contain DNA elements that are bound by transcription factors that are regulated in different signaling pathways (Figure 1.5B). Collectively, these DNA regulatory elements and the transcription factors that bind them cooperatively account for corticotroph- and melanocyte specific transcription.

2.1.1 POMC promoter regulatory elements

Three transcription factors are recruited to DNA regulatory elements of the POMC promoter and are required for its basal cell-specific activity.

2.2.1.1 PITX1

Pitx1 is a member of the homeobox family of transcription factors, in particular, paired/bicoid class of homeodomain proteins. Pitx1 is essential for POMC promoter activity since deletion of Pitx1 DNA binding element abrogate POMC activity [15]. Pitx1 binds a composite regulatory element that also contains a DNA binding sequence for Tpit (it will be discussed below) called Tpit/PitxRE. Pitx1 is important for Tpit binding and action on the promoter as both factors are required for transcriptional activity [16]. Ptx1 is expressed in the ectoderm before pituitary

development and differentiation. It is thus also expressed in other pituitary lineages. Similar to its role in POMC promoter activation, Pitx1 interacts with SF-1 in gonadotroph cells to activate the βLH promoter but not on the αGSU and βFSH promoters [17] [18]. Inactivation of Pitx1 gene has no effect on pituitary differentiation, but inactivation of Pitx2 (second member of Pitx subfamily) leads to arrest of pituitary development [19].

2.2.1.2 TPIT (TBX19)

Tpit is a Tbox transcription factor that binds the POMC promoter at the Tpit/PitxRE as described above. Tpit by its own has low affinity for the Tpit/PitxRE when it is a monomer, but its binding is enhanced by the presence of Pitx1 that forms a heterodimer with it, and to activate synergistically POMC Transcription. At the -7Kb POMC gene enhancer Tpit binds to a palindromic regulatory sequence (TpitREpal) [20]. This sequence is important for the activity of the -7Kb enhancer. Tpit is expressed only in the two pituitary POMC lineages, the corticotroph and melanotrophs [16]. Tpit is a positive regulator for terminal differentiation of these two lineages since its inactivation blocks terminal differentiation of corticotroph and melanotroph. In the IL of Tpit knock out mouse pituitaries, cells fail to differentiate into melanotrophs and more interestingly 10 to 15% of cells change their fate to become gonadotroph that cells express SF1, indicating that Tpit is a negative regulator of the gonadotroph lineage [10].

In humans, mutations in the Tpit gene lead to Isolated ACTH hormone Deficiency (IAD) [16]. those mutations are often missense mutations that affect either the DNA binding domain of Tpit and impair its binding to DNA or they affect the interaction between Tpit and other proteins such as the mutation M86R that is deficient in recruitment of the coactivator SRC2 [21]. Patients with IAD have low basal level of ACTH and glucocorticoids causing hypoglycemia, and adrenal insufficiency leading to death if is not treated with glucocorticoid replacement therapy [22].

2.2.1.3 NEUROD1

NeuroD1 for Neurogenic differentiation 1, also called Beta2, is a member of the basic helix-loop-helix (bHLH) family of transcription factors. It forms heterodimer with other bHLH proteins and binds to specific DNA sequences known as E-boxes and activate transcription. NeuroD1 is

expressed only on corticotroph cells and not on melanotroph cells. In NeuroD1-null animals, the expression of POMC is a delayed and the number of POMC cells slightly reduced [23]. For POMC activation, NeuroD1 forms heterodimers with ubiquitous class A bHLH transcription factors such as E47 (Pan1) and activates transcription in cooperation with Pitx1. Pan1 interact with Tpit, the interaction between all these proteins activates POMC transcription [24] [25]. The importance of Ebox and NeuroD1 was shown by mutation of the NeuroD1 binding site in AtT-20 cells [26].
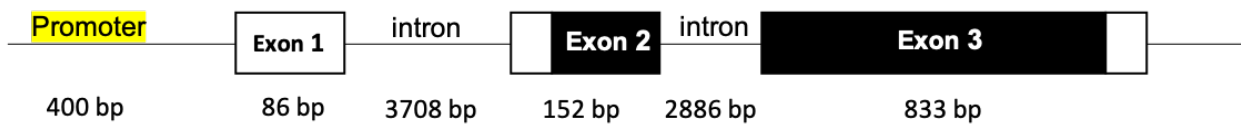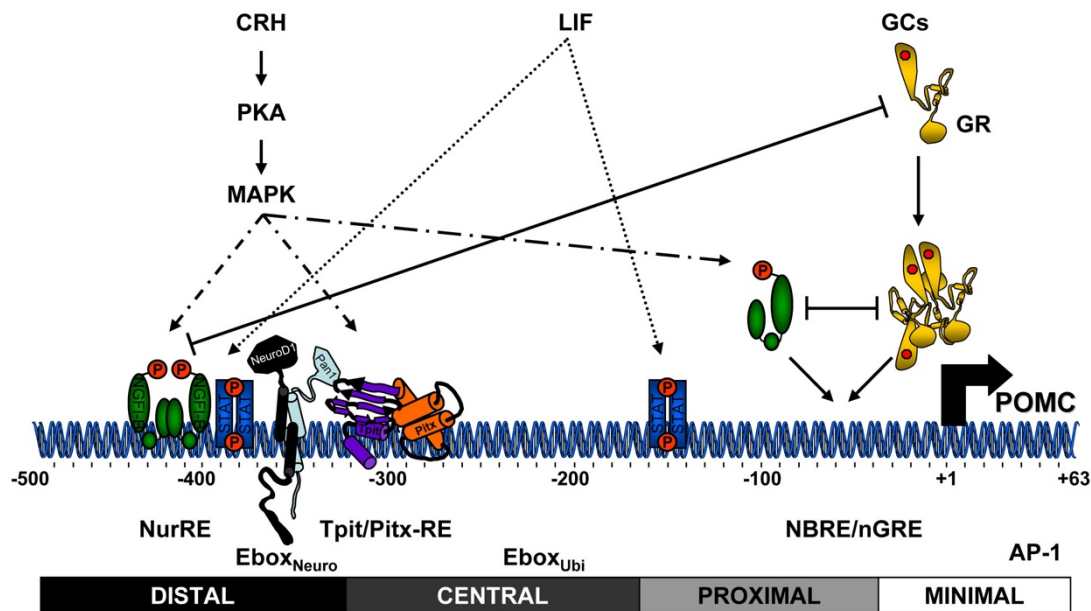
Figure 1.5: Structure of POMC gene and POMC promoter

Structure of POMC gene. (A) Schematic diagram of the POMC gene, composed of three exons (rectangles) separated by two introns (thin lines). Translated regions are shown in black. (B) Regulatory sequence of POMC gene. The POMC promoter is around 500 bp, it is composed of three domains, the proximal, central and distal domain. The CRH activate the POMC promoter via PKA and MAPK pathway leading to NGFI-B and TPIT activation. LIF activates POMC promoter via STAT3 pathway. Glucocorticoids inhibit POMC promoter transcription via direct binding to nGRE or via protein interaction with NGFI-B.

2.2 Hormonal regulation of POMC promoter

POMC transcription is regulated by different signaling pathways that depend on hypothalamic signals. It is activated CRH and repressed by the negative feedback of glucocorticoids. But POMC expression is also affected by other signals such EGF (epidermal growth factor) and LIF (leukemia inhibitory factor) that may have autocrine or paracrine functions.

2.2.1 Activation by CRH

CRH is secreted from PVN and ARC nucleus of the hypothalamus. It consists of 41 amino acid residues. CRH can bind to receptors: CRHR1 and CRHR2. These two receptors are expressed in the pituitary and in other tissues but CRHR1 is the predominant receptor expressed in corticotrophs. CRH binds strongly to CRHR1 than to CRHR2 [27] and activates POMC transcription via protein kinase A (PKA). The two CRH receptors are GPCRs coupled to the Gαs subunit, and they stimulate adenylyl cyclase leading to cyclic AMP accumulation. cAMP activates the mitogen protein kinase (MAPK) pathway and increases cytosolic Ca2+ concentration in corticotrophs which in turn triggers the release of secretory vesicles anchored near the plasma membrane leading to ACTH secretion in the blood [28, 29].

The main mediators of CRH and cAMP stimulation of POMC in corticotrophs are the subfamily of Nur transcription factors. The Nur subfamily consist of three members: Nur77/NGFI-B (NR4A1), Nurr1 (NR4A2), and NOR-1 (NOR1) that are orphan nuclear receptors [30]. Nur factors can bind as monomers to the NBRE target DNA sequence or form homo- or heterodimers that bind to a palindromic response element called NurRE. The stimulatory effect of CRH on POMC promoter gene transcription is mediated through Nur77 and Nurr1 activation and not through the NOR1 activation [31]. CRH stimulation of corticotroph cells leads to nuclear localization of Nur77 and dephosphorylation of its DNA binding domain at Ser316 [32] by PKA resulting on its binding as a homodimer or heterodimer to NurRE in the distal domain of POMC promoter, and to the recruitment of the coactivator p160/Src family [33]. Also, MAPK pathway is implicated in the phosphorylation of Nur77.

Mutagenesis of both half-sites of NurRE results in loss of Nur77 DNA binding and loss of POMC promoter activation [26]. In addition to NurRE, the Tpit/PitxRE regulatory element is also

responsive to CRH and this is mediated by PKA and MAPK pathways. Tpit recruits p160/Src coactivators and exhibits transcriptional synergism with Nur77 [33].

Another cofactor is implicated in the response of the POMC promoter to CRH: Retinoblastoma (Rb). This protein and its related proteins p170 and p130 have an important role in the control of cell cycle. Rb is a tumor suppressor protein: when it is dephosphorylated, it inhibits E2F activity and lead to cell cycle arrest. In addition to its role in cell cycle, it has been shown that Rb is implicated in activation of transcription by nuclear receptors. It enhances SRC coactivator function through direct interaction with Nur77 displaying a transcriptional synergism. Loss of p107 expression by siRNA leads to reduction in CRH responsiveness [34].

CRH may also stimulate POMC transcription via a c-Fos and Jun responsive sequence (AP-1 site) in exon 1 of the POMC gene. Deletion of this AP1 site did not inhibit POMC stimulation by CRH suggesting a collaborative effect on CRH sensitivity [35].

CRH activation of POMC transcription is negatively regulated by the micro RNA miR-375 that is highly expressed in the mouse pituitary gland. miR-375 binds to the 3'-UTR of MAPK-8 and inhibit its mRNA resulting in decrease of Erk1/2 and Nur factor activity and POMC transcription [36].

2.2.2 Activation by LIF

Corticotroph cells are regulated by some inflammatory cytokines in addition to CRH. These cytokines are expressed in the hypothalamus and their expression is increased in response to stress and/or inflammation such as Leukemia inhibitory factor (LIF) and IL6. LIF is a polyfunctional cytokine that induces the terminal differentiation of myeloid leukemia cells. It affects proliferation of primordial germ cells and it activates the HPA axis. LIF is a pleiotropic IL-6 (interleukin 6) family-related cytokine [37].

LIF and LIF receptor (LIFR) are constitutively expressed in the mouse hypothalamus and pituitary, and in human fetal and adult pituitary. Their basal expression is low, but it can be induced by different proinflammatory agents such as lipopolysaccharide, tumor necrosis factor-α (TNF-α), or inhibited by glucocorticoids [38-40].

LIF binds to LIFR and forms heterodimers with the gp130 subunit which is common to all members of IL-6 family. This leads to the recruitment of Janus kinase 1/2 (Jak1/2) that phosphorylates the

gp130 subunit on tyrosine residues, providing docking sites for signal transducers and activators of transcription (STATs) such as STAT3. STAT3 is phosphorylated on tyrosine 705 by JAK, dimerizes with another STAT3 molecules via interaction between the SH2 domains. The STAT3/STAT3 dimer is then translocated to the nucleus and binds to specific DNA-binding motifs.

STAT3 stimulates POMC transcription and ACTH secretion from AtT20 cells after LIF stimulation [41] since overexpression of dominant negative STAT3 mutants abrogates these effects [42].

The maximum activation of POMC promoter activity in AtT20 cells after LIF treatment take place within 2 to 6 hours [43]. Two regions of the POMC promoter are responsible for LIF responsiveness, one is distal (-399/-379) to the transcription start site overlapping in part the NurRE and one is proximal (-166/-96) to the transcription start site. Both sites contribute equally to the stimulation of POMC promoter activity [43]. Mutation of STAT3 DNA-binding sequence (−399/−379) of the POMC promoter abrogate (∼50%) of LIF stimulation of POMC promoter [43].

LIF lead to phosphorylation of STAT1 in addition to STAT3, and both factors can form heterodimers and bind to the POMC promoter to activate it specifically via the binding sequence at (−399/−379) [43].

Also, LIF (via STAT3) induces c-fos and JunB expression and binding to the AP-1 site. Deletion of AP-1 site leads to 20% attenuation of the LIF effect [44]. LIF can synergize with CRH to stimulate POMC transcription and ACTH release [45, 46].

2.2.3 Activation by vasopressin

Vasopressin (AVP) is a hypothalamic peptide secreted from the parvocellular neurons in the paraventricular hypothalamic nucleus [47]. AVP binds to vasopressin receptor 1b (AVP1b) expressed on the corticotroph cell membrane. There are three subtypes of vasopressin receptors: V1 (V1A), V2, and V3 (V1B). They differ by their localization, function and signal transduction mechanisms. VPV1b is mostly expressed on corticotroph cells of the pituitary gland and throughout the brain. AVP stimulates ACTH secretion via protein kinase C. AVP and CRH have a synergetic effect on ACTH secretion [48].

2.2.3 Repression by glucocorticoids

POMC transcription is subject to feedback negative exerted by GCs and their receptor (GR, NR3C1). GCs are steroid hormones that are synthesized in the cortex of the adrenal gland: they are the final output of the HPA axis. GCs modulates various physiological action such as inflammation (important role in reducing inflammation), development, homeostasis, cardiovascular function, and cell proliferation. The natural GCs are cortisol and cortisone. Cortisol is the active GC in humans whereas corticosterone is the main GC in rodents. The enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) is responsible for the conversion of cortisone to cortisol whereas the 11β-HSD2 catalyzes the opposite reaction [49, 50].

2.2.3.1 Glucocorticoids receptor

GR belongs to the nuclear receptor superfamily of transcription factors that includes the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and mineralocorticoid receptor (MR). GR is the principal receptor responsible for the physiological and pharmacological effects of GCs [51-53]. In the absence of GCs, GR is in complex with the chaperone proteins hsp90 and hsp70, immunophilin p59, and phosphoprotein p23 [54]. These chaperone proteins are very important for GR maturation, ligand binding, nuclear transport, and activation. GR is in monomer form in the cytoplasm bound to hsp70. Once the folding process is completed, GR is transferred from hsp70 to hsp90 then, recruitment of p23 leading to maturation of GR into a conformation with high affinity for GC [55-58]. Upon GC binding to GR, GR undergoes a conformational change, dissociates from the cytoplasmic chaperone complex, unmasks its nuclear localization signal (NLS1 and NLS2), becomes active and is translocated into the nucleus. In the nucleus, GR acts through several modes of action: it can activate and repress transcription of genes either by direct DNA binding or indirect recruitment.

2.2.3.2 Activation by glucocorticoids

In the nucleus, GR activated dimerizes and binds to the major groove of DNA via its zinc finger DBD. This dimer binds to a specific palindromic DNA sequences called glucocorticoid response elements (GREs) (Figure 6) represented by the following motif: 5'-TGTACAnnnTCTTGT-3'. Each molecule of GR binds to one half of the palindrome, forming a homodimer on this motif.

Binding of GR to the GRE occurs at regulatory sequences (enhancers and promoters) and leads to conformational change of GR resulting in recruitment of several coactivators and cofactors complexes important for GR action and/or for chromatin structure remodeling. GR interacts with many coregulatory proteins such as p300/CBP, TIF1b, p/CAF, coactivators of the p160 family (SRC1, SRC2 and SRC3) that all possess histone acetyltransferase (HAT) activities, PGC-1a that recruits proteins with HAT activities such as DRIP/TRAP, members of chromatin remodeling complex (Swi/Snf), and cofactors such as TTC5 that protect GR from proteasome degradation [59-61].

In addition, GR can activate transcription via tethering mechanisms in which GR does not bind directly to DNA, but rather binds to another DNA-bound transcription factor. For example, GR tethers to STAT to stimulate b-casein gene expression [62] (Figure 1.6) or GR and AP-1 to interact to stimulate proliferin gene expression [63].

2.2.3.3 Repression by glucocorticoids

GR represses gene transcription by binding to a negative glucocorticoid-responsive element (nGRE). The consensus nGRE sequence is an inverted palindrome separated by 0-2 nucleotides: CTCC(n)0-2GGAGA. Three molecules of GR are recruited to this motif. The mode of action of GR on this DNA sequence and the cofactors recruited are not well defined, but this motif was found on the promoter of the PRL [64], and POMC gene [65].

The POMC promoter nGRE is located in the proximal domain at -63 bp from the TSS. This sequence is required for glucocorticoid inhibition of POMC transcription in vitro since deletion of the GR-binding site abolished glucocorticoid sensitivity [66]

Another mode of repression used by GR is the transrepression mechanism: in this case, GR represses transcription through protein/protein interactions without direct binding to DNA, this mechanism is active at promoters that do not contain GREs (tethering mechanism), or on composite promoters [67] (Figure 6). An example of transrepression is the interaction between GR and AP-1 leading to repression of AP-1 dependent transcription at activity by competition of GR with AP-1 for the p300/CBP coactivators, resulting on limited access of AP-1 to adequate amounts of coactivator [63].

In response to proinflammatory signals, GR can act in synergy with STAT transcription factors such as STAT1, STAT3, STAT5. When GR is tethered to DNA-bound Stat3, this results in transcriptional repression, whereas Stat3 tethering to GR results in synergism [68].

GR Transrepression operates on the POMC promoter. GR inhibits POMC transcription by transrepression of Nur factors on the NurRE at the distal domain of the promoter. The interaction between GR and Nur77 is stabilized by Brg1 (BRM/SWI2-related gene 1), the ATPase subunit of the Swi/Snf complex, and this complex allows recruitment of histone deacetylase 2 (HDAC2) that deacetylate the POMC promoter by removing the acetyl group from histone 4 leading to repression of transcriptional activity of the POMC promoter [69]. GR does not interfere with Nur77 binding to NurRE as showed by chromatin immunoprecipitation (ChIP) technique. In accordance the sensitivity of the POMC promoter to Gc is dependent on Nur77 [70].

A deficiency of nuclear BRG1 and HDAC2 expression was found in 50% of human and canine corticotroph tumors that are resistant to the negative feedback of GC. These observations support the importance of these molecules in the transrepression mechanism of GR [69].
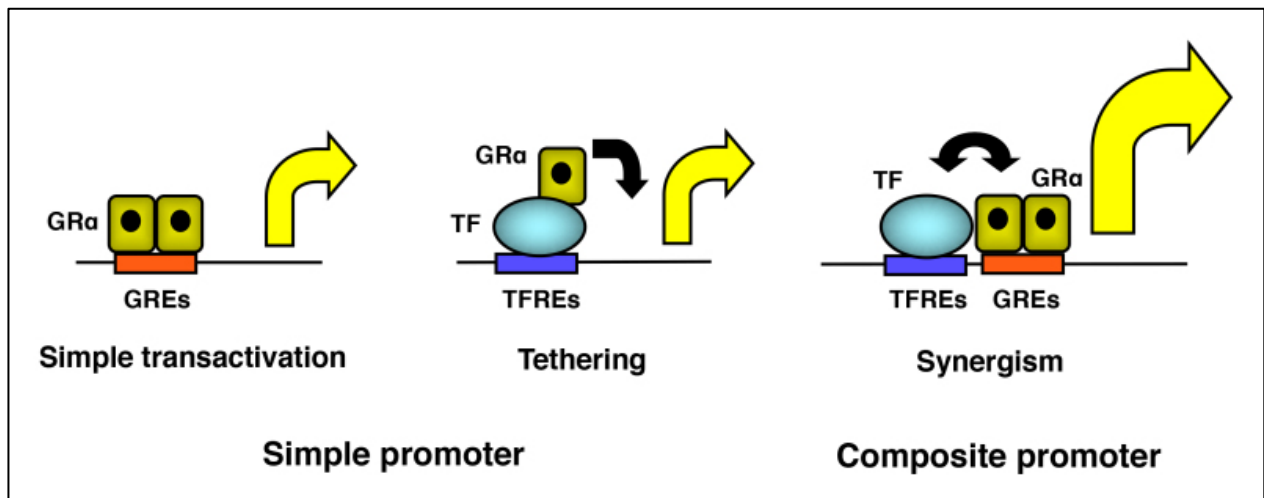
Figure 1.6: Three modes of transcription regulation of GR
from https://www.ncbi.nlm.nih.gov/books/NBK279171
Different mode of transcription regulation by GR. GR can bind either directly to promoter via GRE (GR responsive element) or indirectly via TFRE (transcription factor responsive element). GR also can regulate promoter by synergism with proteins that mediate its binding to GRE.

3. PITUITARY ADENOMAS

Pituitary adenomas are typically benign, slow-growing monoclonal tumors that arise from one cell in the pituitary gland. Most of them are sporadic but some tumors are familial caused by inherited genetic syndrome such as: familial isolated pituitary adenoma (FIPA), multiple endocrine neoplasia types 1 (MEN1) and type 4 (MEN4) and others.

Pituitary adenomas can be classified as functional and nonfunctional adenomas depending on their hormonal activity. Nonfunctional adenomas are not associated with hormonal hypersecretion by contrast to functional adenomas. When tumor cells do not secrete sufficient amounts of hormonal, they are considered as totally silent tumors, or only silent when the amount of hormone produced has no clinical signs associated whith that hormone.

But the World Health Organization (OMS) recommend to classify pituitary adenomas according to their pituitary hormone and transcription factor profile: PIT1 lineage for lactotroph, somatotroph and thyrotroph tumors producing, respectively, PRL, GH, and TSH hormones, TPIT lineage for corticotroph tumors producing ACTH, and SF1 lineage for gonadotroph tumors that produce β-FSH, β-LH hormones.

In some cases, we find plurihormonal adenomas which are tumors producing unusual combination of hormones, they represent 10% to 15% of all adenomas. The most common are tumors that produce GH-TSH-PRL or PRL-TSH.

Pituitary adenomas are formed when there is a loss of balance between cell mass and hormone production resulting in disordered cell proliferation and dysregulated hormone hypersecretion. The initiating cause behind pituitary adenomas development remain unclear, but in transgenic mouse models, activation of proto-oncogenes, and inactivation of tumor suppressor genes can initiate pituitary tumorigenesis [71, 72].

Three genes that that may be related to pituitary adenomas by comparison to normal pituitary tissue were identified which: Pituitary tumor transforming gene (PTTG) is found overexpressed in the majority of pituitary adenomas [73]. A truncated and constitutively phosphorylated form Fibroblast Growth Factor Receptor-4 (FGFR4) was observed [74]. Finally, the Maternally Expressed 3 (MEG3) gene which encode a noncoding RNA that suppresses tumor cell growth is

expressed in all normal anterior pituitary cells but its expression is lost in non-functional adenomas by hypermethylation of the DLK1/MEG3 locus [75].

3.1 Somatotroph adenomas

Somatotroph adenomas produce and secrete excessive GH leading to either acromegaly in adulthood or gigantism during childhood [76]. In 5% of somatotroph adenomas germline mutations was found causing familial syndromes such as:

- FIPA: Familial isolated pituitary adenomas formed due to loss of function mutations in the AIP, Aryl hydrocarbon receptor-Interacting Protein, gene that is suggested to be a tumor suppressor gene. AIP mutations were found also in cases of sporadic acromegaly [77].
- X-LAG: X-linked acrogigantism is caused by microduplication of chromosome Xq26.3 and overexpression of the GPR101, G-protein coupled receptor 101, gene. [78].
- CNC: Carney complex syndrome is caused by mutations in the PRKAR1A gene encoding for the regulatory subunit type I alpha of the protein kinase A [79].
- MEN1 and MEN4: Multiple endocrine neoplasia types 1 and 4. MEN1 syndrome is caused by germline mutations the in MEN1 gene in 85% of cases, and by somatic MEN1 mutations in 15% of patients [80]. MEN1 is a tumor suppressor gene that encodes for the protein menin. Menin has important roles in cell cycle, cell division and transcription regulation [80, 81]. In the MEN4 syndrome, mutations were found in the cell cycle inhibitor gene CDKN1B encoding for p27 [82].
- Paraganglioma/pheochromocytoma syndrome are caused by mutations in SDH gene that encodes succinate dehydrogenase [83-86].

Somatic activating mutation in the gene encoding the α subunit of the stimulatory G-protein (GNAS) was found 40%–60% of sporadic somatotroph adenomas. This mutation results in an increase in GH secretion and cell proliferation [86].

3.2 Lactotroph adenomas

Lactotroph adenomas produce an excess of PRL hormone. They are the most frequent pituitary adenomas as they represent 50% of all pituitary adenomas. Many genetic alterations have been

found in lactotroph adenomas such as abnormalities in cell cycle regulatory genes like CDKN2A, GADD45G, RB1 and p14 [87, 88].

3.3 Thyrotroph adenomas

Thyrotroph adenomas secrete and produce TSH hormone, and they can simultaneously secrete GH and PRL. Thyrotroph adenomas are very rare and they represent less than 1% of all pituitary tumors [89]. No mutations have been associated with thyrotroph adenomas.

3.4 Gonadotroph adenomas

Gonadotroph adenomas are most often non-functioning adenomas that produce β-FSH, β-LH, and α-subunit but that do not lead to any clinical manifestations. Mutations that cause gonadotroph adenoma are not known [89].

3.5 Corticotroph adenomas

Corticotroph adenomas represent 15% of all pituitary adenomas and functioning corticotrope adenomas represent about 4% to 8% of all active anterior pituitary adenomas [90, 91]. Histologically, corticotroph adenomas can be classified in three subtypes: densely granulated corticotroph adenomas (DGCA) which is the most common type of corticotroph adenomas, sparsely granulated corticotroph adenomas (SGCA), and Crooke cell adenomas [91]

Crook's cell adenomas are rare and are associated with aggressive clinical behavior [92]: they are characterized by an accumulation of big amounts of perinuclear cytokeratin filaments in their cytoplasm and they are characterized by the elevated cortisol levels in the serum [92].

The majority of corticotroph adenomas are sporadic representing 95% of cases [93]. Only 5% of patient with MEN1 syndrome have corticotroph tumors, one case of MEN4, one case of CNC, and 20% of FIPA [94-96].

Corticotroph adenomas secrete excessive amounts of ACTH leading to chronic hypercortisolism. Corticotroph adenoma cells are relatively resistant to the negative feedback by glucocorticoids and this represents the hallmark of Cushing's disease (CD) [90].

3.5.1 Cushing's disease

Originally described by Harvey Cushing [97], CD is a rare disorder with an incidence of 1.2 to 2.4 per million per year. It is more frequent in women than men [98-100]. CD is caused mostly by corticotroph microadenomas that are less than 1 cm in diameter, and only 10% of CD cases are caused by macroadenomas [99, 100]. CD is associated with high risks of mortality due to cardiovascular disease if it is not treated [101]. CD patients may develop hypertension, thrombosis diathesis, irritability, obesity, Impaired immunological function, ruddy face, red stretch marks, muscle atrophy, skin fragility, osteoporosis (Figure 1.7).
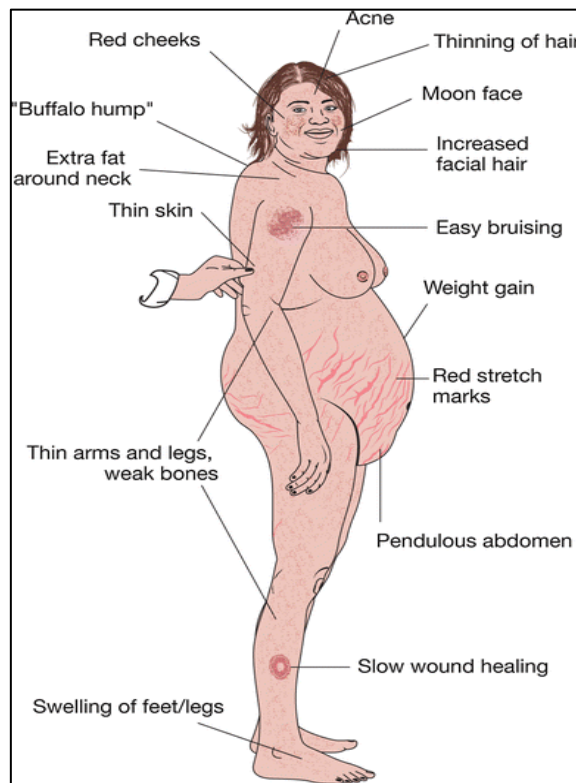
Figure 1.7: Symptoms of Cushing's disease patients

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Representation of different signs and symptoms of Cushing's disease.

The first line treatment of CD is surgical resection of the pituitary adenoma. If the surgery is successful and the adenoma is removed completely, the HPA axis of the patient starts at work normally. Otherwise, the patient may suffer from cortisol deficiency due to loss of normal

corticotroph cells and this can be treated with physiological glucocorticoid replacement. If surgery is unsuccessful with incomplete adenoma removal, this results in persistence of hypercortisolism. In this case, the second line treatment of CD will be medical therapy [102-104]. Different drug therapies have been described to ameliorate the clinical manifestations of the disease. These drugs have three targets: **1-** the adrenals to block cortisol production via inhibition of enzymes responsible for adrenal steroidogenesis (such as Metyrapone, that inhibit 11β-hydroxylase enzyme activity in activating cortisone to cortisol), **2-** the pituitary to block ACTH secretion (dopamine agonists, GABA agonists, somatostatin analogs), or **3-** the glucocorticoid receptor to block its activity (PPAR-γ agonists, Retinoic acid receptor agonists) [90]. These drugs have showed efficiency toward cortisol excess but also, they produce different side effects. Thus, there is a need to develop pharmacological options to control this disease.

3.5.2 Altered protein in Cushing's disease

The mechanisms leading to corticotroph adenoma formation is still not known, but over the years different proteins and genes were found to play a key role in the disease. Studies have identified targets that may be responsible for the hallmark of CD which is resistance to the negative feedback inhibition of Gc, and targets that are suggested to be responsible for the altered proliferation of corticotroph cells.

3.5.2.1 Genetic modifications

It was reported that human corticotroph adenomas may show overexpression of **CRH** and **vasopressin** V3 receptor mRNA and this may be a mechanism to explain ACTH hypersecretion [105]. In contrast, the ACTH receptor (ACTH-R) was absent in 70% of the ACTH-secreting tumors (total of 25 ACTH-secreting tumors) by comparison to normal human pituitary [106]. The mechanism behind the loss of ACTH-R expression is not clear but no mutation has been detected in the ACTH-R gene sequence in those tumors [106].

Cell cycle phases are regulated positively by cyclins that stimulate cyclin-dependent kinases (CDKs), and negatively by CDK inhibitors (CDKI). In corticotroph tumors causing CD, many of these proteins are deregulated. For example, expression of the cell cycle inhibitor **p27** is lost in 33% of human corticotroph adenomas [107]. Mice with p27 knock-out develop hyperplasia in the

intermediate lobe of the pituitary by 12 months, with gain in body weight and growth [107, 108]. p27 blocks the progression of cell cycle from G1 to S phase by inhibiting cyclin E/CDK2 and cyclin D/CDK4/6 complexes [109].

In addition, different cyclins are found to be overexpressed, such as cyclin E. **Cyclin E** form a complex with CDK2 and activates it to regulate the transition from G1 to S phase. The activity of this complex is inhibited by p27. Cyclin E expression is regulated by transcription factors of the E2F family and recruitment of the corepressor complex Rb and HDACs [110]. Cyclin E expression is increased in 70% of corticotroph tumors from patients with Cushing's disease [111]. Cyclin E overexpression leads to increased cell proliferation and hyperplasia of the intermediate lobe [112]. Mice with combined loss of p27 and overexpression of cyclin E showed a synergistic effect resulting in an increase in the frequency, size, and proliferation index of pituitary tumors [112]. Other studies show that loss of function of other CDKI such as **p18** and **Rb** in mice lead to pituitary intermediate lobe tumors [113, 114].

Cables1 (Cdk5 and Abl Enzyme Substrate 1) is a negative regulator the cell cycle progression that is activated in response to GC in corticotroph cells, parallel with Gadd45β and Gadd45γ [115]. Cables1 is a substrate for protein kinases such as CDK3, CDK2, CDK5, 14-3-3 and AKT. It prevents degradation of the cell cycle inhibitors p21 and p27. The tumor suppressor activity of Cables1 is inhibited by AKT phosphorylation and binding of the regulatory protein 14-3-3 [116-121]. Expression of Cables1 was found to be lost in 55% of human corticotrope adenomas, and this is correlated with loss of p27 expression [115]. A recent study in which I participated (Chapter 3) showed that about 5% of human corticotroph adenomas exhibit mutations in the *CABLES1* gene. These mutations are a loss of function mutations, they impair the ability of CABLES1 to suppress proliferation [122] which may contribute to tumor development in these patients.

GADD45γ is a gene that belongs to the family of growth arrest and DNA damage-inducible gene its expression is often lost in human pituitary adenomas [123]. Overexpression of GADD45γ induces p21 expression in HeLa and U2OS cells , and suppresses cell growth in AtT20 cells [123].

Recently, an important progress was made after the discovery of mutations in the *USP8* gene in 25-60% of corticotroph adenomas from patients with CD [124] [125]. The mutations are specific to corticotroph adenomas, and the adenomas are smaller and secrete more ACTH than *USP8* wilt-

type adenomas [124]. USP8 is a ubiquitin-specific protease 8, that mediates deubiquitination of proteins and prevent their degradation. The mutations found in the *USP8* gene are clustered in the 14-3-3 binding motif and this results in exposure of USP8 to proteolytic cleavage leading to increased deubiquitinase activity [124]. One USP8 targets is the epidermal growth factor receptor (**EGFR**). Corticotroph adenomas with USP8 mutations showed increased expression of EGFR, which makes the EGFR pathways constitutively active, leading to stimulation of POMC transcription and ACTH secretion [124]. EGFR was found overexpressed in 60% of corticotroph adenomas [126]. The mechanisms of EGFR inducing POMC transcription and ACTH secretion is still not known it is one aim of Chapter 2 of this thesis.

Recent studies showed that transgenic mice overexpressing human EGFR under the control of the rat POMC promoter form tumors in the anterior and intermediate lobes of the pituitary by 8 months. These tumors are aggressive with features of Crooke's cells and they secrete ACTH. Mice have features of CD including increased body weight, increased ACTH and corticosterone levels, glucose intolerance, and adrenal hyperplasia [127]. EGFR may induce POMC transcription and ACTH secretion via **E2F1**, since it is found to be phosphorylated in CD tumors and its phosphorylation is inhibited by EGFR inhibitors. E2F1 may be an important therapeutic target to treat Cushing's disease [127].

The effects of USP8 may not be limited to EGFR. Studies showed that *USP8* mutations affects expression of different proteins such as upregulation of CDC25A*,* MAPK4 and downregulation of CCND2, CDK6, CDKN1B [128]*.* Another target of *USP8* mutation is the somatostatin receptor SSTR2. Corticotroph adenomas with *USP8* mutations show overexpression of SSTR2 [129, 130].

USP48 is an another deubiquitinase gene that is found mutated in about 23% of corticotroph adenomas, and 16% of the same cohort tested showed mutation in the proto-oncogene **BRAF**. USP48 and BRAF enhance POMC promoter transcription and ACTH secretion from AtT20 cells when expressed ectopically [131].

3.5.3 Glucocorticoid resistance in corticotroph adenomas

The mechanisms of GC resistance in corticotroph adenomas are still not understood, but genetic modifications found in corticotroph adenomas may contribute.

Few studies showed abnormalities in **GR** gene expression that may explain the GC resistance of corticotroph tumors, such as Loss of Heterozygosity (LOH) at the GR gene locus found in corticotroph adenomas of patients with Cushing's disease [132]. A frameshift mutation in exon 2 of GR gene leading to non-functioning protein was found in one corticotroph adenoma [133]. Most corticotroph adenomas showed normal or increased expression of GR which mean GR downregulation cannot explain GC resistance [134], leading us to conclude that mutations in other molecules in relation with GR function or GR repression mechanism may lead to GC resistance.

The 11β-hydroxysteroid dehydrogenases (**11βHSD**) enzyme has two isoforms: 11βHSD 1 and 11βHSD 2, with 11βHSD1 transforming cortisone to cortisol, and 11βHSD2 transforming cortisol to cortisone. Expression of these two isoforms may be misregulated in corticotroph adenomas by comparison to normal pituitary. Indeed, some studies showed that 11β-HSD2 is upregulated in ACTH secreting tumors however it is absent from normal pituitary, this mechanism may contribute to GC resistance: converting cortisol to the inactive cortisone thus, diminishing functional feedback [135, 136].

A recent study showed that the nuclear receptor **TR4** may be responsible for resistance of corticotroph tumor cells to GC. TR4 is the testicular receptor 4 that belongs to the family of orphan nuclear receptors. It regulates various processes such as spermatogenesis, lipoprotein regulation, and central nervous system development [137]. TR4 is expressed in the cytosol of normal corticotroph cells, but it is found overexpressed of human corticotroph adenomas with its redistribution from the cytosol to the nucleus [137]. Overexpression of TR4 in AtT20 cells leads to stimulation of POMC promoter activity, ACTH secretion, proliferation and invasion (TR4 knockdown causes 50% decrease of corticotroph tumor proliferation and 75% reduction in murine corticotroph tumor cell invasion) [137]. TR4 regulate POMC transcription via direct binding to the promoter sequence from −854 to −637bp [137]. The activity of TR4 on the POMC promoter is enhanced after phosphorylation of TR4 at serine 19 by the MAPK pathway [137]. Overexpression of TR4 in AtT20 cells blocks POMC promoter responsiveness to dexamethasone (synthetic analog of glucocorticoids). TR4 binds to the GR N-terminal domain, inhibits GR binding

to POMC and impairs its interaction with cofactors [138]. TR4 may be an important target to treat glucocorticoid resistance and Cushing's disease [138].

Another mechanism may explain glucocorticoid unresponsiveness: It is the overexpression of the **HSP90** protein in human corticotroph adenomas by comparison to normal pituitary [139]. HSP90 is a chaperone protein that is important for the proper folding of the ligand-binding domain of GR. Continued binding of HSP90 to GR impairs GR activity and binding to DNA [139].

The unresponsiveness of POMC to negative feedback of GC may be explained by the loss of nuclear expression of two proteins essential for the GC repression mechanism: HDAC2 and BRG1 [69].

BRG1 is always present to the POMC promoter and its knockdown leads to repression of POMC activity. First, BRG1 is important for the recruitment of GR to the POMC promoter in the presence of GC in order to make a stable complex between GR and NGFI-B and secondly, it is important for the recruitment of HDAC2 to this complex resulting in the decrease of histone acetylation. About 50% of human corticotroph adenomas are either deficient in BRG1 or HDAC2 suggesting that other proteins may be essential for GC repression [69]. The mechanisms behind the loss of BRG1 and HDAC2 expression is still not clear.

3.6 EGFR pathway

Epidermal growth factor (EGF) is a small protein constituted of 53 amino acid residues and it is a potent mitogenic growth factor. EGF is involved in different processes such as differentiation, proliferation, cell growth, survival, metabolism, and tumorigenesis [140].

EGF mediates its effect through the EGF receptor (EGFR) family. This family is composed of four homologous receptors: (ErbB1/EGFr/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors can homo and heterodimerize after EGF binding leading to the activation of their kinase activity and stimulation of intracellular signals. No ligand to date has been identified for ErbB2, it is known to be a co-receptor for the ErbB1, ErbB3 and ErbB4 [141].

EGFR is the best studied among all the members of the EGF receptor family. EGF is not the only ligand that binds to EGFR: five other ligands can bind such as transforming growth factor alpha (TGF-α), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR) and heparin-binding EGF (HB-EGF)

[142]. Constitutive activation of the EGFR pathway leads to tumorigenesis [143]. EGFR is a transmembrane protein that is activated upon ligand binding, leading to activation of the intrinsic tyrosine kinase and autophosphorylation of specific tyrosine residues in its C-terminal domain (Y992, Y1045, Y1068, Y1148 and Y1173): these are docking sites for proteins containing Src homology 2 (SH2). This leads to the activation of several signal transduction cascades principally: MAPK, Akt and STAT3 pathways.

EGFR has a nuclear localization signal allowing it to enter the nucleus [144]. In the nucleus, EGFR act as a transcriptional co-activator. It activates proto-oncogenes and mediate cell cycle progression. EGFR interacts with Mucin-1 (MUC-1) and RNA helicase A (RHA) on the promoter of cyclin D1 and induces its transcription [145-147], suggesting a new role of EGFR in tumor formation.
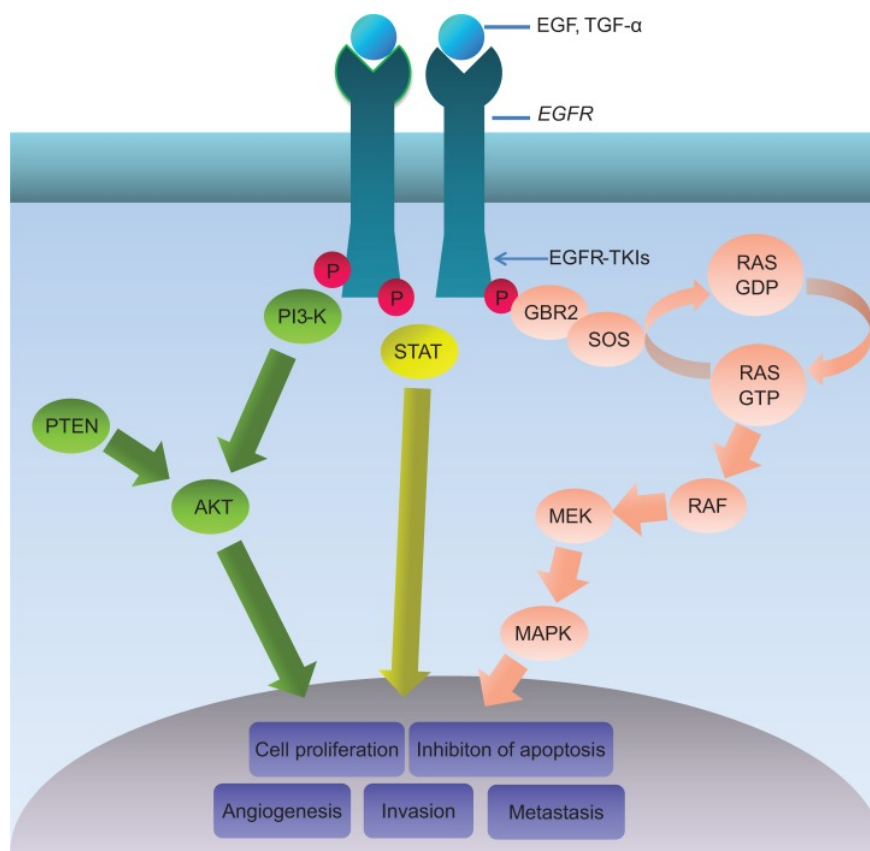
Figure 1.8: EGFR signaling pathways

The binding of EGF to EGFR in the extracellular domain leads to dimer formation and phosphorylation of tyrosine residues in the intracellular domain causing activation of downstream signaling cascade: PI3K/Akt/mTOR pathway, JAK/STAT pathway and Ras/Raf/MEK/MAPK/ERK pathway. EGFR pathways affect cell cycle, cell growth, cell survival and many other biological functions [148].

3.6.1 MAPK pathway

The MAPK pathways regulate diverse processes such as cell proliferation, cell differentiation, and cell death. There are a few parallel MAPK pathways, and each MAPK pathway contains three kinase cascades: MAPKKK, MAPKK, and MAPK.

The MAPKKK (RAF) is activated by interaction with a GTPase protein (RAS) recruited to phosphorylated EGFR by the intermediate of Growth factor receptor-bound protein 2 (GRB2). Raf phosphorylates and activates the next kinase (MAPKK) called MEK. In turn, MEK phosphorylates the MAP kinases ERK, the extracellular signal regulated kinase.

MAP kinases are grouped into three families: ERKs (extracellular-signal-regulated kinases), JNKs (Jun amino-terminal kinases), and p38/SAPKs (stress-activated protein kinases). ERK cascade are responsible for mitogenic signals whereas JNKs and p38 transmit stress signals.

RAF phosphorylates and activates MEK1 and MEK2, the only two substrates of MEK1 and MEK2 are **ERK1** and **ERK2** called also p44 and p42 respectively that are activated by phosphorylation at threonine and tyrosine residue [149]. Activated ERK1 and ERK2 have multiple cytoplasmic and nuclear substrates that regulate diverse cellular functions such as proliferation, differentiation, and cell cycle progression [150]. RSK1 (p90 ribosomal S6 kinase 1) is one of these substrates. RSK1 is phosphorylated by ERK at T573 in the cytoplasm, then it is translocated to the nucleus and activate c-FOS and SRF. RSKs catalyze the phosphorylation of the pro-apoptotic protein BAD inhibiting its apoptotic effect and promoting cell survival [151]. Activation of RSK is not restricted to ERK, it can also be activated by the p38 cascade under stress conditions. In stimulated cells ERK translocates to the nucleus and activates many transcription factors such as the proto-oncogenes c-Fos, c-Myc, c-Jun, ETS, and ELK-1 (ETS Like-1 protein Elk-1) [152-154].

Studies showed that ERK controls G1 progression through positive regulation of transcriptional expression of cyclin D1 via AP1 since the cyclin D1 promoter contains a functional AP-1 binding site [155] [156]. Activation of ERK phosphorylates the proto-oncogene c-Myc and enhance its stability which is necessary to drive cells from G0 to late G1 phase [157]. ERK may also stimulate cell proliferation by downregulating expression of antiproliferative genes such as p21, p27, Tob1, and Ddit3 [158] [159]. In contrast, strong activation of the ERK pathway induces expression of p21 leading to inhibition of Cdk4 and Cdk2 and cell cycle arrest in G1 [160]. ERK1-deficient embryonic

fibroblasts are viable and fertile, and they don't show any defects in embryonic development or growth whereas the ERK2 expression level was not changed suggesting a compensatory effect [161].

JNK for c-Jun N-terminal kinases, is the kinase, phosphorylates c-Jun on its transcriptional activation domain. JNK family consists of three MAP kinases: Jnk1 (MAPK8), Jnk2 (MAPK9), and Jnk3 (MAPK10) [158]. The JNK pathway controls different cellular processes, including proliferation, embryonic development and apoptosis. The main substrate of JNK is c-Jun that interacts with JunB, JunD or c-Fos to activate transcription: they constitute the AP-1 transcription factors [162]. The JNK pathway induces apoptosis by activating apoptosis-related proteins such as BIM and inhibiting antiapoptotic proteins by phosphorylation such as Bcl-2, and Bcl-xL [163, 164].

The **p38** pathway is activated by cellular stress such as UV radiation heat shock or proinflammatory cytokines (IL-1 and TNF α). It regulates different cellular process such as cell proliferation, cell migration, survival, and apoptosis. There are four isoforms of p38: p38 α, p38 β, p38 γ and p38 δ. p38 controls cell cycle progression and induces proliferation arrest through downregulation of cyclin D1 gene transcription, upregulation of p21, GADD45, p53, 14-3-3σ proteins and phosphorylation of Rb [165-168]. In contrast to its tumor-suppressing role, some studies show that p38 can promote tumor formation. P38 was found to be hyperactive in many tumors [169] and in breast tumor cell models, deletion of p38 leads to decreased tumor volume [170].

3.6.2 AKT pathway

The AKT regulates different cellular process including cell survival, growth, proliferation, cell migration and angiogenesis. Stimulation by EGF or other growth factors leads to phosphorylation of phosphatidylinositol 3-kinase (PI3K). PI3K then recruits and activates the serine/threonine kinase AKT protein (protein kinase B). PI3K activity opposes the tumor suppressor PTEN [171]. AKT is partially activated after Thr308 phosphorylation, its full activation requires phosphorylation of S473. The phosphorylation of AKT at Thr308 is catalyzed by phosphoinositide-dependent kinase 1 and 2 (PDK1), while the phosphorylation of AKT at S473 can be catalyzed by

different proteins such as PDK2, integrin-linked kinase (ILK), mechanistic target of rapamycin complex 2 (mTORC2) and DNA-dependent protein kinase (DNA-PK) [172, 173]. AKT and its isoforms are subject to other post-translational modifications such as glycosylation, acetylation, ubiquitylation and SUMOylation [174-177]. AKT has many downstream targets that control cellular functions such as Glycogen Synthase Kinase 3 (GSK3), the Forkhead Box O (FoxO) of family transcription factors, Tuberous Sclerosis Complex 2 (TSC2) and the Mechanistic Target of Rapamycin Complex 1 (mTORC1).

GSK3 is multi-functional Serine Threonine protein kinase that is usually activated in the cell. Its phosphorylation by AKT at S9 and S21 cause its inactivation. GSK3 inhibits BCL-2 family member MCL-1 and Myc. Inactivation of GSK3 by AKT promotes the stabilization of these proteins inducing cell survival and proliferation [178-180].

For FoxO protein, activation of AKT signaling leads to the phosphorylation of FoxO protein on three residues: one of thes is in the nuclear localization sequence and it creates a binding site for 14-3-3 protein resulting in sequestration of FoxO in the cytosol and attenuation of its transcriptional effect. FoxO [181]. FoxO targets are involved in the induction of apoptosis (BIM), cell cycle arrest (p27, p21, GADD45a) and growth inhibition (Sestrin3, BNIP3). Thus, activation of AKT suppresses the FoxO pathway [182].

The AKT pathway also regulates cell growth through activation of the protein kinase complex mTORC1. mTORC1 is activated by phosphorylation. Studies showed that both mTORC1 and mTORC2 can be phosphorylated by S6K and this phosphorylation correlates with mTORC1 signaling [183]. mTORC1 signaling is regulated by PRAS40 protein which is activated by the AKT pathway [184]. mTORC1 activate anabolic processes, such as protein, lipid, and nucleotide synthesis.

3.6.3 STAT3 pathway

STAT3 for signal transducer and activator of transcription 3 is a member of a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6). The genes encoding these transcription factors are grouped on three chromosomes, STAT1 and STAT4 are on chromosome 1, STAT2–STAT6 are on chromosome 2, and STAT3 and STAT5a/STAT5b are linked to chromosome 11 [185]. STATs

transmit signals from cytokine and growth factor receptors at the plasma membrane to the nucleus and they regulate transcription of many genes. STAT pathways regulate critical functions, such as cell differentiation, apoptosis, metastasis, and proliferation. Deregulation in the expression or activity of these STATs proteins are associated with development of many human cancers.

3.6.3.1 The STAT family of transcription factors

STAT proteins consist of 750-850 amino acids and have conserved domains important for their function [186, 187] (Figure 1.9).

The N-terminal domain of STATs is involved in tetramerization and stabilization of STAT–STAT dimer binding to DNA when the activated STATs concentration is small. It helps STATs to bind to a weak STAT binding site by forming a tetramers from a pair of phosphorylated dimers [188].

The coiled-coil domain is between the N-terminal and the DNA binding domains and it contain four long α helices. It is important for interaction with other proteins including IRF-9/p48, CBP/P300 for STAT1, c-Jun, StIP1, and GRIM-19 for STAT3, and SMRT with STAT5A and STAT5B [189-193].

STAT proteins have a DNA binding domain in the center of the molecule that regulates DNA binding specificity, and most of STATs bind to (TTN5AA) DNA sequences. The binding of one STAT and not another to a specific DNA sequence depends on the number of base pair between the palindrome TT-AA, for example when there are 4 base pairs there is selective binding of STAT3 dimers, whereas there is 6 base pairs for selective binding of STAT6, and 5 base pairs are permissive for several STATs [194].

STATs are the only family of transcription factors that contain Src-Homology 2 domains (SH2), that serve mainly for the recruitment of STATs to activated receptor complexes, and this domain is required for STAT homo- and hetero-dimerization. STAT proteins are activated once cytokines or growth factors bind to their receptors. Then, receptors dimerize or oligomerize leading to JAKs phosphorylation on tyrosine residues. The tyrosine phosphorylated JAKs provide a binding site for the SH2 domain of STAT proteins leading to STAT recruitment and their phosphorylation on a single tyrosine residue on their C-terminal domain [195] [196]. After tyrosine phosphorylation,

dimerization takes place between the SH2 domains and the carboxy-terminally localized phosphotyrosine-containing domain. In addition to homodimerization, studies showed that STAT1–STAT2 and STAT1–STAT3 can form heterodimers in response to various cytokines or growth factors, but not STAT4, STAT5a/b and STAT6. STAT5a can heterodimerize with STAT5b.

The critical phosphotyrosine residue for activation is located around amino acid 700 (for STAT1 Tyr 701 and for STAT3 Tyr 705). The SH2 domain is in the carboxyl terminus region and precedes the transactivation domain (TAD). The TAD is important for transcriptional activity of STATs. The TAD contains a PMSP amino acid sequence which is conserved between STAT1, STAT3 and STAT4. Ser 727 phosphorylation is important to achieve maximum transcription activity. Its phosphorylation can be caused by a variety of stimuli and signaling pathways [197, 198]. Ser 727 can be phosphorylated by MAPK suggesting a cross talk between STAT pathway and MAPK pathway.

Mutation of the serine 727 residue to alanine decreases the transcriptional activity of STAT1 and STAT4, prolongs tyrosine phosphorylation and DNA binding of STAT5a, or causes both a decrease in transcriptional activity and sustained tyrosine phosphorylation of STAT3 [199-203].
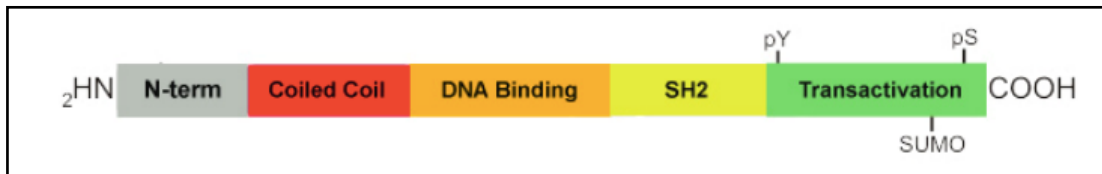
Figure 1.9: Structure of the STAT protein family: Functional domains of STAT proteins [187]
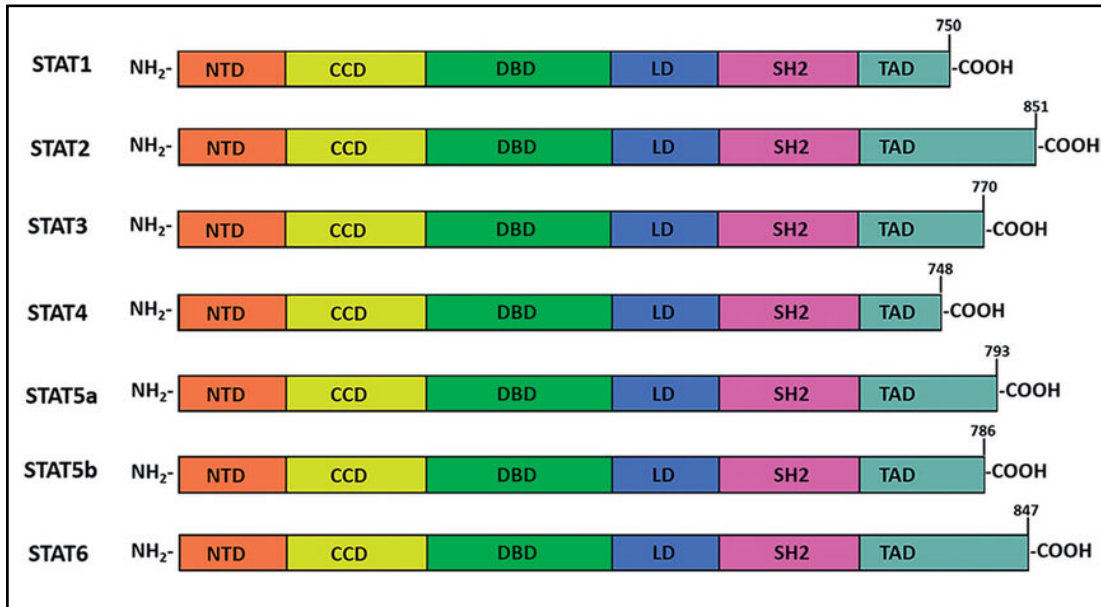
Figure 1.10: Structure of the STAT protein family

STAT proteins are composed of six domains: the N-terminal domain (NTD), coiled-coil domain (CCD), DNA binding domain (DBD), linker domain (LD), Src-homology 2 (SH2) domain and transcription activation domain (TAD). The NTD helps STAT protein dimerization and nuclear import. The DBD is important for DNA binding to specific palindromic sequences in promoters of target genes. The role of helical coiled-coiled domain is to interact with other proteins. The SH2 domain is important for the recognition of tyrosine phosphorylation on receptor subunits. the C-terminal TAD contain tyrosine and serine phosphorylation sites essential for maximal transcriptional activation of STAT [188].

STAT1 has two isoforms, STAT1α (91 kDa) and STAT1β (84 kDa). STAT1β is a truncated version of STAT1α as it lacks the transactivation domain and the serine 727 phosphorylation site in the C-terminus. STAT1β is considered as an inhibitor of STAT1α because it inhibits STAT1α activation by inhibiting tyrosine 701 phosphorylation and DNA-binding activity. Gene knockout studies show that mice lacking Stat1 are viable, but they are sensitive to viral infections and pathogenic agents due to dysregulation in interferon (IFNs) signaling. IFNs are growth suppressive for different cell lines. In the absence of STAT1, IFNs induce expression of the proto-oncogene c-Myc [204]. STAT1 is not expressed in various histological types of tumors such as breast cancer, colorectal cancer, and lung cancer [205-207]. Constitutively active STAT1 increased the transcription and expression of different pro-apoptotic and cell-cycle arrest genes, such as BCL-2, BCL-XL, p27, and p21 [208]. The loss of STAT1 expression or activity in cancers was explained either by protein degradation, promoter DNA methylation, disorder in the activity or expression of STAT1 negative regulator

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(such as SOCS, PIAS, PTPs) or constitutive activation of STAT3 [209]. Studies showed that STAT3 can block the DNA binding activity of STAT1 in human myeloid cells [210].

STAT2 can be phosphorylated on tyrosine residue and it is activated after IFNα/β stimulation. As STAT1, mice lacking Stat2 are viable and develop normally but they show high sensibility to viral and bacterial infections because of the impaired response to IFN α/β [211, 212].

STAT4 and **STAT6** are activated in response to IL-12 and IL-4 respectively. Mice lacking expression of STAT4 show the same phenotype as mice lacking IL-12. Mice lacking expression of STAT6 show defective responses IL-4, and Th2 cells were unable to differentiate in vitro [213-216].

In **STAT5a**, **STAT5b** or STAT5a/b knockout mice, analyses reveal defective mammary gland development, and mice lacking STAT5a/b have the same phenotype as mice lacking the prolactin receptor [217, 218]. STAT5b knockout mice show impaired growth hormone [217, 219].

3.6.3.2 STAT3 protein and its biological functions

There are two isoforms of STAT3: the STAT3α is full length with 770aa, and the STAT3β is a truncated form of STAT3α lacking the 55 residue of C-terminal transactivation domain: it is generated by differential splicing. STAT3β has strongly reduced transcriptional activities as compared to STAT3α indicating the importance of the transactivation domain and the phosphorylation of serine residue in the c-terminal region to achieve the maximal transcriptional activity [220] [221] (Figure 1.11).
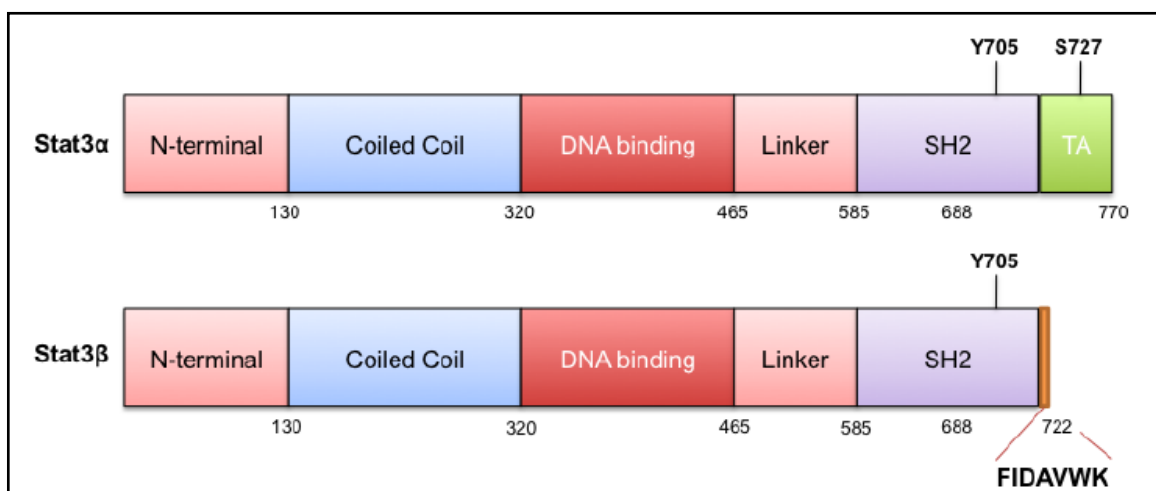
Figure 1.11: Schematic overview of domains of Stat3α and Stat3β
Adapted from [219]

After cytokine or growth factor stimulation, the serine residue is phosphorylated, and this phosphorylation is important for maximal response to IL6. STAT3β or a mutated STAT3α (Ser727Ala) don't respond to IL6. Studies show that the negative charge introduced by the phosphorylation at Ser 727 seems to be important for the interaction of STAT3 with the coactivator p300 since the mutant STAT3α (Ser727Ala) and the STAT3β are unable to interact with p300 [222].

Other studies show that Ser727 of SATA can be phosphorylated by CDK5[223], PKC [224], mTOR [225], and by tyrosine kinase receptors that recognize the LPMSP motif such as EGFR. This phosphorylation can mediate transcription activation independent of Tyr705 phosphorylation [226, 227]. Moreover, some studies show that Ser727 phosphorylation inhibits the effect of Tyr705 phosphorylation of STAT3 [228]. In melanocytic cells, phosphorylated Ser727 without Tyr705 phosphorylation is associated with cell survival activity and nuclear translocation of STAT3 [229]. In melanoma cells, Ser727 was found constitutively phosphorylated and this phosphorylation is mediated by ERK pathway [229].

In addition to Ser727 and Tyr705 phosphorylation, STAT3 can be acetylated on a single lysine residue Lys685 in response to cytokine IL6. The acetylation is mediated by p300 and is reversed by histone deacetylases (HDAC). The STAT3 mutant (Lys685Arg) reveals that Lys685 acetylation is critical for stable dimer formation, DNA binding, and transcriptional regulation of genes that regulate cell growth and cell cycle progression after oncostatin stimulation [230].

Other post-translational modifications can influence STAT3 activity such as Glutathionylation. STAT3 can be Glutathionylated on Cys328 and Cys542 within the DNA binding domain and the linker domain impairing STAT3 phosphorylation [231].

STAT3α null mice are embryonic lethal revealing the importance of this gene during development [232]. Mice lacking both Stat3 isoforms have the same phenotype as STAT3α null mice, whereas STAT3β null mice are viable, fertile, and able to regulate different functional genes, but they are sensitive to endotoxin-induced inflammation [233].

Loss of STAT3α in adult tissues cause failure of cell survival, impaired apoptosis, loss of negative feedback regulation and impaired cell migration [234].Thus demonstrating its importance in all of these cellular systems, [186, 235-237] (Table 1.1).

Once STAT3 is activated by phosphorylation, it dimerizes and translocates to the nucleus where it binds to specific DNA sequence and regulates transcription of its target genes. STAT3 binds as a dimer to a STAT binding element (SBE): TTCCGGGAA. It recruits different co-activators to its transactivation domain, it recruits CBP/p300 that acetylates STAT3 and the histones around the promoter of its target genes to activate transcription. Also, STAT3 can interact with c-Jun, GR, androgen receptor for maximal transcriptional activity.

Table 1.1: Tisue-specefic roles of STAT3 as revealed by conditional gene targeting in mice
Adapted from [233]

Target tissue	Phenotype
Skin	Impaired second hair cycle, wound repair and keratinocyte migration
Thymic epithelium	Age-dependent thymic hypoplasia, hypersensitivity to stress
T lymphocytes	Impaired Il-6-dependent survival and Il-2rα expression
Monocytes/neutrophils	Enhanced inflammatory responses and T_H1 differentiation, chronic colitis
Granulocytes	Enhanced proliferation owing to impaired negative feedback
Mammary epithelium	Defective apoptosis, delayed mammary involution
Liver	Impaired acute phase response
Neurons	Impaired cell survival

3.6.3.3 Regulation of STAT3 pathway

Once activated, the STAT3 pathway is rapidly shut off by negative regulators. This negative regulation is mediated by *Suppressors of Cytokine Signaling (SOCS)* proteins that block continued signaling. They are induced after cytokines or growth factors (EGF) stimulation. These proteins have an SH2 domain by which they bind to phosphorylated tyrosine residue of the receptor or of JAK, in order to inhibit the catalytic activity of JAK and the recruitment of STAT proteins [238].

Protein inhibitors of activated STATs (PIAS): These proteins are nuclear regulators, they are activated after cytokines stimulation and they bind only to the activated form of STAT. PIAS3 is specific to STAT3, and it inhibits STAT3 dimer binding to DNA [238]. PIAS have an E3-ligase-like activity for the small ubiquitin-related modifier SUMO. They conjugate SUMO to target proteins to repress their activity[239] .

Protein tyrosine phosphatases (PTPases) such as TC45 and SHP-2. These dephosphorylate STAT3 and induce its nuclear export. The phosphatase SHP-1 and SHP-2 induce the dephosphorylation of cytokine receptors in order to inhibit signal transduction. The low molecular weight-dual

specificity phosphatase 2 (LMW-DSP2) suppress the phosphorylation and activation of STAT3 induced by IL6 [238, 240, 241].

The STAT3 pathway can be inhibited by the degradation of STAT3 proteins by the ubiquitin–proteasome machinery.

3.6.3.4 Role of STAT3 pathway in cancers

Multiple studies show that aberration in STAT3 activity is associated with the development of different types of human cancers. Constitutively activated STAT3 was found in various types of cancer including leukemias, glioma, ovarian, breast, brain, head and neck cancers [242-247]. STAT3 inhibitors may be effective as anticancer therapies.

Aberrant expression of STAT3 can be caused by deregulation in the stimulatory signals such as mutations in EGFR causing its constitutive activation, mutations in JAKs, or mutations in the negative regulator proteins such as epigenetic silencing of SOCS3.

Studies showed that STAT3 may induce expression of cell cycle regulating proteins such as cyclin D1, cyclin B, cdc2, c-Myc and promote G1/S phase transition in colorectal cancer cells [248-251]. STAT3 was found to be phosphorylated in 19% of bladder cancer tissues. Constitutively phosphorylated STAT3 was found to induce expression of E2F-1 in colon and breast carcinomas. E2F-1 is important for G1/S and G2/M transitions and in inhibiting apoptosis [252]. Cells that express dominant negative STAT3 show induction of p21 mRNA. By comparison to normal cell, transformed cells with constitutively active forms of STAT3 show 3- to 5-fold elevated level in the mRNA for cyclin D1, c-myc, and that inhibition of the STAT3 pathway with a JAK inhibitor, AZD1480, showed decreased tumor growth through downregulation of cell cycle regulators cyclin D1, cyclin D3 and cdc25A [253]. STAT3 induces tumor formation by triggering pro-survival signals in the cell. The expression of Bcl-xL and MCL1, members of the anti-apoptotic Bcl-2-family, depends on constitutively activated STAT3, which means that the blocking activity of STAT3 leads to downregulation of Bcl-xL and MCL expression in multiple-myeloma cells [254]. Studies show that STAT3 represses transcription of the growth arrest and apoptotic protein p53 contributing to cell survival. Another protein that is regulated by STAT3 is HSP70, a chaperone protein that prevents the JNK pathway phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-xL and to

their inactivation. Studies showed that STAT3 can bind to the *HSP70* promoter and mediate expression of anti-apoptotic proteins [255].

Multiple studies demonstrate the central role of STAT3 signaling in angiogenesis, metastasis, and invasion making it a potential target for cancer therapy.

3.6.4 EGFR pathway in normal corticotroph cells

EGF is known for its mitogenic effect, but studies in pituitary cells showed that EGF can alter cell function and cell ability to respond to hormonal signals. For example, in rat pituitary tumor cells (GH3) that produce prolactin and growth hormone, EGFR was found expressed at the surface of those cells. Stimulation with EGF inhibits the ability of GH3 cells to respond to thyroid hormone, suggesting a different role of EGF in addition to its proliferative effects [256].

In cultured bovine pituitary cells, EGF was found expressed in the anterior pituitary with other growth factors [257]. Combined in situ hybridization and immunohistochemistry revealed expression of EGF mRNA in somatotroph and gonadotroph cells, and expression of EGFR mRNA in all subsets of pituitary cells [257]. Under cold stress, EGF mRNA was induced in corticotroph and thyrotroph cells [257]. By in situ hybridization, EGF mRNA was expressed in the anterior and intermediate lobes of rats [258]. In normal human autopsy pituitaries, immunocytochemistry revealed expression of EGF and EGFR in corticotroph cells and EGF-immunopositive cells are stained positive for ACTH [259] suggesting a role of EGF and its receptor in ACTH production from corticotroph cells. Results of a study did in 1987 showed that EGF acts as CRH and AVP in fetal sheep in inducing ACTH secretion [260]. In corticotroph cells, EGF induces ACTH release by increasing the number of POMC cells in an enriched (ACTH,GH and PRL cells) and non-enriched (ACTH cells) culture indicating that EGF action is independent of other pituitary cells [261]. Another study showed that EGF stimulates ACTH production by amplifying the effects of CRH [262]. Treatment of anterior pituitary cells isolated from 2-month-old mice with the EGFR inhibitor RG-13022 decreased the proliferation of corticotroph and lactotroph cells, indicating the presence of active EGFR receptor in those populations [263].

3.6.5 EGFR pathway in tumor corticotroph cells

EGFR pathway activation is associated with a variety of human cancers by activation of mitogenic pathways, perturbation of cell cycle progression, and downregulation of tumor suppressor genes [264]. EGF and its receptor were found expressed in human corticotroph adenomas and carcinomas, and its expression correlates with invasiveness [259]. Approximately 60% of human ACTH-secreting adenomas express EGFR. Human and dog corticotroph tumors overexpress EGFR, and human and dog cultured tumors treated with EGF showed increase in POMC expression. Overexpression of EGFR in a mouse model of corticotroph cells (AtT20) that do not express EGFR, enhances ACTH secretion and basal POMC transcription through the MAPK pathway, while blocking EGFR activity with gefitinib leads to decrease in POMC expression and cell proliferation [265] [266]. In xenograft corticotroph tumor animal model with EGFR overexpression, mice showed features of CD such as hypercortisolemia, hyperglycemia, and weight gain. All of these dysregulations are ameliorated after gefitinib treatment [265]. Most corticotroph adenomas showed expression of phospho-EGFR by comparison to other pituitary adenomas [126] [267]. Furthermore, a specific correlation between EGFR expression and low immunoreactivity of p27 was seen in corticotroph adenomas leading to suggest that EGFR causes corticotroph adenomas formation by downregulating p27 [126].

4. PURPOSE OF THE PRESENT WORK

When I started my PhD in the laboratory, I was interested in Cushing's disease and at that time there was identification of somatic mutations of the gene encoding *USP8* in ≈60 % adenomas. These mutations lead to deregulated USP8 activity and persistent EGFR signalling. Studies have shown that EGFR signaling is essential for the synthesis of POMC, and for the secretion of ACTH and that EGFR was found expressed in ≈60% of human corticotroph adenomas.

The mechanisms by which the EGFR pathway regulates POMC transcription and ACTH secretion is not yet clear as well as the mechanism behind the unresponsiveness or resistance of corticotroph tumors to GC action. Our laboratory has identified two genes (HDAC2 and BRG1) that have an essential role in GC repression, their expression was lost in 50% of human corticotroph adenomas.

The object of the present work was to identify how EGFR controls POMC transcription and assess if EGFR has a role in the resistance of corticotroph tumors to GC action. This is described in Chapter 2.

To achieve this, we used a model of tumor corticotrope cells in culture and by generating a new cell lines and transfection experiments using different constructions of the POMC promoter, we found that the STAT3 pathway has a central role in activating POMC transcription leading to ACTH secretion. We confirmed the implication of STAT3 in corticotroph tumors by immunohistochemistry since we found expression of the activated forms of STAT3 (pSTAT3) in 50% of human adenomas.

Then, we compared the results found with EGF with those obtained with LIF and IL6 that also activate the POMC promoter via STAT3: we found that both stimulators don't induce POMC transcription by the same mechanism. Interestingly, we found that the POMC promoter became resistant to DEX upon EGF induction which mimic the conditions in human corticotroph adenomas. Further, we found that STAT3 is responsible for this unresponsiveness of the POMC promoter to DEX. The mutation of both STAT3 regulatory elements on the POMC promoter does not rescue the repression by DEX; however, the use of a JAK1/2 inhibitor that blocks STAT3 phosphorylation and nucleus translocation restores DEX repression. These were very interesting results since it gives us a new therapeutic target to treat Cushing's disease. These results are

described in chapter 2. The chapter 3 is a collaborative work, and it is published in Endocrine-Related Cancer. It contains results about another mechanism that can contribute to corticotroph tumor formation. CABLES1 is a negative cell cycle regulator that is activated by GC in corticotroph cells. The aim of the study was to investigate the presence of CABLES1 mutations or copy number variations in human samples with CD. Results show the presence of four (4%) missense variants in *CABLES1*: two in a young adults cohort (c.532G > A, p.E178K and c.718C > T, p.L240F) and two from childrens (c.935G > A, p.G312D and c.1388A > G, and p.D463G) with CD. No CNVs were found. Then, we assessed the effect of these missense variants on protein function since the four variants are close to the CDK3 binding region of the CABLES1 protein. We generated conditional tamoxifen-inducible chimeric ERtam-CABLES1 AtT-20 cell, and we measured cell growth after treating cells with DEX. We found that p.E178K, p.L240F, p.G312D and p.D463G *CABLES1* mutants lost the ability to inhibit AtT- 20 cell growth. These mutations are a loss of function mutations.

CHAPTER 2 – MECHANISMS OF GLUCOCORTICOID RESISTANCE IN CUSHING'S DISEASE *(In preparation)*

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Keywords: Corticotroph adenomas, POMC, EGFR, STAT3, Cushing's disease, Glucocorticoid resistance

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1. FOREWORD

Cushing' disease is caused by pituitary corticotroph adenomas that produce excess ACTH leading to hypercortisolism. The cortisol excess leads to severe disorders including diabetes, hypertension, osteoporosis, obesity, cardiovascular diseases and increased risk of mortality if untreated. A hallmark of Cushing's disease is the relative resistance of corticotroph cells to negative feedback regulation by glucocorticoids. Little is known about the mechanism of glucocorticoids resistance. The recent finding of ubiquitin-specific protease 8 mutations in human corticotroph tumors led to increased deubiqutination of the EGF receptor, impairing its downregulation and sustaining EGF signaling. In this work we showed the role of deregulated EGFR pathway in glucocorticoids resistance.

In the present work, we demonstrate that the critical EGFR pathway that activates POMC transcription is JAK-STAT3 pathway. Activated STAT3 appears responsible for unresponsiveness of POMC promoter to glucocorticoids. The mechanism of STAT3 action seems to not be via DNA-bound but rather through protein-protein interaction since mutagenesis of both STAT3 regulatory elements on the POMC promoter does not rescue the repression by glucocorticoids; however, the use of a JAK inhibitor that blocks STAT3 activation restores the ability of glucocorticoids to repress POMC activity. Activated form of STAT3 was found overexpressed in 50% of human corticotroph adenomas highlighting the importance of STAT3 as new therapeutic target to treat Cushing's disease.

1.1 Authors contribution

I performed all the experiments except for:

Figure 2.3F and Figure 2.5B and C which is the work of Yves Gauthier

Figure 2.6 which is the work of Kevin Sochodolsky

The work was supervised by Jacques Drouin and Aurélio Balsalobre

2. ABSTRACT

Cushing's disease (CD) is caused by pituitary corticotroph adenomas that secret excessive ACTH leading to production of large amounts of adrenal glucocorticoids (Gc) and the resulting hypercortisolemia has multiple deleterious effects. The distinctive feature of corticotroph adenomas is their relative resistance to the negative feedback regulation by Gc. Gc resistance prevents the normal inhibition of ACTH secretion and POMC gene transcription. The reasons behind Gc resistance remain largely undefined.

Recent findings showed overexpression of epidermal growth factor receptor (EGFR) in corticotroph adenomas causing increased POMC activity and ACTH secretion. The main objectives of this work were to understand the relationship between deregulated EGF signaling and Gc resistance in the tumorigenesis of CD.

Mapping of EGF action on the POMC promoter revealed that the critical pathway for EGF-dependent activation of POMC is the JAK-STAT3 pathway. We report that activated STAT3 appears responsible for development of Gc resistance at the POMC promoter and STAT3 activation appears to prevent the action of GR. The blocking of STAT3 activity with a JAK inhibitor prevented EGF dependent activation and also restored the ability of Gc to repress POMC activity. Interestingly, 50% of human corticotroph adenomas showed overexpression of activated STAT3. These results clearly support the important role of STAT3 for Gc resistance and that STAT3 may be a potential therapeutic target to treat Cushing disease.

3. INTRODUCTION

Cushing's disease (CD) is a rare endocrine disorder that is caused by pituitary adenomas that secrete excessive amounts of ACTH [99]. The uncontrolled production of ACTH stimulates production of large amounts of adrenal glucocorticoids and the resulting hypercortisolemia has multiple deleterious effects. These ultimately involve diabetes, hypertension, osteoporosis, central obesity, impaired reproductive function and eventually increased mortality if untreated [268]. The first line of treatment for Cushing's disease remains adenectomy although small subsets of CD are responsive to pharmacotherapy and now treated with somatostatin analogs [269]. The pathogenic mechanisms underlying CD remain largely undefined and their unravelling may offer novel therapeutic approaches.

The pituitary adenomas that cause CD are mostly composed of phenotypically differentiated corticotroph cells of the anterior pituitary [270]. These cells express the marker of corticotroph cells, namely the proopiomelanocortin (POMC) precursor gene, and they secrete ACTH that results from processing of POMC [271]. They typically are microadenomas (only a few millimeters in diameter) and their diagnosis has represented a clinical challenge. Whereas state-of-the-art imaging technologies are now of great support to identify adenomas, this is not always sufficient to localize very small microadenomas. The distinctive feature of corticotroph adenomas is their relative resistance to the negative feedback regulation by glucocorticoids (Gc), and this has been the basis of a diagnostic procedure to both confirm the diagnosis of pituitary Cushing's syndrome and to localize adenomas on either left or right sides of the pituitary [272]. CD may also be caused by macroadenomas that can be somewhat invasive and may, as for other pituitary adenomas, be revealed as a result of pressure on the optic tract [273].

Whereas Gc resistance is a hallmark of CD, its severity can vary with subsets of patients showing resistance to moderate or high levels of administered Gc. Gc resistance prevents the normal negative feedback of Gc on ACTH secretion and POMC gene transcription, thus further enhancing ACTH hypersecretion. Although GR mutations are found in small subsets of CD adenomas, loss-of-function GR mutations appear insufficient to cause CD [274]. The development of Gc resistance

thus appears to be an important but contributing factor to CD pathogenesis. Probing of the mechanism of Gc feedback on POMC gene transcription identified mechanisms and proteins that may account for resistance in subsets of CD adenomas. These include the chromatin remodelling proteins BRG1 and HDAC2 that are essential for Gc negative feedback regulation of *POMC* gene transcription and that are no longer expressed in about 50% of CD adenomas [69]. Others have found overexpression of the chaperone HSP90 that is involved in GR processing in subsets of CD adenomas [139]. In addition, the quest for Gc-regulated genes encoding cell-cycle regulators identified the *CABLES1* gene that is Gc-induced and encodes an inhibitor of cell-cycle progression [115, 275]. This gene is mutated in small subsets of adult and pediatric CD [122]. However, it is likely that the above alterations contribute to pathogenic mechanisms without being initiating events.

The first alterations identified in CD adenomas that are likely causative are mutations in a hotspot of the gene encoding *USP8*, a deubiquitinase that has many substrates but primarily the EGF receptor (EGFR) [124]. The frequency of *USP8* mutations varies in different patients' cohorts from 25 to 60% of cases [274]. Since USP8-dependent deubiquitination of EGFR prevents turnover of the protein, this results in increased EGFR and enhanced EGF signalling in corticotroph adenoma cells [124]. Enhanced EGF signalling is manifested by increased MAP kinase signalling: this stimulates *POMC* gene transcription and may provide the molecular basis for ACTH hypersecretion in CD adenomas.

In the present work, we have further dissected the action of EGF in pituitary corticotroph cells and found, as in other systems, that EGF activates not only the MAPK but also the Akt/mTOR and JAK-STAT pathways. Mapping of EGF action on the POMC promoter revealed that the critical pathway for EGF-dependent activation of POMC is the JAK-STAT pathway and different targets of STAT3 action on the POMC promoter were identified. These were compared to the actions of LIF and IL-6 that also activats the JAK-STAT pathway in corticotroph cells. Interestingly, the EGF, LIF and IL-6 pathways exert differential actions on different targets of the POMC promoter. Notwithstanding these differences, we report that activated STAT3 appears responsible for development of Gc resistance at the POMC promoter. Hence, signals that activate STAT3 such as

EGF and LIF results in Gc resistance, whereas CRH-stimulated POMC transcription that is accompanied by MAP kinase activation is sensitive to the repressive action of Gc/GR. We have previously investigated the outcomes of tethering interactions between GR and STAT3 in simple reporter systems and found that STAT3 tethering to DNA bound GR results in synergistic activation of transcription [68]. It thus appears that the context of the POMC promoter presents with a unique environment within which STAT3 activation prevents the action of GR. Altogether, the present work identifies a unique mechanism of Gc resistance through STAT3 and thus offers a novel therapeutic approach for corticotroph adenomas, namely treatment with JAK-STAT inhibitors.

4. RESULTS

In view of the implication of the EGF/EGFR signaling in CD pathogenesis, we investigated in greater detail signaling pathways activated by EGF. We used a model of EGFR-expressing AtT-20 cells in order to assess activation of the different pathways known to be linked to EGFR. These experiments (Figure 2.1) confirmed activation of the MAP kinase (MAPK) pathway following EGF treatment, but also of the Akt/mTOR pathway revealed through Western blot analysis of pAKT, and of the JAK-STAT pathway revealed by Western blot analysis of pSTAT3 (Figure 2.1A). Since prior work on EGF signalling in this system had primarily considered activation of the MAPK pathway, we assessed the importance of each pathway in activation of POMC promoter activity using specific inhibitors (Figure 2.1B-D). These experiments clearly show that only the JAK inhibitor JAKi prevents activation of the POMC promoter by EGF (Figure 2.1D) whereas the AKT/mTOR and MAPkinase inhibitors did not (Figure 2.1B, C). This is a bit surprising since the MAPK pathway was implicated as a mediator of CRH activation of POMC transcription [276]. However, LIF- or IL6-induced STAT3 was previously shown to activate POMC transcription [41].

Prior work had mapped two STAT3 binding sites (centered at positions -384 and -155bp) within the rat POMC promoter, but we first mapped EGF-responsive sequences independently of those using various promoter deletions and mutants. Sets of deletions that represent the proximal, middle and distal parts of the POMC promoter showed that only the proximal region is required

for EGF responsiveness (Figure 2.2A). We then used a series of linker-scanning mutations within known or putative regulatory elements of the proximal promoter to assess EGF responsiveness. This showed that the action of EGF can be entirely ascribed to the STAT binding element (SBE) previously localized between -159 and -151 bp of the promoter (Figure 2.2B). This SBE element was previously shown to be responsive to LIF and STAT3 [41] and a simple reporter plasmid containing three copies of the SBE showed greatly enhanced response to EGF compared to the proximal promoter (Figure 2.2C). This simple reporter (Figure 2.2D) as well as the rat (Figure 2.2E) and human POMC promoters (Figure 2.2F) were used to assess the specificity of their responses to EGF with a specific STAT3 inhibitor, Stattic. This inhibitor prevents EGF activation of all three reporters (Figure 2.2D-F), indicating that STAT3 is an essential mediator of EGF action on the POMC promoter.

We then tested responsiveness to other STAT3 inducing cytokines known to act on the POMC promoter, LIF and IL-6, using the set of promoter deletions (Figure 2.3A). Interestingly, all three subdomains of the promoter respond to LIF or IL-6 stimulation (Figure 2.3A). Accordingly, responsiveness to LIF was greater for reporters containing two subdomains and greatest with the intact promoter. In contrast, the responsiveness to IL-6 was equivalent for all three subdomains individually, in various combinations or all three together in the intact promoter. These results clearly indicate that despite the activation of STAT3 by EGF, LIF and IL-6, their mode of action on the POMC promoter appear to differ substantially. This is further highlighted by the promoter mutation of the proximal SBE that does not affect response to IL6 (Figure 2.3A, construct 9) in contrast to its critical importance for responsiveness to EGF (Figure 2.2B, construct 2). Notwithstanding these differences, we assessed the importance of the STAT pathway for responsiveness to LIF and IL6 and found that both are inhibited by the STAT3 specific inhibitor Stattic (Figure 2.3 B-E). In order to better interpret these results, we performed Western blot analyses of activated phosphotyrosine STAT3 and STAT1 in response to EGF, LIF and IL-6 (Figure 2.3F). These showed very strong activation of pTyr705-STAT3 in response to LIF with more moderate activation in response to EGF or IL-6. In contrast, activated pTyr701-STAT1 was mostly activated in response to EGF, weakly in response to LIF and barely in presence of IL-6 (Figure 2.3F). It is noteworthy that all three ligands activate STAT3, albeit at different levels, whereas STAT1 is

primarily activated by EGF. Despite the lower levels of activated STAT3 in response to EGF and IL-6, these levels appear critical because the action of these ligands is reversed by the STAT3 specific inhibitor Stattic (Figure 2.3 B-E). We next assessed whether the observed levels of activated pSTATs are limiting for activity and whether there is evidence for action as heterodimers between STAT3 and STAT1. Using the simple SBE luciferase reporter in transfection experiments, we found that EGF stimulation is enhanced by STAT3 but not by STAT1 (Figure 2.3G) whereas LIF stimulation is further enhanced by both STATs (Figure 2.3H): this suggests that STAT3-containing dimers (homodimers or heterodimers with STAT1) are active but that the STAT1 homodimers formed after EGF stimulation are not.

Resistance to glucocorticoid action

In view of the association of the activated EGF/EGFR pathway with corticotroph adenomas, we assessed responsiveness of the POMC promoter to dexamethasone (DEX) in EGF-stimulated AtT-20 cells in comparison to the well-documented antagonism between DEX with CRH. Whereas CRH-activated POMC transcription is antagonized by DEX (Figure 2.4A), it was surprising to observe that EGF-stimulated POMC transcription is resistant to DEX repression (Figure 2.4B). Since LIF and IL-6 also activate STAT3 (Figure 2.3), we also assessed the ability of DEX to repress POMC transcription upon stimulation by either LIF (Figure 2.4C) or IL-6 (Figure 2.4D). As for EGF, POMC transcription is resistant to DEX repression stimulated by LIF and IL-6. In order to support the idea that STAT3 is the common culprit in GC resistance, we overexpressed increasing doses of STAT3 with the POMC reporter and assessed the impact of STAT3 overexpression on the ability of DEX to repress transcription. Overexpression of STAT3 further enhanced POMC promoter activity and resulted in complete resistance to DEX (Figure 2.4E). To assess whether STAT3 may be hampering GC repression through a protein-protein tethering type of mechanism, we then assessed the effect of increasing amounts of GR expression on the ability for DEX repression at the POMC promoter. These experiments clearly showed that increasing expression of GR can titer down the action of STAT3 on the POMC promoter (Figure 2.4F). It thus appears that GR and STAT3 can titer each other's action at the POMC promoter but that in normal conditions STAT3 prevails and prevents DEX and GR action on POMC transcription.

Since LIF-induced STAT3 is known to be recruited to both proximal and distal SBEs of the POMC promoter [68] , we compared this recruitment with EGF-induced cells and observed recruitment by ChIP-seq at both sites, albeit of lower magnitude (Figure 2.5A), in agreement with pSTAT3 levels (Figure 2.3F). We then compared these profiles with GR [68] and Tpit [8] recruitment: surprisingly, GR recruitment overlaps with Tpit in the central promoter rather than, as might be expected, over the Nur response element (NurRE) that recruits the GR-antagonising Nur factors [70, 277] or over the nGRE that is a direct GR binding site in vitro [65]. This region of the promoter behaves as a promoter proximal corticotrope-specific enhancer [20] and accordingly, is marked by the Mediator protein Med12 (Figure 2.5A). To better compare the profiles of STAT recruitment to the promoter, we used ChIP-qPCR with an array of primers that extend over the region (Figure 2.5B, C). The profile of STAT3 recruitment (Figure 2.5B) measured by quantitative PCR is similar to ChIP-seq profiles (Figure 2.5A) obtained after LIF and EGF stimulation, with evidence of recruitment at both distal and proximal sites despite EGF-dependent activity relying only on proximal site (Figure 2.3B). The recruitment profile following IL-6 stimulation is similar to EGF-stimulated cells and commensurate with the Western blot data (Figure 2.3F). Surprisingly, the patterns of STAT1 recruitment in response to LIF and EGF are similar to those of STAT3, with recruitment at both sites and stronger recruitment in response to LIF (Figure 2.5C). Since EGF elicits a strong STAT1 activation (Figure 2.3F), we might have expected a predominant STAT1 peak (possibly homodimers) but the similarity of the STAT1 and STAT3 profiles rather suggest that STAT3 is required for recruitment to the POMC promoter in agreement with the blockade by the STAT3-specific inhibitor Stattic (Figure 2.2D-F and 2.3B-E).

The similar recruitment of STAT3 at both distal and proximal sites lead us to ascertain whether both sites are necessary for glucocorticoid resistance. Mutations of either or both sites does not abrogate the ability of EGF to induce GC resistance despite EGF stimulatory action being completely dependent on the proximal site (Figure 2.5 E). Indeed, mutagenesis of the proximal SBE prevents EGF activation of the POMC promoter but this mutant promoter becomes resistant to DEX inhibitory effect. These results suggest that it is not DNA-bound STAT3 but rather STAT3 that may be recruited through protein-protein interactions that is responsible for GC resistance.

The importance of STAT3 for GC resistance was then assessed by blocking its activation with a JAK inhibitor. The treatment of AtT-20 cells with the JAK inhibitor not only prevented EGF dependent activation but also restored the ability of DEX to repress its activity (Figure 2.5F). These results clearly support the role of activated STAT3 for Gc resistance and suggest that this action involves protein:protein interactions rather than direct DNA binding, as observed in tethering mechanisms of trans-repression [68, 277, 278].

Activated pTyr705-STAT3 in CD adenomas

We assessed the activation of STAT3 in a collection of CD adenomas by immunohistochemistry. Corticotroph adenoma cells revealed by immunohistochemistry for Tpit [69] (Figure 2.6A) are also positive for pTyr705-STAT3 with variable penetrance (Figure 2.6B). Overall, we detected pTyr705-STAT3 immunoreactivity in 16 of 33 adenomas samples. However, we did not find a direct relation with the nature of these adenomas (micro or macro) or with their immune-reactive status with regards to expression of BRG1 and HDAC2 [69] and data not shown). Nonetheless, this data indicates robust activation of the STAT pathway in about 50% of CD adenomas.

5. DISCUSSION

Understanding the mechanisms behind the development of corticotroph adenomas in Cushing's disease is an important challenge. In normal physiological conditions the pituitary gland secretes ACTH that is under the negative regulation of Gc produced in the adrenals. In CD, corticotroph tumor cells are relatively resistant to GC action. A hallmark of the disease is thus the resistance of corticotroph tumors to the negative feedback of GC.

In the present work, we explored the role of EGFR in pituitary corticotroph tumors and particularly in Gc resistance. EGFR is often overexpressed in a wide range of tumors such as breast and ovarian cancer [279] [280]. In normal pituitary development, EGFR is expressed, and it was shown that EGF is a regulator of hormone secretion and cell proliferation [281, 282]. In Cushing's pituitary adenomas, EGFR is often overexpressed with the highest incidence by comparison to the other hormone-secreting adenomas [283] suggesting the important role of EGFR signaling in CD tumors.

Here, we found that the three EGFR pathways Akt/mTOR, MAPK and JAK-STAT3 were activated in murine corticotroph cells overexpressing EGFR (Figure 2.1A) and that POMC promoter transcription activity is induced by EGF. We know that the POMC promoter activity is under the control of different hormonal transcription factors such as, GR, STAT3, NF-κB, we identified the transcription factor that mediates EGF effect on POMC promoter activity. By using specific inhibitors for each pathway, we showed that POMC promoter activity depends on the JAK-STAT3 pathway (Figure 2.1B) which requires the SBE sequence from -159 to -151bp on the proximal domain of the POMC promoter (Figure 2.2B). This result is at odds with previous studies that suggested dependency on the MAPK pathway [124, 265] either because it represents the major canonical pathway activated downstream EGFR signaling or because of the effect of a MAPK inhibitor that inhibits POMC promoter activity enhanced by EGF. The same study tested the STAT3 and the STAT1 inhibitor (S3I-201) on POMC promoter activity and found that the EGF-induced POMC promoter is not inhibited by the STAT inhibitor [284] in contrast to our results where we found that both rat (Figure 2.2E) and human (Figure 2.2F) POMC promoter activities are inhibited by the STAT3 specific inhibitor, Stattic. We tested also the S3I-201 in our cells and we found that the EGF-induced activity of POMC promoter is inhibited (data not shown). The difference in results is not immediately obvious but may be related to the serum starvation used in the above study.

STAT3 belongs to the family of signal transducers and activators of transcription (STAT). The STAT family is composed of six members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 and they regulate different pathways [285]. STAT3 was described as in closely associated with cancer progression, proliferation, metastasis and multidrug resistance [285, 286]. The phosphorylation of STAT3 in normal cells is transient, whereas in cancer cells STAT3 was found to be hyperactivated [287]. STAT3 is response to cytokines such as IL6, LIF, IL10, and growth factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF). Once phosphorylated by Janus kinases (JAKs) on its SH2 domain, STAT3 can form homodimers and translocate to the nucleus where it forms complex with coactivators such as p68 to activate transcription of target genes [288] [289]. STAT3 activates POMC transcription following LIF stimulation. LIF that is released from the hypothalamus and produced in the pituitary

gland to provide paracrine and autocrine regulation. STAT3 has two has two binding sites on the POMC promoter one at −380 bp and the other at −150 bp [68].

In contrast to EGF that activates POMC transcription only through the proximal domain SBE (Figure 2.2B), we found that LIF and IL6 act on POMC promoter activity through central and distal domains in addition to the proximal domain that contain a SBE (Figure 2.3A). It was shown that LIF signaling may involve tyrosine phosphorylation of STAT1 and STAT3 to stimulate POMC promoter activity [41]. Our results showed that the pTyr705-STAT3 is activated mostly by LIF in contrast to the pTyr701-STAT1 that is strongly activated by EGF. Further, the activity of SBE reporter is enhanced by STAT3 but not STAT1, after EGF induction, suggesting that in EGF conditions the STAT1 heterodimers is not active (Figure 2.3 G and H) on this reporter [41].

Interesting, we found that the POMC promoter becomes resistant to DEX after EGF treatment (Figure 2.4B) in contrast with the antagonism between DEX and CRH (Figure 2.4A). In our Study, we found LIF and IL6 also causes resistance of the POMC promoter to DEX (Figure 2.4C and D). Since the three cytokines have STAT3 activation in common, we made the hypothesis that it is responsible for the resistance to DEX. To further situate the implication of STAT3 in GC resistance we transfected AtT-EGFR cells with increasing dose of STAT3 or GR and observe that they titrate each other (Figure 2.4E and F). This is typical of mechanisms that rely on protein:protein interactions.

It was previously shown that STAT3 tethering to DNA bound GR results in synergistic activation of transcription [68]. Importantly, GR transrepresses NF-κB and AP-1 dependent-activities on pro-inflammatory target genes and conversely, these two factors inhibit the action of GR following their recruitment by tethering [290] [291]. It was also shown that overexpression of c-Jun prevents the Gc-induced activation of genes carrying a functional Gc response element (GRE) through protein:protein interactions [290]. It thus appears that the context of the POMC promoter presents with a unique environment within which STAT3 activation prevents the action of GR.

To further assess this hypothesis, we used constructs of the POMC promoter with mutations of both SBE motifs. This showed that STAT3 does not need to bind the POMC promoter to cause resistance (Figure 2.5E). We hypothesize that the critical STAT3 is recruited to the promoter

through protein:protein interaction that may involve chromatin remodeling like Brg1 that was previously shown to be essential for GR and HDAC2 recruitment to the POMC promoter [69].

Thus, we used the JAK inhibitor to prevent activation. Importantly, in the cells treated with the JAK inhibitor, the POMC promoter is repressed by DEX (Figure 2.5F) even in the presence of EGF. We think that STAT3 disrupts the transrepression mechanism of GR. We found by ChIP-QPCR, that GR is recruited to the POMC promoter even in the presence of EGF stimulation (Figure 1-Annex) similar to the recruitment of GR and STAT3 to the POMC promoter after LIF and DEX treatment. We propose that resistance to DEX is due to disrupted HDAC2 recruitment by STAT3.

In order to assess this hypothesis, we assessed the level of acetylated histone H4 over the promoter using ChIPqPCR and found that acetylation is inhibited by DEX. But when we treated cells with EGF and DEX, the acetylation levels are no longer inhibited (preliminarily results, data not shown).

Collectively, these observations support a role of STAT3 as disrupting the transrepression mechanism of GR and blocking HDAC2 action on the POMC promoter (Figure 2.7). These results highlight the potentially useful role JAK inhibitors for treatment of corticotroph adenomas. In this work we used the JAK inhibitor: AZD1480 that is a novel pyrazol pyrimidine ATP-competitive inhibitor of JAK1 and 2 kinases, with IC50's of 1.3 and <0.4 n, respectively. It inhibits STAT3 phosphorylation in vitro and in xenograft model of human solid tumors and multiple myeloma [292, 293]. The JAK inhibitor has anti-tumor activity by decreasing proliferation and increasing apoptosis of colorectal cancer cells [294] and in our work, we found that the JAK inhibitor inhibits the proliferation of AtT-EGFR cells induced by EGF (Figure 2-Annex).

Due to the importance of STAT3 in GC resistance, we assessed the expression of pSTAT3 in a subset of human corticotroph adenomas samples. We found that STAT3 is overexpressed in 50% of cases. This finding suggests that STAT3 may explain GC resistance is some CD cases. In addition, the involvement of other mechanisms in the other 50% of cases such as downregulation of BRG1 and HDAC2 two regulators of chromatin remodeling that are part of the GR trans-repression complex [69].

Taken collectively, our data highlight the importance of STAT3 in GC resistance in CD. Further, understanding of STAT3 pathway and its target proteins may offer insights into mechanisms of steroid resistance in other diseases.

6. CONCLUSION

Based on our results, we believe that EGFR signaling enhance POMC transcription via STAT3 pathway, and that STAT3 is responsible for Gc resistance in corticotroph adenomas by impairing GR tranrepression mechanism. STAT3 may be an important therapeutic target to treatment CD.

7. MATERIALS AND METHODS

7.1 Cell culture and transfection

AtT-20/D16v-F2 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. To generate stable transgenic AtT-20 EGFR cell populations retroviruses were packaged using the Platinium-E Retroviral Packaging Cell Line (Cell Biolabs, catalog #RV-101) and infections performed as described in [295]. Selection of cell populations was made with 400 µg/mL Geneticin (Gibco, #11811-031). The resistant colonies were pooled in order generate populations with hundreds of independent colonies.

For transient transfections, EGFR expression plasmid was constructed in the expression vector JA1394 [296]. The coding sequences corresponding to EGFR were provided by Dr. Marily Theodoropoulou (Ludwig-Maximilians-University of Munich). We used the retroviral vectors pLNCX2 to generate stable cell populations expressing EGFR.

AtT-EGFR cells were maintained in DMEM supplemented with 10% fetal bovine serum added with antibiotics. The cells were plated in 12-well plates and transfected with 500 ng of luciferase reporter constructs using the Lipofectamine reagent. The second day after transfection, cells were stimulated for 24 h with either H2O, PBS or DMSO as control, with CRH 10^{-7} M, with dexamethasone (DEX) 10^{-7} M (Sigma-Aldrich), and/or LIF 10ng/ml (Millipore), and/or EGF 100ng/ml (PeproTech), and/or IL6 20ng/ml (Stem Cell Technology) and/or Stattic 5uM (Sigma-Aldrich), PD98059 1uM (Sigma-Aldrich), Rapamycine 0,5nM (Millipore Sigma), JAKi 0,5uM

(AZD1480: Sellechchem) as indicated. To assess STAT3 or GR responses, we co-transfected 500 ng of luciferase reporters with increasing concentrations of either STAT3 (0–400 ng) or GR (0–500ng) effector plasmids, and empty pSP64 plasmid to make up a constant amount of transfected plasmid DNA. Transfection data shown are as means ±SE. of three experiments each performed in duplicates (Figure 2.2A,B; Figure 2.3A, G,H; Figure 2.4A,B,C,D; Figure 2.5E,F) or are expressed as mean ± SE of one representative of three independent experiments with similar results (Figure 2.1B,C,D; Figure 2.2C,D,E,F; Figure 2.3B,C,D,E; Figure 2.4E, F).

7.2 Luciferase reporters

The POMC-Luc constructs used in Figure 2.2A and B are described in [297]. The trimeric reporter of SBE for STAT3 binding and the mutant trimeric SBE used are described in [68]. The plasmid construct with double SBE mutations of the proximal and/or the distal domain of POMC promoter was created using as template the plasmid with the proximal SBE mutation using the following primers: forward primer, 5'-GTGATATTTACCTCCAAAAGGAACGAAGGCAGATGGACGCACACAGG-3'; and reverse primer, 5'-CCT GTGTGCGTCCATCTGCCTTCGTTCCTTTTGGAGGTAAATATCAC-3'. All plasmid constructs were confirmed by DNA sequencing.

7.3 Western blotting

Total extracts were prepared by solubilizing cells in 50 mM Tris-HCl, pH 8, 170 mM NaCl, 50mM NaF, 0.5% Nonidet P-40, 10% Glycerol, 1mM DTT, 1mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin and 1μg/ml pepstatinA, and collecting the supernatants after centrifugation. Then, the supernatants were analyzed on SDS-PAGE. Western blots were revealed using STAT3 (9139, Cell Signaling), pY-STAT3 (4113, Cell Signaling), pS-STAT3 (9134, Cell Signaling), STAT1 (9172, Cell Signaling), pY-STAT1 (9167, Cell Signaling), pS-STAT1 (9177, Cell Signaling), AKT (9272, cell Signaling), pAKT (9271, Cell Signaling), pp44/42 (4376, Cell Signaling), α Tubulin (sc-32293), and GAPDH (8245ab) antibodies.

7.4 ChIP sequencing

AtT-EGFR cells were treated with H2O as control, with EGF 100ng/ml, with LIF 10ng/ml or with IL6 20ng/ml. High throughput sequencing of chromatin immunoprecipitation (ChIP-Seq) was performed as described previously [68] using antibodies against STAT3 (sc-482X), pY-STAT3 (sc7993-R), GR (sc-1004; Santa Cruz), Tpit (in-house antibody 1250) and Med12 (Bethyl A300-774A). Data analysis was performed as described previously [68].

7.5 Immunohistochemistry

Human corticotrope adenoma sections of patients with CD were paraffin-embedded and assessed by immunohistochemistry as described previously [69] using pY-STAT3 antibody (9145, cell signaling) and TPIT (in- house antibody 1250). The use of human CD samples in the present work was approved by the Human Ethics Committees of Institut de Recherches Cliniques de Montréal and of Assistance Publique-Hôpitaux de Marseille.
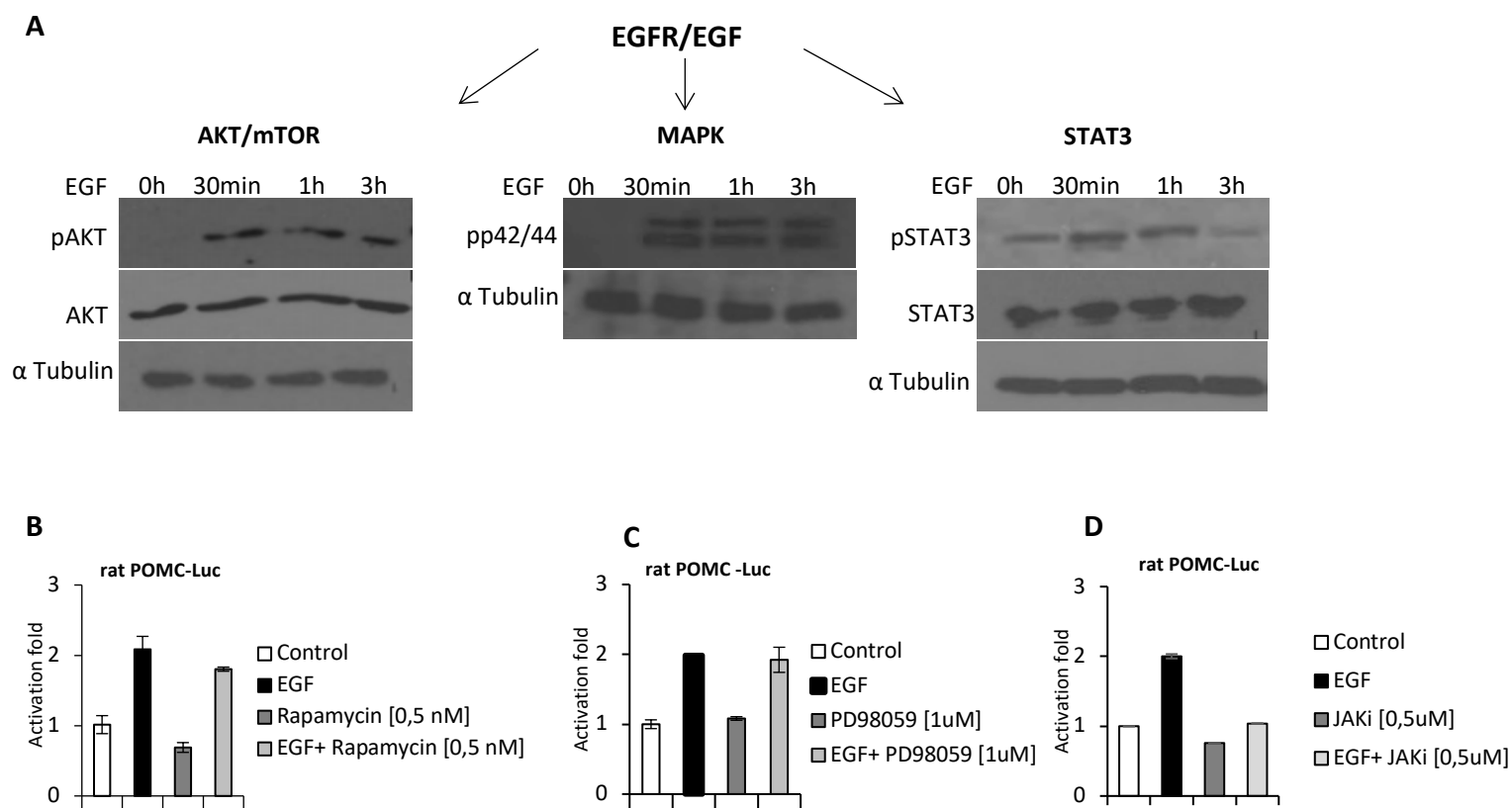
Figure 2.1 : EGFR signaling enhance POMC transcription via JAK

(**A**) Representative immunoblots of AtT-20 cells overexpressing EGFR for pAkt (S473), total Akt, pp42/44 (Thr2020/Tyr204), pSTAT3 (Y705), total STAT3 and α Tubulin, cells were treated with 100ng/ml EGF for 30 min, 1 hour, and 3 hours. (**B**) Effect of EGF, AKT/mTOR inhibitor (rapamycin 0,5nM), (**C**) Erk1/2 kinase inhibitor (PD098059 1uM), and (**D**) JAK inhibitor (JAKi 0,5uM) on POMC transcription. POMC-luc were transfected into AtT-EGFR cells using lipofectamine. Cells were treated with control (white columns), with 100ng/ml EGF alone (black columns), with the inhibitors alone (dark grey columns) or with EGF and the inhibitors (light grey columns). Cells were treated with the inhibitors 1 hour prior the induction with EGF for 24 hours. Results are expressed as fold induction of luciferase activity over control. Results are expressed as mean ± SE of one representative of three independent experiments with similar results. Results are expressed as fold induction of luciferase activity over control.
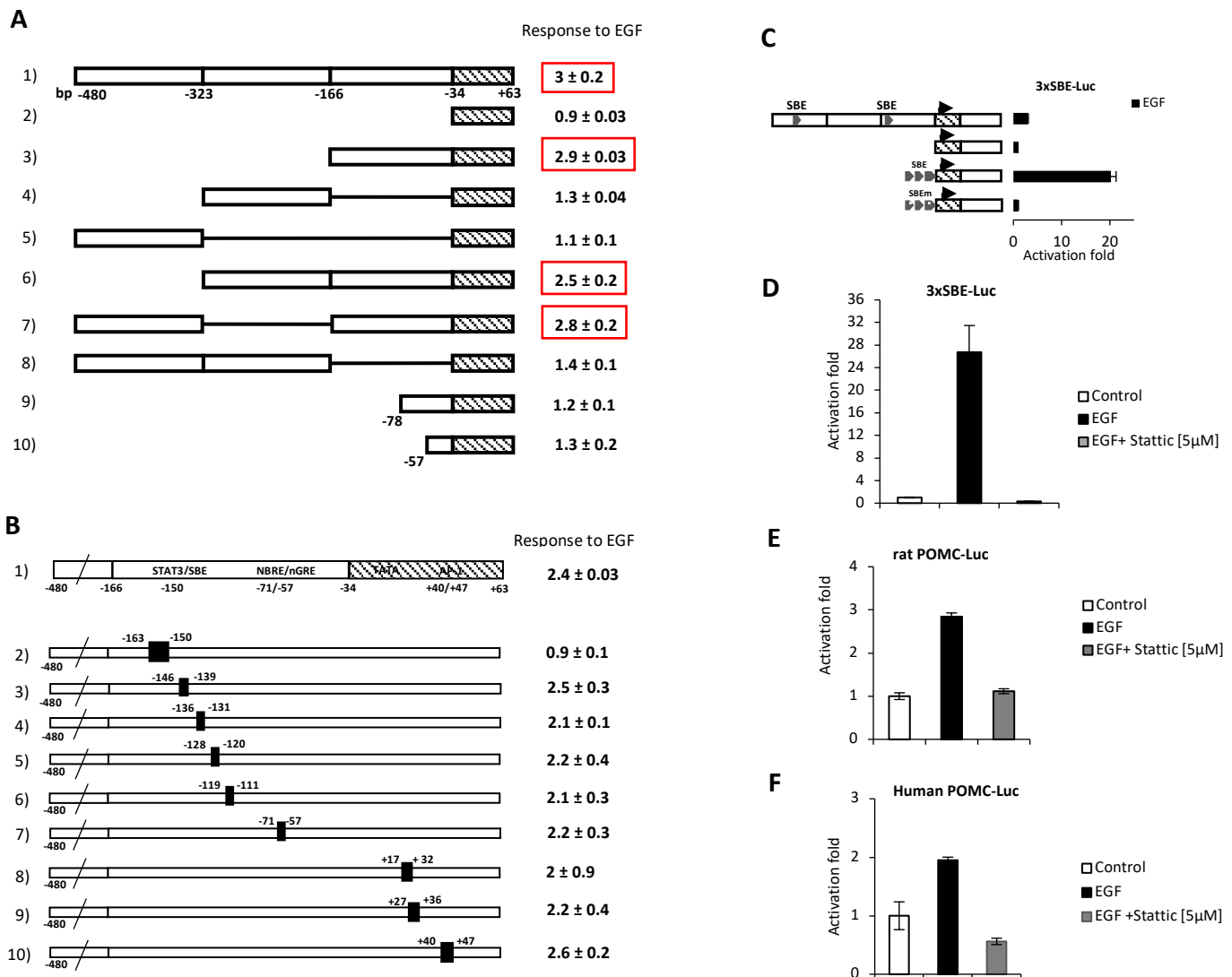
Figure 2.2 : EGF-responsive element on the POMC promoter is SBE

(**A**) The response of the three domains of the POMC promoter to EGF was tested alone or in combination upstream of the rat minimal POMC promoter fragment. Cells were stimulated with 100ng/ml EGF for 24 hours. (**B**) AtT-EGFR cells transfected with replacement mutagenesis of POMC promoter and treated with 100ng/ml EGF for 24h. The data represent the means ± SE of at least three experiments each performed in duplicate. (**C**) Reporters containing either three copies of WT SBE or mutant SBE. (**D**) Activity of reporter containing three copies of WT SBE is assessed by transfection of AtT-EGFR cells treated with EGF ± STAT3 inhibitor Stattic [5uM]. The data represent the means ± SE of at least three experiments each performed in duplicate. (**E**) Activity of rat-POMC promoter or (**F**) human-POMC promoter is assessed by transfection of AtT-EGFR cells treated with EGF ± STAT3 inhibitor Stattic [5uM]. Results are expressed as mean ± SE of one representative of three independent experiments with similar results. Results are expressed as fold induction of luciferase activity over control.
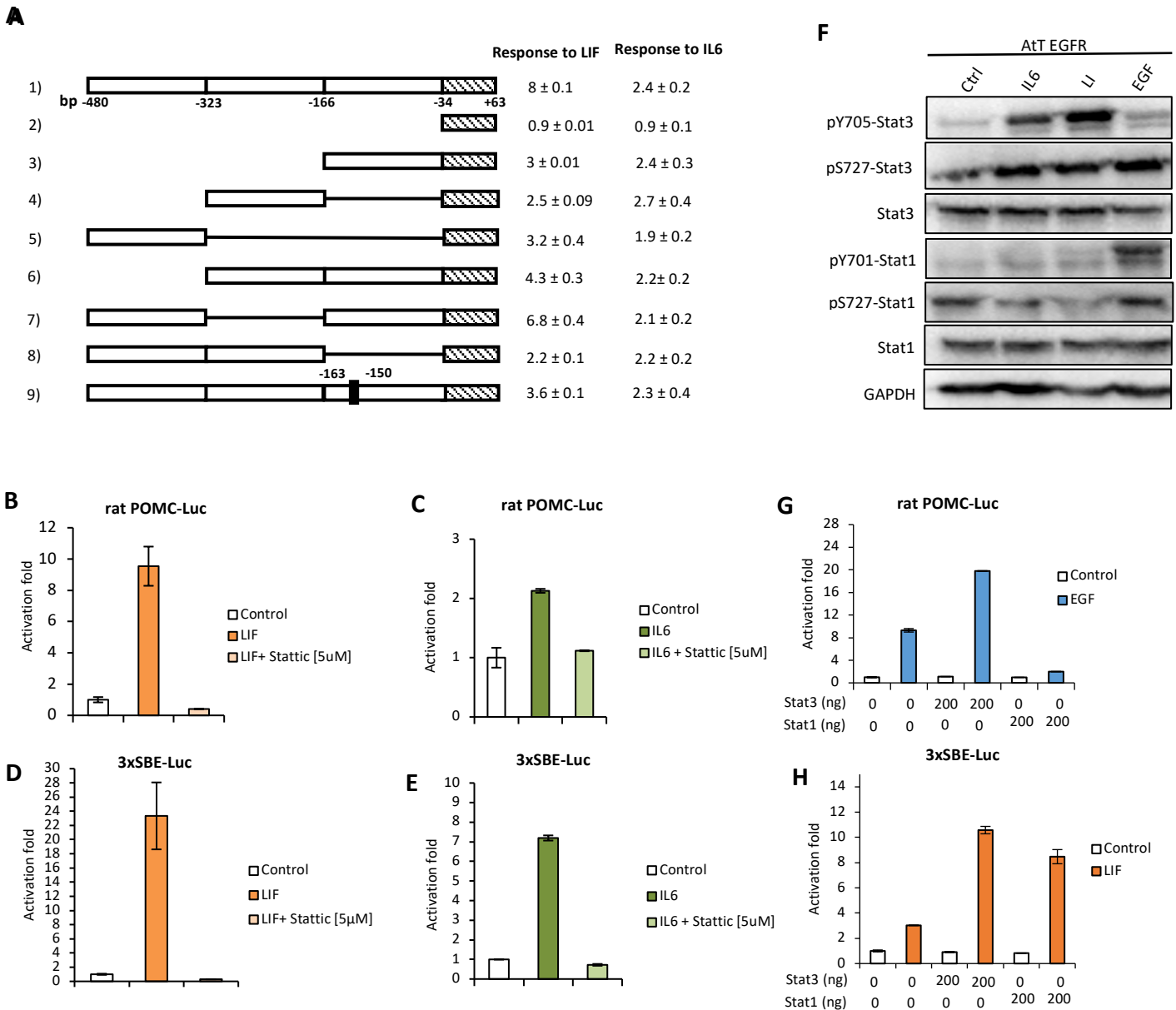
Figure 2.3 : Response of POMC promoter to LIF and IL6

(**A**) The response of the three domains of the POMC promoter to LIF was tested alone or in combination. Cells were stimulated with 10ng/ml of LIF or IL6 for 6 hours. (**B** and **C**) Activity of POMC promoter in response to LIF 10ng/ml or IL6 20ng/ml ± STAT3 inhibitor, Stattic [5uM]. (**D** and **E**) Activity of the trimeric SBE is assessed by transfection of AtT-EGFR cells treated with LIF or IL6 ± STAT3 inhibitors, Stattic [5uM]. (**F**) Western blot analysis of STAT3, pS-STAT3, pY-STAT3, STAT1, pS-STAT1, pY-STAT1 expression after EGF, LIF or IL6 treatment for 30 minutes. GAPDH is used as an endogenous control. (**G**) AtT-EGFR cells were co-transfected with full-length rat Pomc-Luc and 200ng of STAT3 or 200ng of STAT1 expression vectorS then treated with EGF or LIF (**H**) for 24 hours. Results are expressed as mean ± SE of one representative of at least three independent experiments with similar results. Results are expressed as fold induction of luciferase activity over control.
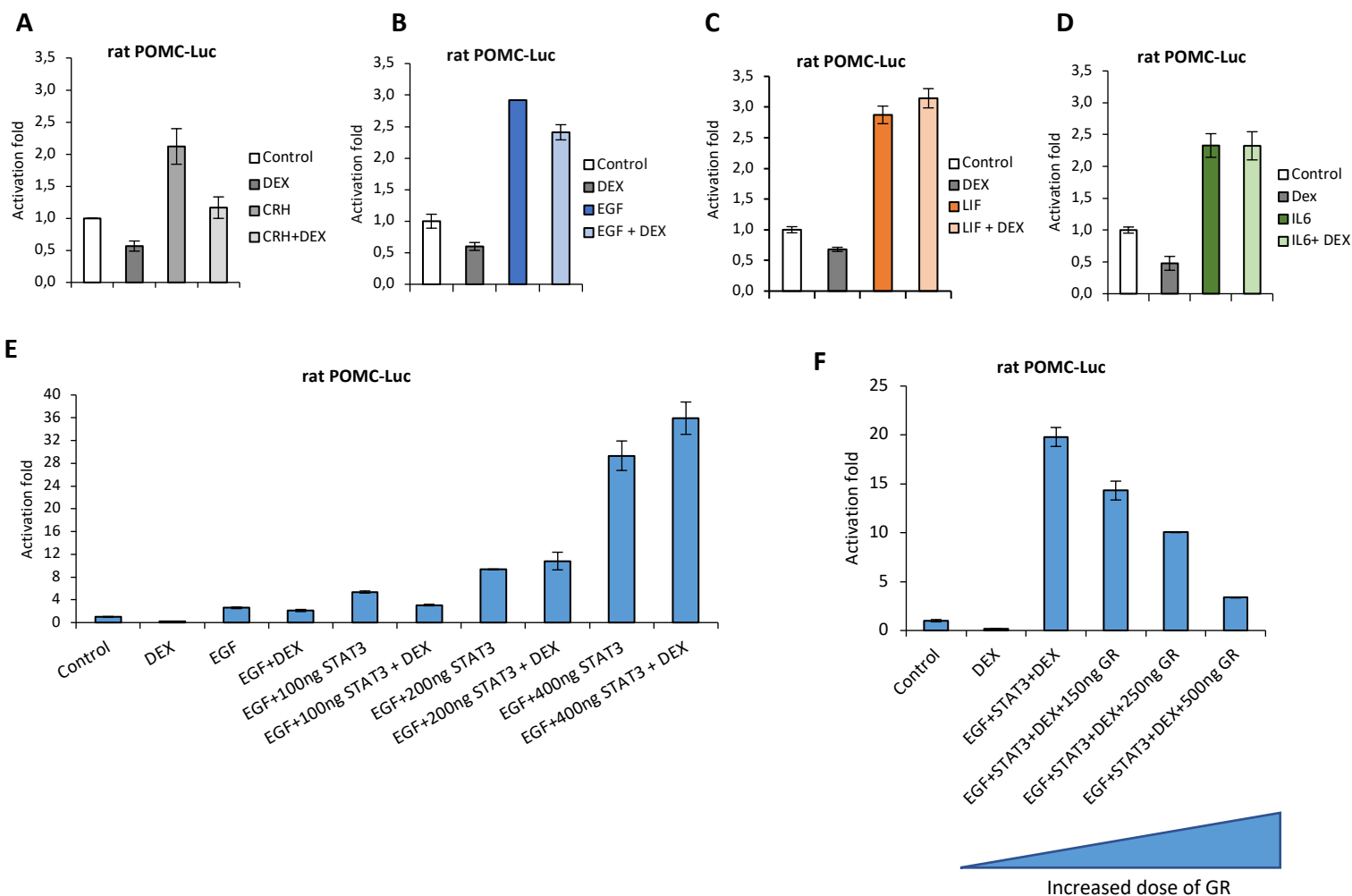
Figure 2.4 : STAT3 is responsible for the resistance of POMC to Gc and STAT3 and GR compete on the POMC promoter

(**A**) AtT-EGFR cells transiently transfected with full-length rat Pomc-Luc were treated with Control, CRH 10^{-7}M, (**B**) EGF 100ng/ml, (**C**) LIF 10ng/ml, (**D**) IL6 20ng/ml ± DEX 10^{-7}M, for 24 hours before the luciferase assay. Results are expressed as fold induction of luciferase activity over control. Results are expressed as mean ± SE of one representative of at least three independent experiments with similar results. (**E**) AtT-EGFR cells were co-transfected with full-length rat Pomc-Luc and increased dose of STAT3 expression vector then treated with EGF, DEX, or both f for 24 hours. (**F**) AtT-EGFR cells were co-transfected with full-length of rat Pomc-Luc, increased dose of GR expression vector, and 200ng of STAT3 expression vector then treated with EGF, DEX or both for 24 hours. Results are expressed as fold induction of luciferase activity over control (no treated cells). Results are expressed as mean ± SE of one representative of at least three independent experiments with similar results.

A

Control	[0-621]
Stat3-LIF	[0-441]
Stat3-EGF	[0-441]
GR-DL	[0-1030]
Tpit	[0-827]
Med12	[0-863]

POMC

3950000 3951000 3952000 3953000 3954000 3955000 3956000 3957000 3958000 3959000 3960000 3961000

B

Recruitment of Stat3 to POMC promoter

Enrichment fold

- LIF
- Control
- EGF
- IL6

C

Recruitment of Stat1 to POMC promoter

Enrichment fold

- Control
- EGF
- LIF

D

JA300
-480   -323   -166   -34   +63

JA301
-480   -323   -163 -150   -166   -34   +63

JA2790
-387 -379
-480   -323   -166   -34   +63

JA2791
-387 -379   -163 -150
-480   -323   -166   -34   +63

Activation fold

- Control
- DEX
- EGF
- EGF+DEX

83

E

rat POMC-Luc

[Bar chart with y-axis labeled "Activation fold" ranging from 0,0 to 3,5; x-axis categories: Control, DEX, EGF, EGF+DEX, JAKi, EGF+JAKi, EGF+JAKi+DEX]

Figure 2.5 : Restoration of Gc repression by blockade of Stat3 activation

(**A**) ChIP-Seq data for STAT3, GR, Tpit, Med12 proteins from AtT-EGFR cells untreated (control) and after 30 minutes of DEX, LIF, EGF or DEXLIF. (**B**) ChIP-qPCR for STAT3 or (**C**) STAT1 recruitment on POMC promoter at different positions from AtT-EGFR cells untreated (control) and after 30 minutes of EGF or LIF treatment. Results of STAT3 are expressed as mean ± SE of four independent experiments, and Results of STAT1 are expressed as mean ± SE of four independent experiments for Control, and EGF, whereases the excrement for LIF represent one experiment. (**D**) POMC promoter constructs with replacement mutations, each mutation is indicated by a black box is fused to the luciferase reporter gene were assessed for activity by transfection into AtT-EGFR. Cells are treated with EGF, DEX or both for 24 hours. (**E**) Activity of POMC promoter is assessed by transfection of AtT-EGFR with full-length rat Pomc-Luc and treated with EGF, DEX, DEX+EGF ± JAKi [0,5uM]. Results are expressed as fold induction of luciferase activity over control (no treated cells). Results are expressed as mean ± SE of four independent experiments each performed in duplicate.
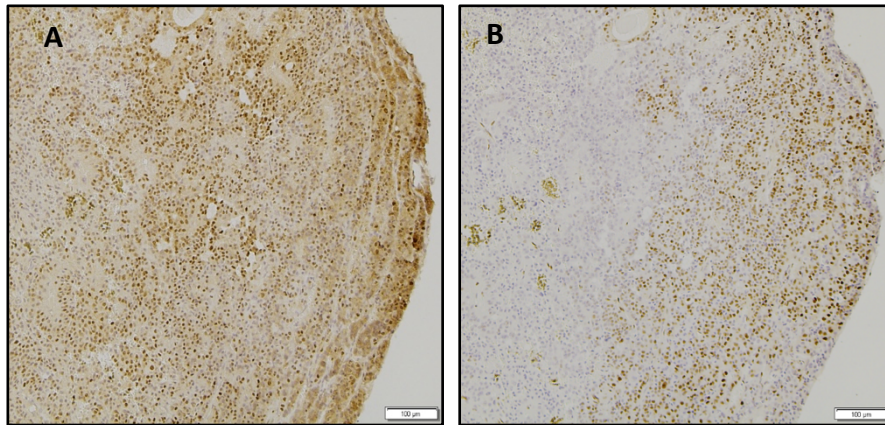
TPIT 10X   **pY-STAT3 10X**

pY-STAT3	number of adenomas	%
+	16	48,5
-	17	51,5
total	33	100

Figure 2.6 : Activated pSTAT3 in Cushing disease

Immunohistochemical analysis of the overexpression of STAT3 in corticotroph adenomas.
(**A**) nuclear TPIT expression is used as marker of tumor, (**B**) expression of pY-STAT3. The data
presented are for one patient, the summary for the other patients is in (**C**), 16 of 33 patients
(48,5%) present with abnormal expression of STAT3.
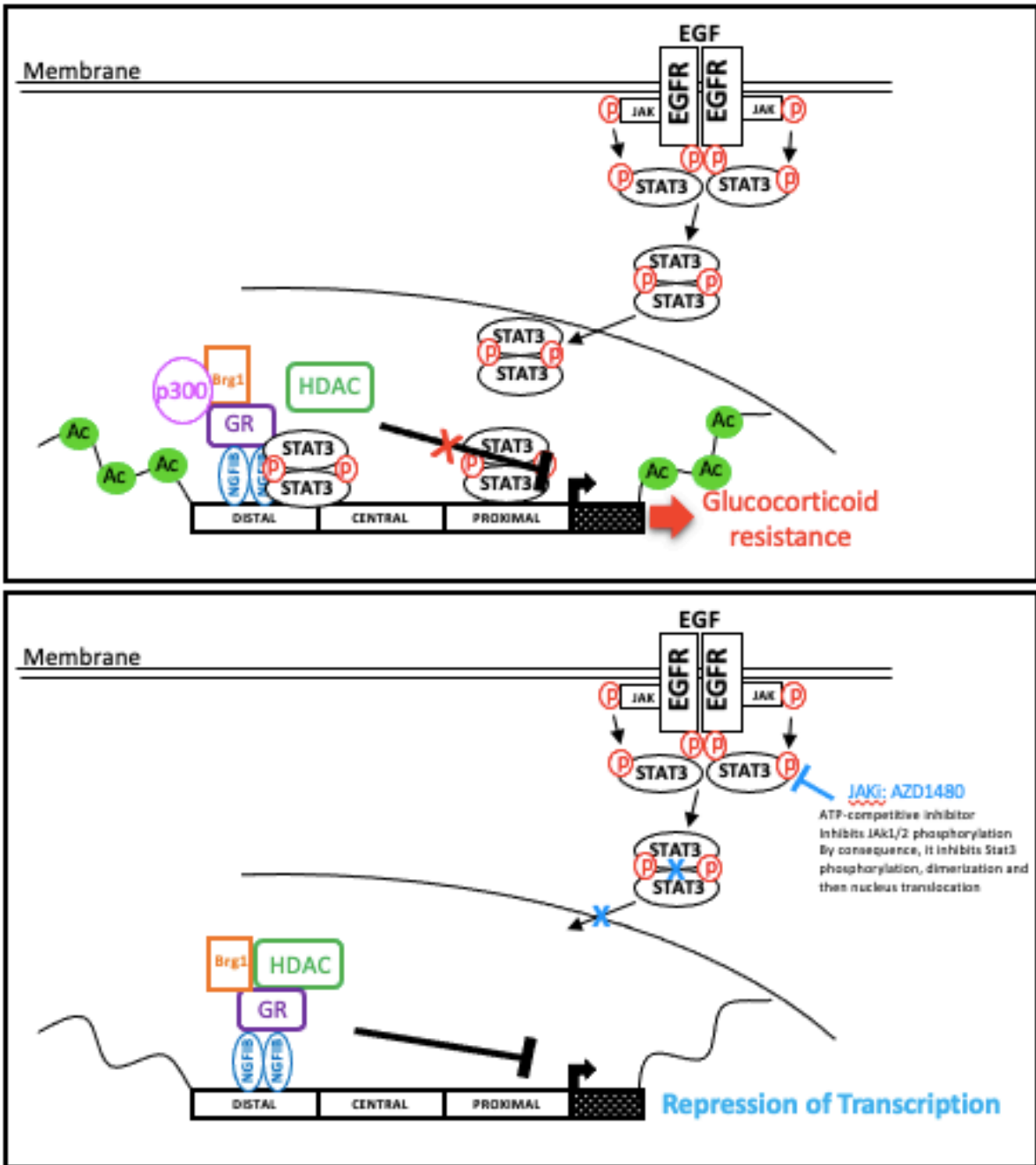
Figure 2.7 : Mechanism of STAT3 action in glucocorticoids resistance

CHAPTER 3 – LOSS-OF-FUNCTION MUTATIONS IN THE CABLES1 GENE ARE A NOVEL CAUSE OF CUSHING'S DISEASE

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1. FOREWORD

The results presented in the chapter 3 show the presence of four missense variants of the cell cycle inhibitor gene CABLES1, these variants are found in two adults and two pediatric patients suffering from Cushing's disease. The four alterations affect relatively very conserve residue among spices and are located very close to the motif that interacts with CDK3.

Since CABLES1 is a cell cycle inhibitor and it is activated with glucocorticoids, we did a functional assessment of the putative CABLES1 mutations. We generated conditional tamoxifen-inducible chimeric ERtam-CABLES1 proteins WT form or with the four alteration and inserted in AtT20 cells. Results show that the CABLES1varients identified in our patients affect the ability of CABLES1 to inhibit cell growth in response to glucocorticoids. These mutations are a loss of function mutations.

1.1 Authors contribution

The next chapter is a collaborative work with equal contribution of our laboratory and the laboratory of Dr. Constantine A Stratakis, and it is already published in Endocrine-Related Cancer in 2017. I did the experiments about the functional assessment of putative Cables1 mutations.

2. ABSTRACT

The CABLES1 cell cycle regulator participates in the adrenal–pituitary negative feedback, and its expression is reduced in corticotropinomas, pituitary tumors with a largely unexplained genetic basis. We investigated the presence of CABLES1 mutations/copy number variations (CNVs) and their associated clinical, histopathological and molecular features in patients with Cushing's disease (CD). Samples from 146 pediatric (118 germline DNA only/28 germline and tumor DNA) and 35 adult (tumor DNA) CD patients were screened for CABLES1 mutations. CNVs were assessed in 145 pediatric CD patients (87 germline DNA only/29 germline and tumor DNA). Four potentially pathogenic missense variants in CABLES1 were identified, two in young adults (c.532G > A, p.E178K and c.718C > T, p.L240F) and two in children (c.935G > A, p.G312D and c.1388A > G, and p.D463G) with CD; no CNVs were found. The four variants affected residues within or close to the predicted cyclin-dependent kinase-3 (CDK3)-binding region of the CABLES1 protein and impaired its ability to block cell growth in a mouse corticotropinoma cell line (AtT20/D16v-F2). The four patients had macroadenomas. We provide evidence for a role of CABLES1 as a novel pituitary tumor-predisposing gene. Its function might link two of the main molecular mechanisms altered in corticotropinomas: the cyclin dependent kinase/cyclin group of cell cycle regulators and the epidermal growth factor receptor signaling pathway. Further studies are needed to assess the prevalence of CABLES1 mutations among patients with other types of pituitary adenomas and to elucidate the pituitary-specific functions of this gene.

3. INTRODUCTION

In recent years, germline defects in multiple genes have been linked to the pathogenesis of pituitary adenomas, both with syndromic and isolated presentation [298]. In contrast with other types of pituitary tumors, corticotropinomas are infrequent in patients with 'classic' multiple endocrine neoplasia and have rarely been described in the setting of familial isolated pituitary adenoma (FIPA) [299, 300]. Consequently, the germline abnormalities leading to corticotroph cell tumorigenesis remain largely unknown. On the other hand, somatic mutations in the USP8 gene hotspot are highly prevalent among corticotropinomas in adult and pediatric patients, but they have not been detected in other types of pituitary adenomas [301, 302]. Therefore, it might be that the majority of the corticotropinomas are caused by disruptions in molecular pathways that are not shared by other pituitary tumor types.

The CABLES1 (Cdk5 and ABL enzyme substrate 1) gene (18q11.2) is a negative regulator of cell cycle progression that is activated in corticotroph cells in response to glucocorticoids [303]. The physiological negative feedback exerted by glucocorticoids on the corticotroph cells is often impaired in corticotropinomas, and, concordantly, CABLES1 protein expression is lost in around half of such tumors [303, 304]. CABLES1 gene inactivation by allelic loss, aberrant splicing or promoter hypermethylation has been observed in different types of human cancers but, to our knowledge, it has not been explored in pituitary adenomas before [305-307]. Moreover, there are no human phenotypes reported in association with CABLES1 germline mutations (http://omim.org/entry/609194, accessed: 28-03-17). Therefore, we investigated the presence of CABLES1 gene mutations and copy number variations (CNV) in a large group of patients with Cushing's disease (CD).

4. RESULTAT

4.1 Genetic findings

The pediatric CD cohort was composed of 72 males and 74 females, with mean age at first symptoms of 10.3 ± _3.3 years and age at diagnosis of 12.5 ± _3.4 years, for a median delay between disease onset and diagnosis of 26 months (IQR: 12–38.3). The median pituitary tumor size was 4 mm (4–7); 88.1% (126/146) of the patients had microadenomas and 8.2% (12/146) had macroadenomas. Four patients (2.7%) had two or more adenomas and in four cases, the tumor was not identified, despite response to surgical treatment in three of them. The first line of treatment was transsphenoidal surgery in all the cases, except for one patient who developed spontaneous apoplexy of the pituitary adenoma. Ninety-seven percent of the patients (141/146) had sporadic presentation; among them, we identified two simplex patients with mutations in *AIP* and *PRKAR1A*, as previously reported [299, 308]. Out of the five familial cases, three were members of families with a multiple endocrine neoplasia phenotype (two of them with *MEN1* mutations) as previously described [299] and two had a FIPA phenotype (family history of prolactinomas), but tested negative for *AIP* mutations. None of the patients had a family history of CD. The adult cohort consisted of 35 CD patients, including 25 females and 10 males. Mean age at diagnosis was 44 years (range: 16–83). A microadenoma was found in 22.9% (8/35) and a macroadenoma in 77.1% (27/35) of the patients. Seventeen patients (48.6%) presented with overt CD, whereas clinical signs were limited or absent for the rest of them. Four missense CABLES1 single-nucleotide variants of interest (each in one patient) were identified among the 182 patients studied (Table 1), affecting exon 1 (c.532G > A, p.E178K and c.718C > T, p.L240F), exon 3 (c.935G > A, p.G312D) and exon 7 (c.1388A > G, p.D463G), respectively (Figure 3.1 and table 3.1) these missense variants were selected because of being either novel (p.D463G) or very infrequent in the general population (p.E178K, p.G312D) and/or because they were predicted to affect the protein structure (p.L240F, p.D463G). The ExAC database reported a frequency of 2.8% for the p.E178K variant: this might be an overestimation, since coverage for *CABLES1* exon 1 was low in the ExAC and gnomAD databases [309], while the 1000 Genomes database reported a much lower frequency. No pathogenic associations were reported for these variants in ClinVar or The Human Gene Mutation Database [310, 311]. In addition, other CABLES1 variants (five of them

not previously reported in public databases) were identified in the pediatric cohort (Supplementary Table 2). Given the methods used for screening, we could not accurately assess the frequency of intronic variants. No CNVs were found in the samples analyzed by ddPCR.

4.2 Clinical, genetic and histopathological features in patients with putative CABLES1 mutations

We identified putative CABLES1 mutations in four sporadic female patients: two young adults (Table 3.2, cases 1 and 2) and two pediatric cases (cases 3 and 4). All these putative mutations were found in the heterozygous state in the germline DNA samples, and no LOH was detected in the pituitary adenomas. The four tumor samples were negative for USP8 hotspot mutations. All these patients had large corticotropinomas (3/4 with extrasellar extension) with high Ki-67 proliferation index, and three of them required a second transsphenoidal surgery (and radiotherapy in one case) to achieve disease control. Patient 1 presented at age 27 years with clinical manifestations due to the mass effect of her tumor (macroadenoma extending to the sphenoid and cavernous sinuses). She was diagnosed with a clinically non-functioning pituitary adenoma and two transsphenoidal surgeries followed by radiotherapy, were required to achieve control. Histopathological analysis established the diagnosis of a silent corticotroph adenoma, with microscopic invasion of the dura mater, bone and respiratory mucosa, Ki-67 index of 6% and 2 mitoses per 10 high-power fields. Patient 2 presented with a clinical phenotype consistent with hypercortisolemia and was diagnosed with CD at age 32 years. Her pituitary macroadenoma with parasellar extension to the cavernous sinus was surgically removed. The resected tumor had a Ki-67 index of 8% and 7 mitoses per 10 high-power fields, but invasion of surrounding structures could not be assessed in the specimen. Since the patient was lost to follow-up, we have no data of her current status. Patient 3 was diagnosed with CD at age 16 years, after a one-year history of hypertension, Cushingoid features and weight gain (weight: 131.4 kg, height: 160 cm, BMI: 51.3 kg/m2). She had elevated midnight cortisol (24.6 µg/ dL), and urinary free cortisol (UFC, 437 µg/dL, ×11 ULN), unsuppressed serum cortisol (20.6 µg/dL) after a low-dose dexamethasone suppression test (LDDST) and her ACTH doubled during a CRH stimulation test (55.3–113 pg/mL). Her macroadenoma with suprasellar extension was resected, but a 15-month remission period

was followed by recurrence, requiring a second surgery and remaining hypercortisolemic afterward. Genetic screening identified paternal inheritance of her putative *CABLES1* mutation, but despite being a carrier, her father was apparently unaffected.

Patient 4 had a congenital polycystic non-functional kidney, and her family history included one case of gastric cancer. She was evaluated at age 10 2/12 years because of decreased growth velocity (height 132 cm, −1 s.d.), hypertension, hirsutism and Cushingoid features, which developed in the previous 26 months. She had elevated ACTH (19.1 pg/mL) and midnight cortisol (19 µg/ dL), failed a LDDST (cortisol 17 µg/dL) and had central hypothyroidism and dyslipidemia. Bilateral petrosal sinus sampling under CRH stimulation resulted in 3:1 central to peripheral ACTH ratio, confirming CD. Her pituitary adenoma without extrasellar extension was successfully resected, and the patient achieved remission. The *CABLES1* alteration found in the patient was absent in her mother's DNA. As a sample from the father was not available, we cannot determine whether the variant was inherited or appeared *de novo*.

Compared with samples without *CABLES1* alterations, samples from Patients 1 to 4 displayed reduced CDKN1B cytoplasmic and, more importantly, nuclear expression by immunohistochemistry (Figure 3.2). No differences were found in the CABLES1 staining between samples with and without putative *CABLES1* mutations, although it was weaker in the tumors than in areas of non-tumoral pituitary (data not shown). This finding was not unexpected, as none of the samples displayed LOH for the variants identified, and points toward an alternative mechanism for reduced CABLES1 activity in the corticotroph cells.

4.3 Functional assessment of putative *CABLES1* mutations

The conditional tamoxifen-inducible chimeric ERtam- CABLES1 proteins inserted by retroviral transduction were expressed at similar levels among the different AtT- 20/D16v-F2 cell pools (Fig. 3A). As expected, [303], WT CABLES1 inhibited the growth of AtT 20/D16v-F2 cells, but not as much as treatment with the synthetic glucocorticoid dexamethasone (10−7 M) or their combination (Figure 3.3 B). This result was expected, as AtT-20/D16v-F2 cells express *CABLES1* in response to dexamethasone, but *CABLES1* is only one of a group of dexamethasone-controlled cell cycle regulators [303]. In contrast, p.E178K (Figure 3.3C), p.L240F (Figure 3.3D), p.G312D

(Figure 3.3E) and p.D463G (Figure 3.3F) CABLES1 mutants lost the ability to inhibit AtT- 20/D16v-F2 cell growth. In summary, all the CABLES1 variants identified in our patients affect a core property of CABLES1: its ability to inhibit cell growth in response to glucocorticoids.

5. DISCUSSION

The *CABLES1* gene has been recently identified as a key mediator of the regulatory feedback loop of glucocorticoids on the corticotroph cells [303]. We have identified a small, but not negligible number (2.2%, 4/182) of patients with *CABLES1* alterations. The four putative mutations identified in our patients displayed reduced ability to block corticotroph cell proliferation in response to dexamethasone stimulation, supporting a role for *CABLES1* as a regulator of the corticotroph cell growth. Ours is the first study to identify a human phenotype associated with *CABLES1* germline mutations.

The CABLES1 protein was originally described as an interacting partner and substrate of the cyclin-dependent kinase-3 (CDK3) [312, 313] . It plays an important role in neural development during embryogenesis, as inferred from *in vitro* studies and from the mouse and zebrafish models of gene inactivation [116, 314, 315]. *CABLES1* is highly expressed in the nucleus of proliferating cells, where it is tightly regulated throughout the cell cycle, peaking at mid-late G1 [312, 313, 316]. In addition to CDK3, CABLES1 is a substrate for other protein kinases, including CDK2, 14-3-3, AKT, CDK5 and ABL, also serving as an adaptor protein for the last two [116, 316] (Shi *et al.* 2015*b*). Another important function of this protein is to stabilize regulators of the cell cycle, such as CDKN1A (P21), CDK5R1 (P35) and TP63, preventing their degradation [116, 314, 317] (Shi *et al.* 2015*a*). CABLES1 also interacts with TP53 and TP73, triggering apoptosis [318].

The CABLES1 protein is widely expressed among tissues and is lost in a variety of human cancers (endometrial, ovarian, colorectal, lung and squamous cell carcinomas) [319]. *CABLES1* gene inactivation promotes cell proliferation and survival, as well as tumor formation in vitro and replicates the human neoplasms in mouse models [116, 316, 319, 320]. The tumor suppressor activity of CABLES1 is inhibited by 14-3-3 or AKT-mediated phosphorylation (Shi et al. 2015b). Interestingly, CABLES1 could provide a link between two important molecular mechanisms

disrupted in corticotropinomas: dysfunction of the CDK/cyclin-dependent cell cycle regulation and activation of the epidermal growth factor receptor (EGFR) pathway [321] [284].

The RAC-alpha serine/threonine-protein kinase (AKT1), one of the main effectors of EGFR, can inactivate CABLES1 by phosphorylation, neutralizing this way the regulatory effect of CABLES1 over the CDK/cyclin complexes [284] (Shi et al. 2015b). Although a characteristic phenotype cannot be inferred from such a small number of patients, we found some noteworthy features. The four patients had youngonset macroadenomas, in agreement with previous observations suggesting that germline mutations in pituitary adenoma-causative genes are relatively frequent among patients with such characteristics [299, 322]. The tumors in the two adults were particularly large and aggressive, suggesting that, perhaps, they had arisen years before their diagnoses.

Considering that our patients had sporadic disease presentation and given that corticotropinomas rarely course asymptomatic in the long term, we could assume an incomplete penetrance for *CABLES1*-associated CD, which could explain the low frequency of these cases. In this sense, it is important to emphasize that incomplete penetrance is a frequent finding among kindreds with pituitary adenoma-associated germline alterations [323, 324]. The study of large pedigrees with multiple cases of CD might be necessary to identify additional cases. However, the finding of multiple patients with CD in the same family is quite an infrequent phenotype: only 7.9% of the *AIP* mutation-negative FIPA families include CD, and only 1.4% of the families are 'homogeneous' for corticotropinomas [325]. On the other hand, as we could not establish the pattern of inheritance in three of the cases, *de novo* mutations cannot be confirmed or ruled out. A caveat of our study is that we could not rule out that the *CABLES1* mutations found in the adult patients were somatic defects, as germline DNA samples were not available from these cases. It is however noteworthy that the father of patient 3 and the mother of patient 4 carry the *CABLES1* mutations: this may suggest that *CABLES1* mutations may predispose to CD and potentially be associated with very rare cases of familial CD, with incomplete penetrance.

Another interesting feature is the absence of somatic *USP8* mutations in our patients. This is the most common genetic defect so far identified in corticotropinomas, being found in around 40% of the tumors [301, 302]. We have recently identified such mutations in one-third of our pediatric

CD patients [326]. The absence of this somatic defect somehow mirrors the absence of *GNAS1* mutations in patients with somatotropinomas due to *AIP* mutations, suggesting that the genetic defects causing tumors due to germline predisposition are different than those found in non-inherited pituitary adenomas [325].

The histopathological analysis of the samples revealed other common features among the four cases. CABLES1 expression ranged from low to moderate, but it was not completely absent in any of the tumors and was predominantly nuclear. However, the nuclear CDKN1B (P27) staining was particularly affected, compared with other corticotropinomas. Human germline *CDKN1B* mutations are associated with approximately 2% of the cases of *MEN1* mutation-negative multiple endocrine neoplasia [327]. Cases associated with *CDKN1B* mutations display a heterogeneous phenotype, referred to as MEN4, encompassing parathyroid and pituitary adenomas, neuroendocrine tumors and various benign and malignant tumors [328, 329]. Pituitary tumors have been reported in eight MEN4 patients so far, only one of them with CD [329-331]. However, *Cdkn1b*-knockout (KO) mice develop, among other phenotypic abnormalities, ACTH-secreting hyperplasia or adenomas of the pituitary *pars intermedia* with full penetrance [332-334].

More importantly, nuclear CDKN1B immunostaining is significantly reduced in pituitary adenomas of all types, particularly in corticotropinomas, in association with high Ki-67 expression, and is lost in pituitary carcinomas [335, 336]. This low expression is very likely not due to mutations or deletions, since these alterations have not been detected at the somatic level but caused by posttranslational events [337]. The best-characterized mechanism for this in CD is increased CDKN1B phosphorylation by cyclin E (upregulated in corticotropinomas), which inactivates the protein and triggers its degradation, although 14-3-3 and AKT-mediated phosphorylation have the same effect [112, 335, 338]. We observed that CDKN1B nuclear expression was particularly low in tumors with putative *CABLES1* mutations. As CABLES1 is known to stabilize and prevent the degradation of other cyclin-dependent kinases, we could speculate that our findings are due to increased CDKN1B degradation as a result of impaired CABLES1 function. This is in agreement with the rough correlation between CABLES1 and CDKN1B, but not cyclin E expression by

immunohistochemistry in CD patients [303]. Further experiments are required to confirm this mechanism, although they are beyond the scope of the present article.

We acknowledge the limitations of our study. Given that our centers are tertiary referral hospitals, aggressive or atypical cases of CD might be overrepresented in our cohorts. Although only a minority of the patients had macroadenomas, the frequency was higher than what is reported in the literature [339]. The adult CD cohort included only patients for whom tissue was available, which means macroadenomas would be overrepresented. While these selection biases might preclude any inference on the prevalence of *CABLES1* variants in the general population of patients with CD, it was probably the uniqueness of our cases, which allowed us to identify such uncommon genetic alterations. Also, we do not have a firm basis to explain why the loss of function of a 'generic' tumor suppressor such as *CABLES1* caused a pituitary-specific phenotype in our patients. We could speculate that the missense variants found in these cases might cause loss of crucial pituitary-specific protein– protein interactions, but further studies are required to better characterize the function of this tumor suppressor in the corticotroph cells.

Our results provide evidence for a role of *CABLES1* as a novel pituitary tumor suppressor. The function of the CABLES1 protein as a regulator of different elements of the molecular pathways that control the cell cycle progression and apoptosis links genes previously known to have a role in the pathogenesis of CD. A more detailed characterization of such regulatory networks will allow a better understanding of the genetic basis of corticotropinomas, providing grounds for the search of novel therapeutic targets.

6. CONCLUSIONS

We have identified four potentially pathogenic missense *CABLES1* variants as a novel, although infrequent, cause of CD in children and young adults. The CABLES1 protein functions as a glucocorticoid-responsive regulator of the effect of growth factor receptor activation on cell cycle control in the corticotroph cells. The putative *CABLES1* mutations found in our patients impair the ability of this tumor suppressor to control cell proliferation and might influence the function of CDKN1B by regulating its subcellular localization and/or degradation. Further studies are required to characterize in detail the function of this tumor suppressor in the corticotroph cells.

7. MATERIALS AND METHODS

7.1 Pediatric cushing's disease cohort

We studied 146 pediatric (<18 years at diagnosis) patients with CD who are part of a large cohort evaluated at the outpatient clinic and/or admitted for clinical work-up and treatment at the National Institutes of Health (NIH) Clinical Center between 1997 and 2017 and recruited under the research protocol 97-CH-0076 (ClinicalTrials.gov: NCT00001595). The Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Review Board approved this study, and informed assent/consent was obtained from all the patients and their parents/guardians. Clinical data were obtained directly from the patients and/or from the Clinical Research Information System. Parents and siblings of the patients were also recruited, when available.

For all the individuals, DNA was extracted either from a peripheral blood sample using the Maxwell 16 Blood DNA Purification Kit in a Maxwell 16 Instrument (Promega AS1015 and AS3050) or from saliva using the Oragene-Dx collection kit and the PrepIT-L2P DNA extraction kit (DNA Genotek OGD-500 and PT-L2P-45), according to the manufacturer's protocols. When available, stained histopathological sections from the corticotropinomas were retrieved from the Department of Pathology. After either manual delimitation of the tumor area or microdissection, DNA was extracted from unstained sections using the Pinpoint Slide DNA Isolation System (Zymo Research D3001). Screening for germline mutations in *AIP*, *CDKN1B*, *CDKN2C MEN1* and *PRKAR1A* in 74 of these patients, and *GPR101* in 34 of them, as well as somatic *GPR101* in 23 corticotropinoma DNA samples from these patients has been reported before [299, 340].

Germline DNA samples from 98 patients and tumor DNA samples from 28 of them were submitted for whole-exome sequencing (WES) at the University of Minnesota Genomics Center. Targeted capture libraries were generated using the Agilent QXT v5 + UTRs kit for both germline and tumor samples. The germline samples were sequenced on an Illumina HiSeq 2000 platform producing 100 bp paired-end reads, while the tumor samples were sequenced on a HiSeq 2500 platform producing 125 bp paired-end reads. FASTQ files were processed using the steps delineated in the Broad Institute's Genome Analysis Toolkit (GATK) best practices [341], including using BWA-MEM [342] for alignment, GATK for quality recalibration and indel realignment and

GATK HaplotypeCaller for genotyping. The median number of on-target reads generated per sample was 41 million, resulting in median target coverage of 54× (78% of targets covered at >20×). For *CABLES1*, the median percent of the 10 coding exons covered at 20× was 100% (average = 91%). The only exon with reduced coverage was exon 1, with 43% of samples having 20× coverage. ANNOVAR [343] was used to determine the effect of coding variants using both the RefSeq and UCSC gene sets, including putative amino acid changes, distance to intron–exon boundary, the creation or removal of a stop-codon and location within known non-coding RNAs. Nonsynonymous variants were annotated based on their computationally predicted deleteriousness using information from dbNSFP. All variants were annotated for their frequency and presence in multiple variant collections (e.g., dbSNP, 1000 Genomes and internal WES datasets totaling over 10,000 samples). Targeted bioinformatic analysis ruled out rare mutations (present in <1% of any variant collection) in other known pituitary adenoma-associated genes (*AIP*, *CDKN1B*, *GPR101*, *MEN1* and *PRKAR1A*). For all the samples, a manual check of the WES raw data for *CABLES1* was performed, using the Integrative Genomics Viewer 2.3.72 platform (Broad Institute) [344].

In addition, 48 other patients were screened for germline *CABLES1* variants by Sanger sequencing. The primers included in Supplementary Table 1 (see section on supplementary data given at the end of this article) were used to amplify the coding regions and exon–intron junctions by PCR (GoTaq Green Master Mix, Promega M7123) and for Sanger sequencing (BigDye Terminator 3.1 Cycle Sequencing Kit, Thermo Fisher Scientific 4337456). Sequences were analyzed using the SeqMan Pro 11.1.0 (DNASTAR) software. The Alamut Visual 2.9 software (Interactive Biosoftware) was used for the annotation, *in silico* prediction and to search the frequency in public databases of all the variants identified. Four algorithms (Align GVGD, PolyPhen-2, SIFT and Mutation Taster) were used for missense variants and five (Splice Site Finder, MaxEnt, NNSplice, GeneSplicer and Human Site Finder) were used for splicing variants. Variants were considered probably damaging or affecting splicing when the majority of the algorithms agreed; otherwise, they were considered variants of uncertain significance (VUS). Sanger sequencing confirmed all the variants identified by WES. Parents of the patients with *CABLES1* variants of interest were screened *ad hoc*, and loss of heterozygosity (LOH) was investigated in the corticotropinomas by Sanger sequencing.

7.2 Collection of corticotropinomas from adult patients

Fresh frozen corticotropinoma tissue samples were obtained from 35 adult patients from Cochin Hospital and Foch Hospital operated for CD between 2009 and 2016. The local ethical committee approved the study, and all the patients provided written informed consent. RNA was extracted using the RNeasy kit (QIAGEN), and then reverse-transcribed. All the samples were submitted for RNA sequencing (RNA-seq) at the genomic platform of Cochin Institute. The libraries were prepared following the TruSeq mRNA protocol (Illumina RS-122-2101), starting from 1 µg of high-quality total RNA. Paired-end (2 × 75 bp) sequencing was performed in an Illumina NextSeq 500 instrument. Reads were aligned using the STAR software [345]. Sequencing variants were annotated using ANNOVAR (Wang *et al.* 2010*a*) and then filtered for exonic nonsynonymous variants, with frequency <1% in the general population (based on the 1000 Genomes database) (The 1000 Genomes Project Consortium *et al.* 2015). Variants of interest were confirmed by Sanger sequencing in DNA extracted from representative sections of paraffin-embedded tissues, as described previously.

7.3 Copy number variation analysis

CNV analysis was performed in 116 germline and 29 corticotropinoma DNA samples using FAM-labeled assays binding *CABLES1* exon 1, intron 3–exon 4 and exon 10 (TaqMan CNV assays Hs07536236_cn, Hs02003953_cn, and Hs00413958_cn, respectively, Thermo Fisher Scientific) a VIC-labeled *RPP30* (*Rnase P*) assay (Thermo Fisher Scientific 4403326) as an internal control, and the ddPCR SuperMix for Probes (no dUTP) (Bio-Rad 1863024) in a QX200 Droplet Digital PCR System (Bio-Rad). Results were analyzed with the Quanta Soft software 1.7.4.0917 (Bio-Rad).

7.4 USP8 screening

For patients with potentially pathogenic *CABLES1* variants, the primers 5'-CTTCCACCCCTCCAACTCAT-3' _and 5'-TGGAGTTACTGTTGGCTTCCT-3' _were used to amplify and sequence a region of 146 bp covering the *USP8* mutational hotspot in corticotropinoma DNA, as described above. Patients with *USP8* mutations from our cohort have been reported elsewhere [301, 326].

7.5 Immunohistochemistry

In corticotropinomas from patients with putative CABLES1 mutations and in two corticotropinomas with no mutations, immunohistochemical staining for ACTH (1:1000 rabbit polyclonal anti-ACTH antibody, Abcam 74976), CABLES1 (1:1000 rabbit polyclonal Abcam ab75535) and CDKN1B (1:100 rabbit polyclonal Santa Cruz sc-528) was performed as follows: deparaffinization for 30 min in Histo-Clear (National Diagnostics HS-200), sequential 5-min washes with 100, 95, 70 and 50% ethanol, antigen retrieval with citratebased antigen unmasking solution (Vector H3300) for 20 min in a steamer, blocking for 1 h with 10% normal goat serum (Jackson ImmunoResearch Laboratories 005-000-121) in 1× PBS/0.1% Triton X-100, incubation with primary antibody overnight at 4°C, incubation with 1% H_2O_2 for 5 min, and then for 1 h with 1:1000 Biotin-SP AffiniPure Goat Anti-rabbit IgG and for 30 min with 1:500 Peroxidase Streptavidin (Jackson ImmunoResearch Laboratories 111-065-144 and 016- 030-084, respectively). Samples were developed by incubation for 1 min with ImmPACT DAB peroxidase (HRP) substrate (Vector SK-4105) and counterstained with Gill's hematoxylin I (American MasterTech Scientific HXGHE1PT) and Dako Bluing Buffer (Agilent Technologies CS70230-2). Sequential washes in a 50–100% ethanol gradient and Histo-Clear were done before mounting with Cytoseal XYL (Thermo Fisher Scientific 8312-4). Images were acquired using a Leica DMRX optical microscope, attached to an Olympus DP72 camera, and processed with the CellSens Dimension 1.6 software (Olympus).

7.6 Expression plasmids

The mouse *Cables1* cDNA (variant 1: NM_001146287.1) was obtained from IDT and inserted downstream of the tamoxifen-inducible form of the estrogen receptor (ERtam) ligand-binding domain into a 3xFlag-tagged retroviral expression vector derived from pLNCX2 [346]. Mutagenic primers were used to introduce the *Cables1* point mutations p.E139K (p.E178K in human, 5'-GCCACGAGTCCTCGGGAAACCCTCACAACCAC-3'_and 5'-GTGGTTGTGAGGGTTTCCCGAGGACTCGTGGC-3'), p.L201F (p.L240F in human, 5'-GGGTCAAGGGG-TAGATTTAATTCCTTTACTCAGGG-3' _and 5'- CCCTGAGTAAAGGAATTAAATCTACCCCTTGACCC-3'), p.G273D (p.G312D in human, 5'-GTCGAACACTTTCAGATTCTCCTAGACCAAAG-3' _and 5'-

CTTTGGTCTAGGAGAATCTGAAAGTGTTCGAC-3') and p.D424G (p.D463G in human, 5'-GACCCAAACCTCCTGGGTGACCCCCAGTGGCC-3'_and_5'-GGCCACTGGGGGTCACCCAGGAGGTTTGGGTC-3') in the plasmid, using the QuikChange site-directed mutagenesis kit (Agilent Technologies 200519) and the KOD hotstart DNA polymerase (Millipore 71086). The vectors were transfected into EcoPackTM 2-293 cells (Clontech 631506) to produce the viruses, which were then used to transduce AtT-20/ D16v-F2 cells (ATCC CRL-1795).

7.7 Cell culture, transfections and growth curves

The transformed AtT-20/D16v-F2 cells were grown in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum and antibiotics. Pools of at least 1×10^3 stable cell colonies were obtained as described [295] for pLNCX2-3xFlag-ERtam-Cables1 WT, pLNCX2-3xFlag-ERtam-Cables1 p.E178K, pLNCX2- 3xFlag-ERtam-Cables1 p.L240F, pLNCX2-3xFlag-ERtam- Cables1 p.G312D, pLNCX2-Flag-ERtam-Cables1 p.D463G or pLNCX2-3xFlag-ERtam empty vector as control. Cables1 protein levels were assessed by Western blot using 1 μg mouse monoclonal anti-Flag (SIGMA F1804) antibody. For growth curves, 7×10^5 cells per well were plated in 6-well plates and treated in duplicate with 400 nM tamoxifen (SIGMA T5648), 10^{-7} M dexamethasone (SIGMA D4902), both or vehicle (100% ethanol). Viable cells were counted every 12–18 h for 4 days, starting 4 h after plating, and medium was replaced with fresh medium at every time point.

7.8 Statistical analyses

All the analyses were carried out using the GraphPad Prism 7.0 software (GraphPad Software). Parametric data are presented as mean ± _standard deviation (s.d.) and non-parametric data are presented as median ± _interquartile range (IQR). Gene variant frequencies in the study population were compared with the frequencies reported in public databases using the Fisher's exact test. For the cell growth experiment with WT CABLES1, cell counts at each time point were compared among experimental conditions using one-way ANOVA with Dunnett correction for multiple comparisons. For the rest of the experiments, cell counts at different time points were compared between ethanol and tamoxifen treatments using multiple *t* tests, with Holm–Sidak correction for multiple comparisons. Results were considered statistically significant when $P < 0.05$.

8. ACKNOWLEDGEMENTS

A

c.532G>A   c.718C>T   c.935G>A   c.1388A>G

NM_001100619.2 {

NM_138375.2 {

NM_001256438.1 {

B

p.E178K p.L240F p.G312D   p.D463G

S168   S287 S313   T415   Cyclin-like domain (478-617)

Interaction with TDRD7 (1-109)   Interaction with CDK3 (179-492)

C

p.E178K

Homo sapiens	Q8TDN4	166	FASPLGAGRASGEDWQPPRPAPLAACAQLQLLDG
Pan troglodytes	H2QEC2	165	FASPLGAGRASGEDWQPPRPAPLAACAQLQLLDG
Callithrix jacchus	F7I2D5	143	PGSPLGAGRTSGEDWQLPRSAPFAASAQSQLKDG
Canis lupus familiaris	F1P9I2	164	FLSPLGAGRASGEDPQPPRPAPPASCAPPQLPDA
Equus caballus	F6TB29	110	FSSSLGAGRASGEDWPLPRPAPIAACAQPQQPDG
Sus scrofa	F1SBB7	158	-------PRASGEDLQLPRPALLAACGQPQLPNG
Mus musculus	Q3U148	133	------VPRVLGEPSQPPRSAPAVTGAQLQLPDG
Rattus norvegicus	F1LUR9	82	------VPRVLGEPSQPPRSAPAVTGAQLQLPDG
Bos taurus	F1MZY0	1	---------------------TGVQPQPPDG
Danio rerio	A0A0R4IWM8	88	FMAGTVATSA--AAAAPPPPD----------PDG

p.G312D

Homo sapiens	Q8TDN4	286	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Pan troglodytes	H2QEC2	285	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Callithrix jacchus	F7I2D5	263	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Canis lupus familiaris	F1P9I2	284	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Equus caballus	F6TB29	230	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Sus scrofa	F1SBB7	241	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Mus musculus	Q3U148	247	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Rattus norvegicus	F1LUR9	196	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPENF
Bos taurus	F1MZY0	97	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKNF
Danio rerio	A0A0R4IWM8	176	ISQRSSLETLEDIEENAPLRRCRTLSGSPRPKSF

p.L240F

Homo sapiens	Q8TDN4	226	GSGTPGSGSGSRGRLNSFTQGILPIAFSRPTSQN
Pan troglodytes	H2QEC2	225	SSGTPGSGSGSRGRLNSFTQGILPIAFSRPTSQN
Callithrix jacchus	F7I2D5	203	GSGTPGSGSGSRGRLNSFTQGILPIAFSRPTSQT
Canis lupus familiaris	F1P9I2	224	GSGTPGSGSGSRGRLNSFTQGILPIAFARPTSQN
Equus caballus	F6TB29	170	GSGTPGSGGGSRSRLNSFTQGILPIAFSRQTSQN
Sus scrofa	F1SBB7	195	--------------XNSFTQGILPIAFSRQTSQN
Mus musculus	Q3U148	187	GSGTPGSTSGSRGRLNSFTQGILPIAFSRQNSQN
Rattus norvegicus	F1LUR9	136	GSGTPGSASGSRGRLNSFTQGILPIAFSRQNSQN
Bos taurus	F1MZY0	37	SSGTPGGGSGSRGRLNSFTQGILPIAFSRQTSQN
Danio rerio	A0A0R4IWM8	119	SSPFSAVPPATRGRLQTYTQGILPVSYARQSSQN

p.D463G

Homo sapiens	Q8TDN4	463	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Pan troglodytes	H2QEC2	462	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Callithrix jacchus	F7I2D5	440	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Canis lupus familiaris	F1P9I2	461	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Equus caballus	F6TB29	407	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Sus scrofa	F1SBB7	418	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Mus musculus	Q3U148	424	DDPQWPCGKHKRVLTFPSYMTTVIDYVKPSDLKK
Rattus norvegicus	F1LUR9	347	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Bos taurus	F1MZY0	248	DDPQWPCGKHKRVLIFPSYMTTVIDYVKPSDLKK
Danio rerio	A0A0R4IWM8	355	DDPQWPCGKHKRVLIFPSYMTTVIEYVKPSDLKK

Figure 3.1: Missense CABLES1 variants of interest: effects on CABLES1 transcripts and proteins

(A) Three transcripts of CABLES1, NM_001100619.2, NM_138375.2 and NM_001256438.1, which differ in their first exon, are translated into proteins of 633, 368 and 306 amino acids, respectively. The former (NM_001100619.2,) is the 'canonical' sequence (UniProt Q8TDN4-1). For each transcript, mRNA is schematized in blue and the coding sequence in green. The CABLES1 variants identified in Patients 1 and 2 (p.E178K and p.L240F), respectively, affect exon 1 of the reference transcript, but not the other transcripts. The variant detected in Patient 3 is located in exon 3 (p.G312D) and the variant from Patient 4 (p.D463G) affects exon 7. There is no information in the literature about the expression of these transcripts in the pituitary gland. (B) The domains of the CABLES1 protein are incompletely characterized and no 3-D structure is available that represents the majority of the protein sequence. The 633 amino acid (65.7 kDa) isoform of the protein contains an N-terminal site for interaction with TDRD7 and a central large sequence necessary for interacting with CDK3. Four residues are targets for phosphorylation: S168, S287, S313 and T415 (Shi et al. 2015, Uniprot Consortium 2015)[313]. The C-terminal end of the protein contains a cyclin-like domain, and for this reason, CABLES1 has been included in the cyclin superfamily, although it differs in functions with cyclins. The alterations found in Patients 1–4 are included in

(p.L240F, p.G312D, p.D463G) or very close to (p.E178K) the motif that interacts with CDK3. (C) The CABLES1 gene is highly conserved among species, and the variants found in our patients affect relatively (p.E178K, p.L240F) and highly (p.G312D, p.D463G) conserved residues.

| | **ACTH** | **CABLES1** | **CDKN1B** |

Control, Patient 1 (p.E178K), Patient 2 (p.L240F), Patient 3 (p.G312D), Patient 4 (p.D463G)
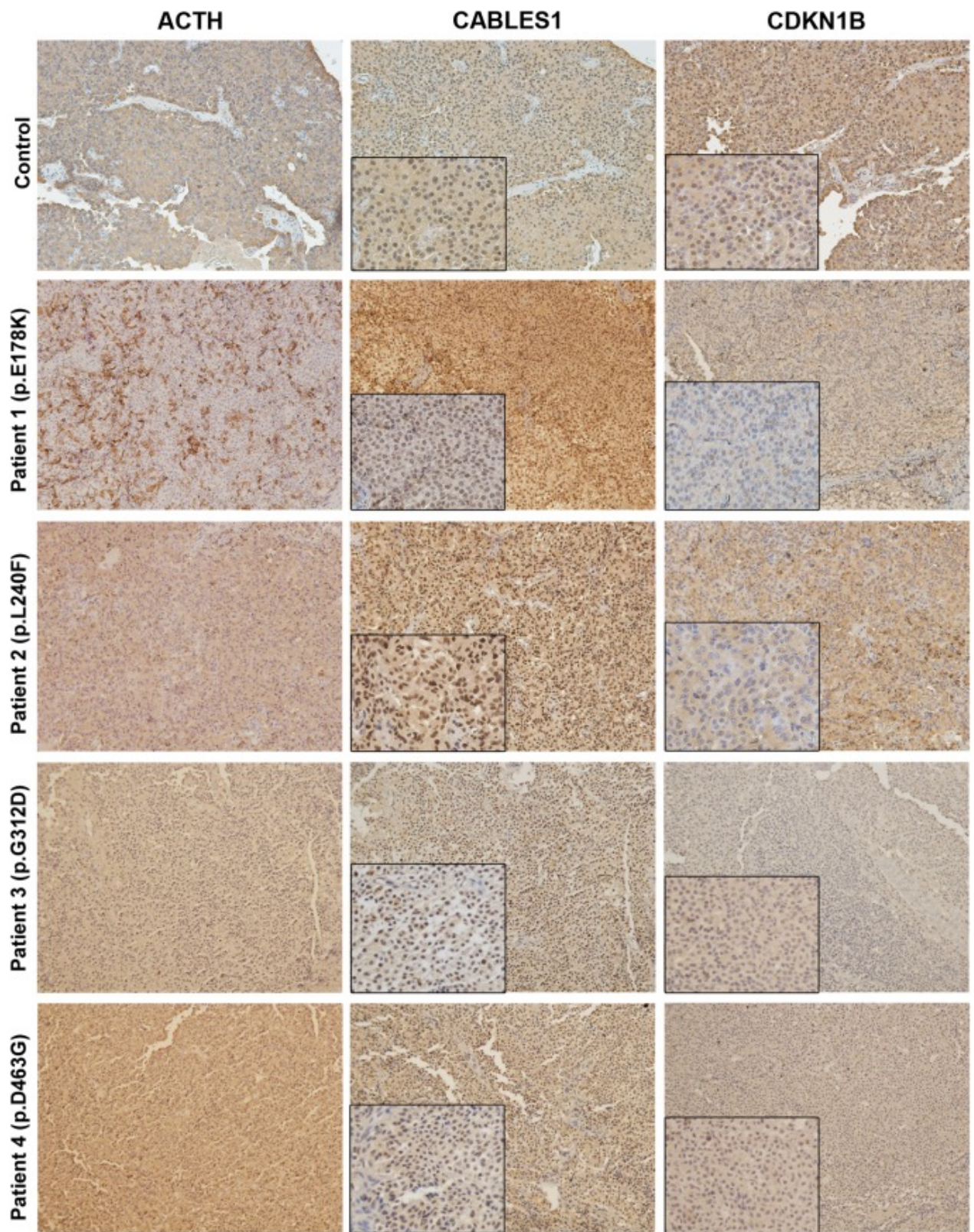
Figure 3.2: ACTH, CABLES1 and CDKN1B expression in corticotropinomas

We compared ACTH, CABLES1 and CDKN1B immunostaining in samples from the four patients with putative CABLES1 mutations with samples from two patients negative for such alterations (one representative example is presented). ACTH immunostaining was observed in the vast majority of the cells in all the cases, except for Patient 1, for whom only 50% of the cells were immunoreactive (immunoreactivity for other pituitary hormones was ruled out). All the samples displayed positive CABLES1 cytoplasmic and, predominantly, nuclear immunoreactivity. In specimens that contained areas of non-tumoral pituitary, reduced CABLES1 staining was observed in the corticotropinomas, compared with the surrounding tissue (Supplementary Fig. 1), in concordance with previous data (Roussel-Gervais et al. 2016). Nevertheless, we did not observe differences in the CABLES1 immunostaining between samples with and without putative CABLES1 mutations. In contrast, while in controls CDKN1B staining was moderately intense in the cytoplasm of the great majority of the cells and in the nucleus of 50–60% of them, only weak cytoplasmic staining and very few cells with nuclear staining were observed in the cases with putative CABLES1 mutations (<10% cells with nuclear staining for Patients 1 and 2 and 30–40% cells with weak nuclear staining for Patients 3 and 4). Magnification in all the images: 10×, inserts: 20×.
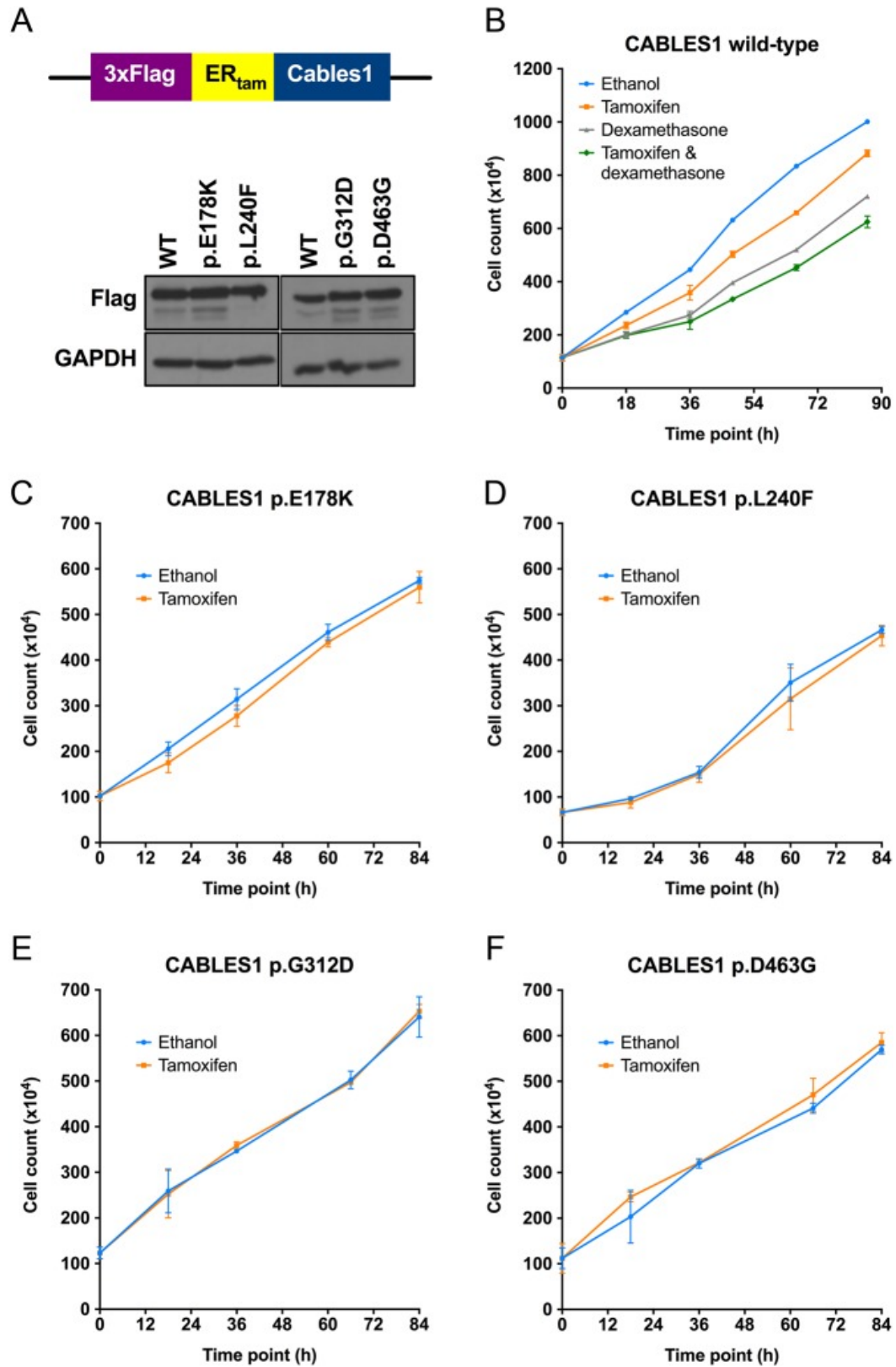
Figure 3.3: CABLES1 mutant proteins have lost their growth inhibition activity

(A) Schematic representation of tamoxifen-inducible chimeric CABLES1 proteins fused to the ERtam ligand-binding domain. Upon stable transduction of AtT-20 cells, wild-type (WT) and mutant ERtam-CABLES1 proteins are expressed at similar levels as revealed by Western blot against the Flag epitope. (B) Growth curves of AtT20/D16v-F2 cells expressing WT ERtam-CABLES1 treated with vehicle (ethanol), tamoxifen, dexamethasone or both, as indicated. All the conditions rendered statistically significant differences in cell counts at all time points. To analyze exclusively the effect of CABLES1 on cell growth, only the tamoxifen treatment was used for experiments with the mutant proteins. (C, D, E and F) Growth curves for AtT20/D16v-F2 cells expressing CABLES1 p.E178K (C), p.L240F (D), p.G312D (E) or p.D463G (F) mutant protein in vehicle and tamoxifen-treated cells. There were no statistically significant differences in cell count at the different time points, meaning that these variants have lost the ability to suppress cell growth.

Table 3.1: Potentially pathogenic CABLES1 variants identified in patients with Cushing's disease

Patient ID (race/ethnicity)	DNA change (Ref Seq GRCH37/hg19: NM_001100619.2)	Protein change	dbSNP ID	Location in gene	Variant type	MAF among subjects in this study (%)[a]	Control MAF (%)				P value				In silico prediction
							ExAC[b]	gnomAD[b]	1000 genomes	NHLBI EVS	MAF vs ExAC	MAF vs gnomAD	MAF vs 1000 genomes	MAF vs NHLBI EVS	
Patient 1 (Caucasian)	c.532G > A	p.E178K	rs200098768	Exon 1	Missense	0.2762	2.8436	0.9898	0.2796	n/a	0.0043[c]	ns	ns	n/a	Benign
Patient 2 (Caucasian)	c.718C > T	p.L240F	rs79793507	Exon 1	Missense	0.2762	0.0825	0.0783	0.2995	0.2799	ns	ns	ns	ns	Probably damaging
Patient 3[d] (Hispanic)	c.935G > A	p.G312D	rs774334448	Exon 3	Missense	0.2762	0.0083 (0.0779)	0.0061 (0.0417)	n/a	n/a	0.0324 (ns)	0.0232 (ns)	n/a	n/a	VUS
Patient 4 (Hispanic)	c.1388A > G	p.D463G	NA	Exon 7	Missense	0.2762	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Probably damaging

MAF, minor allele frequency; n/a, not available; ns, not significant; VUS, variant of uncertain significance.

aMAF among the screened patients. bThe frequency of variants located in the 5' of the gene might not be accurate in the ExAC and gnomAD databases, as that region is poorly covered in those datasets. cMore common in ExAC than in our dataset. dPopulation-specific MAF and P value of comparisons provided in parenthesis, when available.

Table 2.2 : Patients with putative CABLES1 mutations.

Patient ID	Cohort	CABLES1 variant	Gender	Origin and ethnicity	Clinical presentation	Age at disease onset	Age at diagnosis	Tumor size (mm)	Ki-67 IHC (%)	Treatment	Disease status
Patient 1	Adult CD	c.532G > A (p.E178K), heterozygous	F	French (Caucasian)	NFPA (silent corticotroph adenoma)	27	27	25	4	TSS (×2), RT	Stable residual tumor
Patient 2	Adult CD	c.718C > T (p.L240F), heterozygous	F	French (Caucasian)	Cushing's disease	32	32	15	5	TSS	Unknown
Patient 3	Pediatric CD	c.935G > A (p.G312D), heterozygous (no LOH in tumor)	F	Guatemalan (Hispanic)	Cushing's disease	15	16	19 × 11 × 14	>5	TSS (×2)	Active
Patient 4	Pediatric CD	c.1388A > G (p.D463G), heterozygous (no LOH in tumor)	F	Mexican-American (Hispanic)	Cushing's disease	8	10	10 × 3 × 12	n/a	TSS	Remission

IHC, immunohistochemistry; n/a, not available; NFPA, non-functioning pituitary adenoma; RT, radiotherapy; TSS, transsphenoidal surgery.

CHAPTER 4 – DISCUSSION

Cushing's disease is a serious hormonal disorder that can cause mortality if it is not treated. Transsphenoidal or transnasal surgery is the first line of treatment, but it is not always successful, and patient may need another surgery, radiotherapy, adrenalectomy, or medical treatment which is not always safe nor efficient in many cases [90]. For this reason, understanding the pathogenic mechanisms of CD is crucial to discover therapeutic targets.

1. MODELS OF CD

Generating mice model of CD is an important tool to understand the disease and to develop therapeutic targets to treat it, but until now there is only a few animal models that recapitulate human CD pathogenesis.

CRH transgenic mice model were generated in order to test whether the overproduction of CRH may lead to Cushing's syndrome. These mice showed Cushing phenotype such as elevated ACTH and GC overproduction but they don't develop pituitary tumors [347]. Also, EGFR transgenic mice model were created, and they recapitulate features of human CD. The authors of this study used a specific POMC promoter to direct expression of EGFR in POMC cells of the AL of pituitary. They modified the rPOMC promoter by putting multiple copies of Tpit and NeuroD1 binding sites at the 5' end of the 480 bp promoter to enhance corticotroph specificity. Results showed 65% of mice developed pituitary tumors by 8 months, but in addition to the tumors with AL origin there were tumors with IL origin [92, 127].

Heterozygotic mice Rb1+/− knock-out models were created and found to develop pituitary tumors only in the IL with no Cushing phenotype [348] . In our work, we tried to generate mouse models of CD by knockout of GR from POMC expressing cells, but we did not observe any tumor mass or hyperplasia despite that the mice were between 12 and 14 months. This means that resistance to GC is not the first step that leads to corticotroph tumor formation (data in preparation). The only models until now that have transcriptional components similar to human corticotroph tumors are the corticotroph tumor AtT20 cells and the transgenic zebrafish expressing Pttg under the POMC promoter control [349, 350]. Transgenic zebrafish with Pttg overexpression develop corticotroph tumors with partial GC resistance and other characteristic

of human corticotroph adenomas [321]. Recently, a group has developed a human ACTH-secreting corticotroph tumoroid model, they characterize the cells that derived from fresh surgically resected human pituitary corticotroph tumor tissues by single cell RNA-sequencing and then they defined different culture nutriments necessary to maintain ACTH secretion for up to 4 months in vitro [351]. These human corticotroph cell model can be used to identify new molecular targets and to test different therapeutics.

2. RESPONSE OF CORTICOTROPH CELLS TO DIFFERENT HORMONES AND SIGNALING PATHWAYS

POMC transcription is under the control of different hormones and signaling pathways. Any disorder in this control may lead to tumor formation.

2.1 Cytokines and POMC transcription

The HPA axis is under the control of neuroendocrine hormones and cytokines that constitute the immune-neuroendocrine connection. The cytokines are released in response to immune stress such as infection and inflammation. Cytokines were shown to stimulate ACTH production through CRH secretion or by acting directly on corticotroph cells to activate POMC transcription [352, 353]. Cytokines such as LIF and IL6 are the most studied. These cytokines activate POMC transcription through activation of the STAT3 transcription factor that has two binding sites on the POMC promoter, one at −380 bp and the other at −150 bp (Figure 1.5B). Our detailed mapping of LIF and IL6 response on POMC promoter showed that both distal and proximal domains of the promoter are responsive, and we found that the central domain is also responsive to both LIF and IL6. This suggests the implication of other factors in addition to STAT3 for activation of POMC promoter transcription (Figure 2.3A). We know that in the central domain there is a binding motif for Ets transcription factors such as ETV1 and ETV5. Some studies showed that the downregulation of ETV1 resulted in the inhibition of IL6/STAT3 pathway and that ETV1 overexpression leads to the stimulation of this pathway [354]. Moreover, the knockdown of ETV5 induces expression of genes

associated with inflammation response and IL-6 production in macrophages [355]. Such interactions may be present at the POMC promoter.

2.2 EGFR pathway and POMC transcription

EGF is expressed in the human anterior pituitary gland. EGFR was found to be expressed in human corticotroph cells since the use of EGFR inhibitor RG-13022 decreased the percentage of BrdU labeled cells immunoreactive for ACTH [126, 356]. A disorder in EGFR expression can leads to tumor formation. EGFR may accumulate in corticotroph adenomas as a result of somatic mutations in the *USP8* gene that enhance its deubiquitinating activity. All USP8 mutations identified are clustered within the 14-3-3 binding motif, inhibiting thus 14-3-3 binding and increase deubiquitination activity of USP8 toward its cellular substrate EGFR. Corticotroph adenomas with USP8 mutations have persistent EGF signaling [301, 302].

It was shown in many studies that EGFR activates POMC transcription. All studies show that the MAPK pathway or the most pathway activated in corticotroph adenomas [266, 284]. In our studies we found that the MAPK and the AKT pathway substrates (pp42/44 and pAKT respectively) are expressed and activated but they are not a functional component of EGF action to induce POMC transcription since the block of these two pathways by specific inhibitors did not disrupt EGF stimulation of POMC promoter activity (Figure 2.1 A-D) . Indeed, we showed that the JAK/STAT3 pathway is the crucial pathway to activate POMC promoter transcription in AtT20 cells overexpressing EGFR. Inhibition of the STAT3 pathway by specific STAT3 inhibitor abrogate EGF-induction of POMC promoter indicating that STAT3 is a mediator of EGF action. Activation of the POMC promoter by STAT3 after EGF stimulation is exerted only by the SBE of the proximal domain (-150bp site) (Figure 2.2 A). However, we observed by ChIP that STAT3 can bind both the proximal and the distal sites of POMC promoter equally well (Figure 2.5 A). This means that STAT3 activity in the distal domain is blocked by another factor. We suggest that an EGF-dependent targets may be recruited and interfere with STAT3 activity. The transcriptome analysis of AtT-EGFR cells treated with EGF for 18h reveals that EGF activates transcription of bHLH factors such as Hes1. Hes1 can regulate transcription either by active repression through direct binding to N-box motif or by passive repression through binding to activator bHLH transcription factors and sequesters

the proteins away from normal homo-and heterodimeric partner making them non-functional. On the POMC promoter very close to the SBE distal there is a binding motif for NeuroD1, thus we suggest that Hes1 can bind to NeuroD1 and as a result it blocks the activity of the complex composed by STAT3, NeuroD1, NGFIB, and GR, since all these factors bind very close motifs. This hypothesis needs to be assessed.

The role of STAT3 in tumorigenesis was widely studied but little is known about the role of STAT3 in pituitary adenomas. In somatotroph adenomas, STAT3 causes GH hypersecretion by binding to GH promoter. Treatment with STAT3 inhibitors reduced GH secretion and attenuated somatotroph tumor growth in rat xenograft models [357]. This study showed that GH enhances STAT3 activity which in turn stimulates GH production indicating a positive feedback loop between STAT3 and GH in somatotroph tumors [357]. In addition, a mutation in the fibroblast growth factor receptor 4 (FGFR4) is accompanied by enhanced STAT3 activation in AtT20 cells, increased POMC expression with relative resistance to dexamethasone inhibition through diminished GR phosphorylation and nuclear translocation [358].

3. STAT3 AND GLUCOCORTICOID RESISTANCE

During inflammatory responses, IL6 has a synergistic effect with GC signaling. IL6 activates STAT3, and STAT3 may interact with GR to act through either an IL-6-responsive element or a GC-responsive element [359]. In primary human B cells and in a B cell line, GC induce IL10 through inducing STAT3 binding to IL10 promoter leading to gene expression [360]. It was shown that inducing B cells by GC led to up-regulation of IL10. GC activate IL10 transcription not via binding to its GRE motif but via recruitment of STAT3 to its site on IL10 promoter. This result was confirmed by using trimeric STAT-motif and by over-expressing of dominant-negative STAT3 that blocks GC induction of IL10 transcription [360] .

 In the case of the POMC promoter, our laboratory has identified a trans-repression mechanism between GR and NGFI-B that account for GR inhibition of POMC transcription. GR recruitment to the POMC promoter requires BRG1 which, once recruited, forms a complex with NGFI-B and HDAC2 leading to the deacetylation of the POMC promoter on histone H4 [69]. In the present work, we found an alteration of the tranrepression mechanism in AtT-EGFR induced by EGF. We

found that the POMC promoter becomes resistant to DEX after EGF treatment, in contrast with the antagonism between DEX and CRH. The question that we asked here is: is STAT3 responsible for the unresponsiveness of the POMC promoter to DEX. To answer this question, we first assessed whether IL6 and LIF mimic the effect of EGF and cause POMC resistance to DEX since both of these signaling pathways activate POMC transcription via STAT3. We found that following IL6 and LIF activation, the POMC promoter becomes unresponsive to DEX, supporting the model that STAT3 may cause promoter resistance.

In order to better comprehend the mechanism of resistance by STAT3, we tried to identify which phosphorylated form of STAT3 is expressed in AtT-EGFR cells after stimulation with EGF, LIF, and IL6. We showed that pSer727-STAT3 is induced equally by the three signaling pathway in contrast with the pTyr705-STAT3 that is increased mostly by LIF.

We know that STAT3 can bind DNA as homodimers or heterodimers (with STAT1 or STAT5) and activate POMC promoter transcription in LIF-inducing conditions [41, 361]. For this reason, we assessed the expression of activated forms of STAT1 (pTyr701-STAT1 and pSer727-STAT1) after stimulation with EGF, LIF, and IL6. We found that STAT1 is activated strongly by EGF (pTyr701-STAT1 and pSer727-STAT1) weakly by LIF and not by IL6. This result lead us to make the hypothesis that STAT3 may heterodimerize with STAT1, bind to the distal domain of POMC promoter, but not to the proximal domain, and the complex STAT3-STAT1 heterodimer enabling activation of POMC transcription, in contrast to the proximal domain where STAT3 binds as homodimer (this hypothesis needs to be validated). STAT1 was recently proposed to oppose the STAT3 tumorigenic actions [362]. The fact that is activated strongly with EGF may suggest that STAT1 has compensatory role on STAT3 activity in AtT-EGFR cells. Using the simple SBE luciferase reporter in transfection experiments, we found that EGF stimulation is enhanced by STAT3 but not by STAT1 (Figure 2.3G) whereas LIF stimulation is further enhanced by both STATs (Figure 2 3H): this suggests that STAT3-containing dimers (homodimers or heterodimers with STAT1) are active but that the STAT1 homodimers formed after EGF stimulation are not, supporting the idea that STAT3-STAT1 heterodimer inhibit activation of POMC transcription. This observation is supported by the ChIPqPCR data for STAT3 and STAT1 recruitment on POMC promoter showing that the two

STATs are recruited approximelty at the same positions but with different level (Figure 2.5B and C) .

With respect to the mechanism of resistance, our results indicate that STAT3 is the common candidate to block GC action and cause unresponsiveness of the POMC promoter since it is the common protein activated by the three signaling pathways.

To better understand the role of STAT3 in GC resistance, we transfected AtT-EGFR cells with increasing doses of STAT3. We observed that upon stimulation with EGF, POMC promoter activity is dose-dependently increased by STAT3 and is always resistant to Dex (Figure 2.4E). This means that either GR cannot bind any more to the POMC promoter or that STAT3 affects the complex of GR transrepression. The question thus is: is STAT3 binding mutually exclusive with GR binding to the POMC promoter. We transfected AtT-EGFR cells with either increasing doses of GR and found that they compete each other's action (Figure 2.4 E and F). We initially thought STAT3 may prevent GR binding to its transrepression complex causing the unresponsiveness of POMC promoter, but by ChIP-qPCR we demonstrated that under EGF conditions, the binding of GR is not affected (Figure 1-Annex), similarly the results of the ChIP-seq under LIF condition also showed that GR is able to bind POMC promoter in presence of STAT3 (Figure 2.5A). It thus appears that the POMC promoter presents with a unique environment within which STAT3 activation prevents the action of GR in contrast to the interaction between GR and STAT3 that has been previously described in which the STAT3 tethering to DNA bound GR results in synergistic activation of transcription [68].

In order to better understand the mechanism of STAT3 action, we generated mutant constructs of the STAT3 binding motif in POMC promoter either in the proximal domain, the distal domain or both. The results showed that the STAT3 binding sites are not required to have resistance to GC since the POMC promoter construct with double mutations of the SBEs is not activated by EGF but also is not repressed by DEX (Figure 2.5E). We think that the presence of STAT3 itself is responsible the GC resistance. To assess this hypothesis, we treat cells with a JAK1/2 inhibitor. The JAK inhibitor inhibit phosphorylation of STAT3 and thus, STAT3 cannot dimerize and cannot be active. The result was very interesting: with JAK1/2 inhibition, we restore the repression by Gc that was blocked by STAT3 (Figure 2.5F).

The question then is: how can the active form of STAT3 cause resistance of the POMC promoter to Gc?

In EGF conditions, GR can bind the POMC promoter, so the one possibility is that pSTAT3 interferes between GR and its trans-repression complex which may mean that it blokes HDAC2 recruitment. The hallmark of GR action is the recruitment of HDAC2 to the promoter resulting in decreased histone acetylation. Like other promoters, the activity of the POMC promoter is associated with histone H4 acetylation [69, 363]. We therefore assessed the level of acetylated histone H4 over the POMC promoter by ChIP qPCR. In preliminary results, we observed that DEX does not reduce acetylated H4 in presence of EGF. Again, these data highlight the essential role of EGF/STAT3 in causing resistance of POMC promoter to GC.

STAT3 thus may be an interesting therapeutic candidate for CD and JAK inhibitor may have a potentially useful role for corticotroph adenomas treatment. Many STAT3 inhibitors were described and they are currently in use. STAT3 is largely believed to be one of the key oncogenes and crucial therapeutic targets. Many cancers or many primary human cancer tissues present with overexpressed and/or constitutively active STAT3. Further, STAT3 was found to be responsible for treatment resistance or chemoradiotherapy resistance [364]. This highlights the necessity to develop and use small-molecule inhibitors that target STAT3 in order to overcome drug resistance.

4. MISEXPRESSION OF STAT3 IN CUSHING DISEASE

Pituitary corticotroph adenomas are resistant to Gc feedback and secrets excessive levels of ACTH resulting in hypercortisolism [365]. All CD patients do not response to low dose of DEX, and a subset of patients are completely resistant to DEX even at high dose. Due to the importance of STAT3 in Gc resistance, we assessed the expression of pSTAT3 in a subset of human corticotroph adenomas samples. We found that STAT3 is overexpressed in 50% of cases (Figure 2.6). This finding suggests that STAT3 can explain in part the mechanism of Gc resistance is some CD cases, but also the potential involvement of other mechanisms in the other 50% of cases such as downregulation of BRG1 and HDAC2, two regulators of chromatin remodeling, and part of the GR trans-repression complex [69]. Other mechanisms have also been proposed previously for the

loss of GC feedback, including the loss of heterozygosity (LOH) at the GR gene locus, the increased level of 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), the enzyme converting cortisol to the inactive cortisone [366-368].

The overexpression of STAT3 in corticotroph adenomas can be caused by mutations in the USP8 genes causing EGFR accumulation and persistent activation of the EGFR pathway, but it can also be associated with other mechanism such us high levels of LIF or IL6. In our panel of patients, we did not assess expression of pEGFR and only 25% of our CD series have *USP8* mutation.

Taken collectively, our data lead to the proposal of mechanisms (Figure 2.7) for POMC response to EGF and for GR resistance. In this model, EGF/EGFR activate the STAT3 pathway, leading to STAT3 recruitment to POMC promoter. This causes alteration in the GR trans-repression mechanism associated with blockade of HDAC2 action. STAT3 inhibitors are thus a promising new therapy for the treatment of CD: The STAT3 inhibitor used in our work show inhibition of AtT-EGFR cells induced proliferation with EGF (Figure 1-Annex).

5. CELL CYCLE PROTEINS AND CORTICOTROPH TUMORS

Cell cycle progression is under the control of different CDKs. Progression during G1 depend on cyclin D (D1, D2 and D3)-CDK4/6. Transition from G1 to S require the CDK2-cyclin E (E1 and E2) complex formation. Cyclins A and B activate CDK1 and CDK2 at the end of S and during G2. Cell cycle progression is under the control of negative regulators, the cyclin-dependent kinase inhibitors (cdki's): INK4 and Cip/Kip families. The INK4 family include p16, p15, p18, and p19 that inhibit the cyclin D-cdk4/6 complexes, whereas the Cip/Kip family include p21, p27, and p57 that inhibit the cyclinE-CDK2 complex [369, 370].

Corticotroph cells are the first pituitary lineage to reach terminal differentiation by expression specific transcription factors such as Tpit [16]. During normal pituitary development, cell cycle proteins cyclin E, p27, Rb, and E2F1 are expressed at different level in embryonic and/or adult cells or in corticotroph adenoma. The pituitary is very sensitive to genetic disorder of cell cycle regulators such as RB, p27, or p18 and this appears implicated in pituitary tumorigenesis. Mice with RB, p27, or p18 ablation develop IL tumor with complete penetrance [113, 371-374].

Corticotroph adenomas are responsible for CD. These adenomas are unresponsive to GC. The negative feedback of GC is critical to inhibit corticotroph cells proliferation since GC enhances the expression of the negative cell cycle regulator CABLES1 [304].

In this work, we identified mutations in the CABLES1 gene of CD patients. These alterations were found in 2.2% (4/181) of cases and they are loss-of-function mutations since they inhibit CABLES1's ability to block corticotroph cell proliferation in response to dexamethasone stimulation (Figure 3.3).

Expression of CABLES1 is reduced in ~55% of corticotrope adenomas and this is strongly correlated with the loss of p27Kip1 expression [115]. CABLES1 expression is lost in 60-70% of sporadic human colorectal cancers. Also, human endometrial cancer samples exhibit marked decreases in CABLES1 mRNA and Cables1–/– mice develop endometrial cancer [375]. Human non–small cell lung cancer show loss of CABLES1 expression in 45% of cases [116, 306, 316, 319, 320]. These observations indicate that CABLES1 has an important role as tumor supressor.

CABLES1 interacts with different CDK proteins and regulate their phosphorylation by acting as an adaptor protein with CDK and non-tyrosine kinase receptor. For example, during development of the CNS, CABLES1 has an important role. It is a substrate of c-Abl kinase. Active c-Abl leads to Cdk5 tyrosine phosphorylation, and this phosphorylation is enhanced by CABLES1 [116]. It was shown that CABLES1 enhances CDK2 Tyr15 phosphorylation by Wee1 causing a decease in CDK2 kinase activity. The activity of CABLES1 is under the control of the cell survival kinase AKT. AKT phosphorylates CABLES1 at two residues, T44 and T150: this phosphorylation leads to the recruitment and direct binding of the regulatory protein 14-3-3 to CABLES1 preventing apoptosis induction by CABLES1 [121].

Corticotropinomas with CABLES1 missense mutations have moderate to low CABLES1 expression. By trying to find correlations with other cell cycle regulators, our collaborators found that the nuclear CDKN1B (P27) staining was affected, and no defects were detected in CyclinE [122], in agreement with a previous study done in our laboratory in which we found no correlations between loss of CABLES1 expression in corticotroph adenomas and CyclinE. It is known that CABLES1 stabilizes P21 thus, they suggested that impaired CABLES1 function causes CDKN1B degradation [376].

The absence of USP8 mutation in the DNA of the four patients with CABLES1 mutation does not exclude the possibility that CABLES1 can be a substrate of USP8 and may affect its stability or its action. Since USP8 mutations increase deubiquination and recycling of the EGFR, it is possible that the loss of CABLES1 is caused by activation of AKT pathway. Thus, we assessed the expression of CABLES1 by western blot after EGF stimulation and found that the expression of CABLES1 is not affected. However, since we don't have a good antibody against phosphorylated CABLES1, we cannot assess if CABES1 is active or not (data not shown). Collectively these data support an important role of CABLES1 as a pituitary tumor suppressor.

6. GENERAL CONCLUSION AND PERSPECTIVES

This work identified the targets of the EGFR pathway that induce POMC transcription. As showed in Chapter 2, EGFR signaling induces POMC transcription via the JAK/STAT3 pathway in corticotroph tumor cells and blocking of this pathway with specific inhibitors, either for JAK1/2 or STAT3, attenuate POMC promoter induction. Based on this result, we assessed the role of STAT3 in glucocorticoid resistance. We found that under EGF stimulation and/or by overactivation of STAT3, corticotroph cells became resistant to glucocorticoids, and that the POMC promoter becomes unresponsive to the inhibitory effect of Dexamethasone. STAT3 affects the transrepression mechanism of GR without affecting GR recruitment to the POMC promoter. The treatment of cells stimulated by EGF with the JAK inhibitor restores the response of cells to glucocorticoids. We further showed expression of activated form of STAT3 in human corticotroph adenomas tissues. The STAT3 pathway could be targeted to treat patients with Cushing disease. Many STAT3 inhibitors are available and used in the clinic. It will be important to validate these results by generating mouse models and treat them with STAT3 inhibitors as corticotroph tumors is developed, and it will be interesting to assess the role of STAT3 in glucocorticoid resistance in other cell types to demonstrate whether this mechanism of action is specific to the POMC promoter or corticotroph cells, or whether it represents a general mechanism of resistance to steroid receptors.

In the Chapter 3, we identified 4 loss of function mutations in the *CABLES1* gene in adult and pediatric CD patients. These mutations affect CABLES1's activity to inhibit cell proliferation. In correlation with other cell cycle proteins, the expression of CDKNB1 is very low in human corticotroph tumor tissues. These finding highlight the likely role of CABLES1 as a tumor suppressor in CD.

We found that STAT3 pathway activates corticotroph cell proliferation: further analysis of STAT3 targets and STAT3 cell cycle targets will help to understand its role in tumorigenesis of Cushing's

disease. The relation between the STAT3 pathway and CABLES1 should also be clarified in order to develop a unified scheme of CD pathogenesis.

Annex
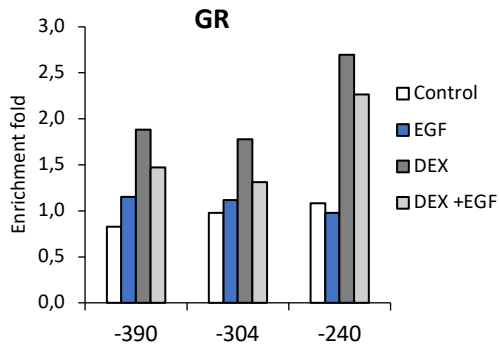
Figure 1-Annex: Recruitment of GR to POMC promoter is not affected by EGF

ChIP-qPCR data for GR protein from AtT-EGFR cells untreated (control) and after 30 minutes of DEX, EGF or both to assess recruitment of GR to POMC promoter at -390bp, -304bp, -240bp positions.
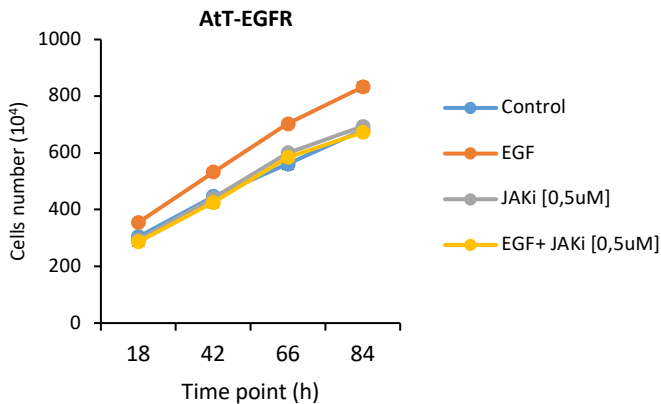
Figure 2-Annex: EGF stimulation of cell proliferation is inhibited by JAK inhibitor

Growth curves of AtT-EGFR treated with control, EGF 100ng/m, JAK inhibitor [0,5uM] or both, as indicated. The stimulatory effect of EGF on cell proliferation is statistically significant, JAK inhibitor inhibit EGF-inducing proliferation.

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