

Ludwig Maximilian University, Munich
Faculty of Biology

Max Planck Institute of Psychiatry
Neuroendocrinology Group

**Regulation of pituitary growth hormone synthesis by
NAD⁺ dependent deacetylase Sirt1**

Dissertation

Submitted by Jose Luis Monteserin Garcia

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Erstgutachter: Prof. Dr. Rainer Landgraf
Zweitgutachter: Prof. Dr. Anja Horn-Bochtler
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ABBREVIATIONS

ABC	Avidin-Biotin Complex
AMP	5' adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ADP	Adenosine-5'-diphosphate
AICAR	5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide
ATP	Adenosine-5'-triphosphate
Cito aa3	Cytochrome c oxidase
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
CREB	cAMP response element binding protein
DAB	3,3-Diaminobezidine
DEPC	Diethyl-pyrocarbonate
EDTA	Ethylendiamintetracetic
FAD	Flavin adenine dinucleotide
FCS	Foetal Calf Serum
FFA	Free fatty acids
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GSK3 β	Glycogen synthase kinase-3 subunit β
hGH-N	Human growth hormone-normal
I-2	Inhibitor 2 (specific for PP1)
IGF-I	Insulin-like growth factor-I
Met	Metformin
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
ONPG	Ortho-nitrophenyl- β -galactoside
PCR	Polymerase chain reaction
Pit1	Pituitary transcription factor 1
PP1	Protein phosphatase 1
PRL	Prolactin
rGH	Rat growth hormone
rGHRH	Rat growth hormone releasing hormone
RSV	Resveratrol
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Polyacrylamide electrophoresis gel with sodium dodecyl sulphate
Sir2	Silent information regulator 2
Sirt1	Sirtuin 1

SUMMARY

Animal size is very variable even among individuals of the same species and is determined not only by genetic but also environmental factors. Nutrient availability is a crucial environmental factor, which determines the growth and development of tissues, organs and ultimately, the whole organism's growth and metabolic homeostasis. Organisms have evolved mechanisms that sense changes in energy availability in order to keep the homeostasis. Two of the most important energy sensors are 5' adenosine monophosphate-activated protein kinase (AMPK) and the protein deacetylase Sirt1. Both are active at high 5' adenosine monophosphate (AMP) and nicotinamide adenine dinucleotide (NAD⁺) conditions, which reflect low energy levels, and regulate important aspects of cell and organism metabolism. To achieve this intracellular energy sensors have to coordinate with hormonal stimuli that control these processes in multicellular organisms. In mammals, one of the most important hormones driving organism growth and metabolism is the growth hormone (GH).

This work shows that both AMPK and Sirtuin 1 (Sirt1) suppress GH synthesis from pituitary cells. Sirt1 effect was the most prominent, therefore this study went on to investigate how the deacetylase crosstalks with the hormonal signalling cascade regulating GH transcription. Sirt1 was detected in the anterior pituitary in somatotroph cells and pharmacological activation of Sirt1 decreased GH levels *in vivo* and *in vitro*. Sirt1 suppressed GH promoter activity from a plasmid, revealing that its effect was not merely mediated by histone deacetylation. GH transcription is stimulated by the cAMP cascade through the cAMP response element binding protein (CREB) dependent transcription of the pituitary transcription factor 1 (Pit1). Herein it is shown that Sirt1 suppresses Pit1 transcription by inhibiting CREB activity. CREB has to be phosphorylated and acetylated to reach the maximal activity, and Sirt1 is decreasing both using its deacetylase activity. A novel cascade unveils in which the Sirt1 deacetylase triggers CREB dephosphorylation in a pathway involving glycogen synthase kinase-3 subunit β (GSK3 β) and subsequent protein phosphatase PP1 activation. The drop in phosphorylated CREB levels results in suppressed Pit1 and GH transcription.

Altogether, this work reveals a new mechanism by which the energy sensor Sirt1 regulates GH transcription in somatotroph cells. Coordinated systemic response adjusts the organism needs to the environmental nutrient offer. The herein proposed pathway shows a model in which energy changes may modify hormonal levels that regulate whole body metabolism and adapt the organism to the current environmental demands.

ZUSAMMENFASSUNG

Die Größe von Tieren ist sehr variabel, selbst unter Individuen der gleichen Spezies, und wird sowohl durch genetische als auch durch Umweltfaktoren bestimmt. Die Verfügbarkeit von Nährstoffen ist ein wichtiger Umweltfaktor, der das Wachstum und die Entwicklung von Geweben, Organen und letztlich des gesamten Organismus und dessen Stoffwechsellhomöostase bestimmt. Organismen haben Mechanismen entwickelt, die Veränderungen in der Verfügbarkeit von Energieträgern erkennen, um die metabolische Homöostase aufrecht zu erhalten. Zwei der wichtigsten Energiesensoren sind die 5'Adenosinmonophosphat-aktivierte Proteinkinase (AMPK) und Protein-Deacetylase Sirtuin 1 (Sirt1). Diese beiden Enzyme sind bei hohen Konzentration von 5'Adenosinmonophosphat (AMP) und Nicotinamidadenindinucleotid (NAD^+) besonders aktiv. Hohe NAD^+ - und AMP-Werte sind charakteristisch für einen niedrigen intrazellulären Energiestatus und sind wichtige Moleküle für die Regulation des Stoffwechsels von Einzelzellen und vielzelligen Organismen. In letzteren müssen intrazelluläre Energiesensoren mit hormonalen Stimuli koordiniert agieren, um diese metabolischen Prozesse zu kontrollieren. In Säugetieren ist das Wachstumshormon eines der wichtigsten Hormone, das Wachstum und Stoffwechsel reguliert.

In der vorliegenden Arbeit wurde gezeigt, dass sowohl die AMPK als auch Sirt1 die GH-Synthese in Hypophysenzellen unterdrücken. Die Wirkung von Sirt1 war dabei deutlich stärker, weshalb in dieser Studie erforscht wurde, wie die Deacetylase mit der hormonellen Signalkaskade interagiert, die die GH-Transkription reguliert. Sirt1 wurde in somatotropen Zellen des Hypophysenvorderlappens nachgewiesen, und die pharmakologische Aktivierung von Sirt1 reduzierte die GH-Konzentration *in vivo* and *in vitro*. Sirt1 unterdrückte die GH-Promotoraktivität in einem Reporterplasmid, woraus man schließen kann, dass dieser Effekt nicht alleine durch Histon-Deacetylierung vermittelt wird. Die GH-Transkription wird durch die cAMP-Kaskade stimuliert und zwar durch die cAMP *response element binding protein* (CREB) abhängige Transkription des *Pituitary Transcription Factor 1* (Pit1). Es konnte gezeigt werden, dass Sirt1 durch Inhibition der CREB-Aktivität die Transkription von Pit1 unterdrückt. CREB muss phosphoryliert und acetyliert vorliegen, um seine maximale Aktivität zu erreichen, und Sirt1 unterdrückt diese beiden posttranslationalen Proteinmodifikationen durch ihre Deacetylaseaktivität. Eine neue Signalkaskade wurde entdeckt, über die Sirt1 die Dephosphorylierung von CREB auslöst. An diesem neuen Signalweg ist die *Glycogen Synthase Kinase-3 Subunit β* (GSK3 β) und nachfolgend die Aktivierung der *Protein Phosphatase PPI* beteiligt. Die Senkung der Konzentration an

phosphorylierten CREB führt zur Unterdrückung der Pit1-Transkription und damit vermutlich auch der GH-Transkription.

Zusammengefasst wird in dieser Arbeit ein neuer Mechanismus gezeigt, durch den der Energiesensor Sirt1 die GH-Transkription in somatotropen Zellen reguliert. Koordinierte systemische Reaktionen passen die Bedürfnisse eines Organismus an das Nährstoffangebot der Umwelt an. Der hier vorgeschlagene Signalweg zeigt ein Modell, in dem Änderungen des Energiestatus, zur Modifikation von Konzentrationen von Hormonen führen, die den Stoffwechsel eines Organismus regulieren und diesen dadurch an die aktuellen Umwelтанforderungen anpassen.

1 INTRODUCTION

1.1 Growth Hormone (GH)

GH is pivotal for the growth of the organism by promoting organ and soft tissue growth. It promotes bone growth in young by triggering chondrocyte division. In addition, GH increases the gastrointestinal absorption of calcium further contributing to bone growth, mineralization and strength. The importance of GH is evident in children with inborn GH deficiency that present with abnormally short stature (dwarfism) and delayed puberty (Reynaud et al., 2004). In contrast GH excess leads to gigantism in young subjects, and in adults, in whom bone lineal growth does not any longer take place due to the merge of epiphysis with the diaphysis, it leads to severe bone and soft tissue deformities (Ayuk & Sheppard, 2006).

GH is the primary positive regulator of hepatic IGF-I secretion, which mediates most of its physiological effects (Bichell et al., 1992; Wurzbarger et al., 1993; Clemmons & Underwood, 1991). IGF-I induces growth on almost every organ in the body, especially in skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cells, and lung (Guler et al., 1988).

In addition to its growth promoting action, GH is a major anabolic peptide hormone inducing protein synthesis. To meet the energetic needs for this process, GH triggers free fatty acids (FFA) synthesis by upregulating lipoprotein lipase that is an enzyme involved in lipolysis (Møller et al., 1990; Fryburg et al., 1992). GH induced protein synthesis and drop in protein catabolism, reduces the nitrogen that is present in blood and urine in the form of urea (Møller et al., 2009; Ganong, 1994). The net result of these actions is positive nitrogen balance, increased lean body mass and energy expenditure. Administration of GH in GH deficient adults increases their lean body mass and metabolic rate and decreases body fat (Jørgensen et al., 1989) and plasma cholesterol (Beshyah et al., 1995; Salomon et al., 1989). The loss of muscle and bone density and increase in fat tissue in the elderly is attributed to the decline of GH levels during aging (Gil-Ad et al., 1984; Arvat et al., 2000).

A downside of GH induced FFA levels is the reduction in glucose uptake (Møller et al., 1991). In addition, GH opposes to insulin action in the liver by directly suppressing glucose uptake and activating hepatic gluconeogenesis and glucose release. These diabetogenic properties of GH were initially observed in hypophysectomized dogs which are insulin sensitive and hypoglycemic (Houssay, 1936). Ames and Snell dwarf mice which lack GH (Sornson et al., 1996), are insulin sensitive despite increased body fat mass (Hsieh et al., 2002) and the same is true for the GH receptor knockout (GHRKO) or Laron mice (Liu et al., 2004). In addition, patients with Laron syndrome who present with dwarfism because of a mutation in the GH receptor constituting them resistant to GH action, are insulin sensitive and do not suffer from diabetes (Guevara-Aguirre et al., 2011). In contrast, transgenic mice overexpressing GH are insulin resistant (Bartke, 2005). Similarly patients with acromegaly due to GH secreting pituitary adenoma have insulin resistance and type 2 diabetes despite the increased lean mass and reduced body fat mass (Giustina et al., 2003).

The metabolic effects of GH have suggested a putative role for GH in aging. In fact Ames, Snell and GHRKO mice live much longer than their wild type counterparts (Coschigano et al., 2003; Bartke, 1998; Schaible & Gower, 1961; Brown-Borg et al., 1996; Bartke, 2000; Coschigano et al., 2000; Zhou et al., 1997), while mice overexpressing GH or treated with GH have a shorter lifespan (Bartke, 1998; Groesbeck et al., 1987). Similarly acromegalic patients present with higher mortality (Dekkers et al., 2008; Melmed, 2009). The decreased energy expenditure in animals without GH and subsequent lack of reactive oxygen species can explain in part this observation (Brown-Borg et al., 2009). Nevertheless, the pro-aging effects of GH are mediated by IGF-I, which is an evolutionary conserved regulator of life span (Bartke & Brown-Borg 2004; Holzenberger, 2004). Mutations in the insulin/IGF-I receptor orthologue DAF-2 in worms and flies double their life span (Tatar et al., 2001; Kimura et al., 1997; Kenyon et al., 1993). Similarly IGF-I receptor heterozygous knockout mice live 30 % longer than their wild type littermates (Holzenberger et al., 2003).

Summarizing, GH is important regulator of diverse physiological processes. During development it is pivotal for the organism growth, while in adult it ensures bone maintenance and muscle strength. However high GH levels are also accompanied by increased insulin resistance. In addition there is increasing evidence for its positive association with cancer either directly or indirectly through IGF-I which is a major growth factor contributing in tumorigenesis (Khandwala et al., 2000). These characteristics of GH are probably responsible for the observed negative correlation with organism lifespan.

1.2 The pituitary gland

GH is primarily produced in the pituitary gland. The pituitary gland or hypophysis is a small endocrine gland situated at the base of the brain in a small bony cavity (sella turcica). It weights approximately 0.6 g in humans. Anatomically it can be divided in the posterior pituitary (neurohypophysis) and the anterior pituitary (adenohypophysis). An intermediate lobe is found in many animals, but it is rudimentary in humans (fig. 1).

The posterior pituitary is composed of nerve fibers extending from the hypothalamus with their nerve endings, and pituicytes that are modified glial cells. The posterior pituitary stores and releases oxytocin, which plays an important role during childbirth and breastfeeding and antidiuretic hormone (ADH), which facilitates the kidneys to reabsorb water regulating in that way blood pressure.

The anterior pituitary is composed of endocrine cells that synthesize important hormones regulating a variety of physiological processes in vertebrates. These hormones can be classified into three groups based on structural and functional similarity: the proopiomelanocortin family, the glycoprotein hormone family and the GH family.

With techniques such immunohistochemistry and electronic microscopy, it is possible to distinguish five different types of endocrine pituitary cells:

- Somatotroph cells that produce GH.

- Lactotroph cells secreting prolactin (PRL), which controls milk production during lactation in mammals.
- Thyrotroph cells secreting thyroid-stimulating hormone (TSH), which is important for the physiological growth and function of the thyroid gland.
- Gonadotroph cells synthesizing follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates spermatogenesis in the testes and follicular growth in the ovaries.
- Corticotroph cells in which proopiomelanocortin (POMC) is transcribed. POMC cleavage products are the adrenocorticotrophic hormone (ACTH), which stimulates glucocorticoid production from the adrenal cortex, lipotropic hormone (β -LPH), corticotrophin-like immunoreactive peptide (CLIP), endorphins, enkephalin, and melanocyte-stimulating hormone (α -MSH). α -MSH synthesized from the corticotrophs of the intermediate lobe plays an important role in animals that have the ability of changing color to camouflage.

In addition to the endocrine cells, three to five percent of all the cells in the anterior pituitary are folliculostellate cells (Allaerts et al., 1990). These cells have thin cytoplasmatic projections that grow between the surrounding endocrine cells.

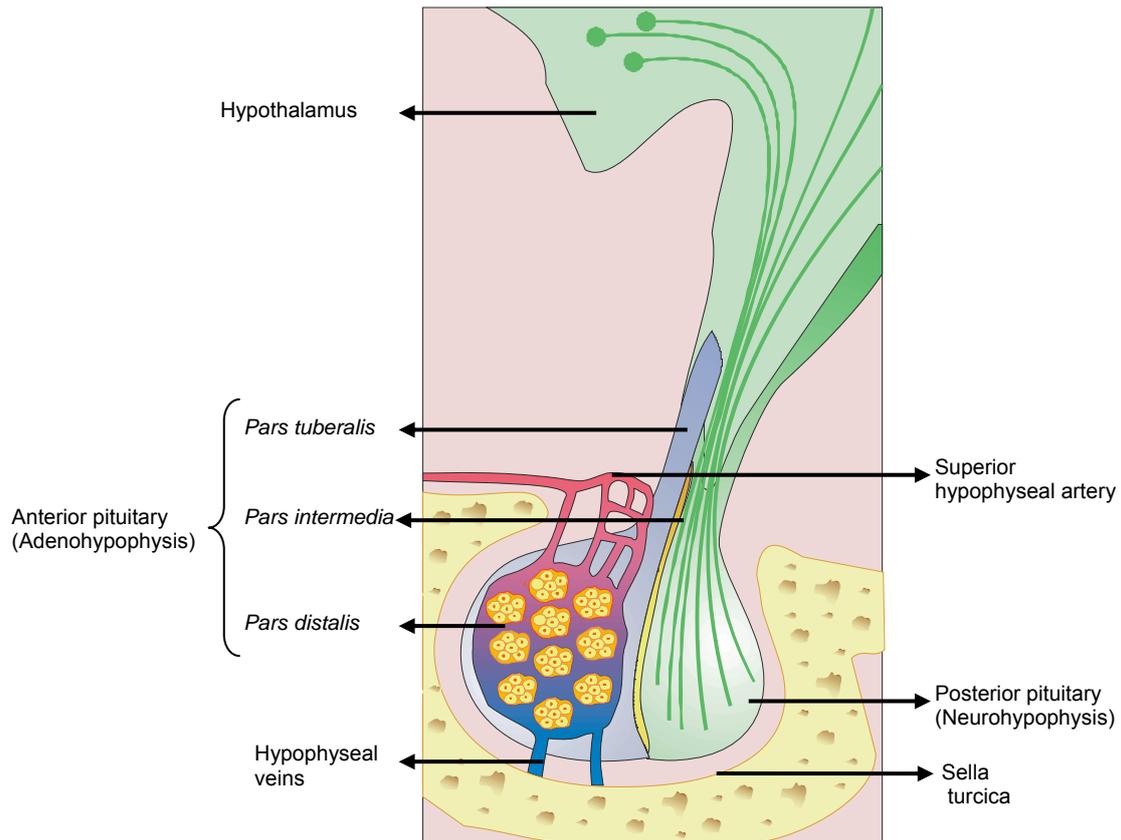


Figure 1. Pituitary gland. Showing the hypophysis location within the invagination of the sphenoid bone, the sella turcica. The two lobes, anterior (adenohypophysis; in blue color) and posterior (neurohypophysis in green) are indicated. The pars intermedia (in yellow color) is rudimentary in humans.

1.3 Somatotroph development

Pituitary gland development starts when oral ectoderm cells from the roof of the pharynx express specific transcription factors like the Rathke's pouch homeobox (Rpx), the paired box gene 6 (Pax6), the pituitary homeobox (Ptx) 1 and 2, and the LIM homeobox genes (Lhx) 3 and 4. The oral ectoderm forms a pocket (Rathke's pouch) that invaginates into the ventral diencephalon. This happens at embryonic day 8.5 in the mouse. The cells of the anterior wall of Rathke's pouch differentiate under the influence of other transcription factors (Fig. 2) and proliferate to form the adenohypophysis, while the posterior wall forms the *pars intermedia*. The diencephalon turns into the pituitary stalk and the neurohypophysis. In adult pituitary, rudiments of the Rathke's pouch can be found in the *pars intermedia*.

The development of the somatotroph lineage starts with the expression of transcription factor prophet of Pit1 (Prop-1). Subsequent expression of pituitary transcription factor 1 (Pit1) and estrogen receptor (ER) α completes the differentiation of the somatotroph lineage (Asa & Ezzat, 1999; Cohen, 2000; Fig.2). Transgenic animal models lacking Prop-1 and Pit1 expression and humans carrying mutations in these genes suffer from hypopituitarism (Mullis, 2007).

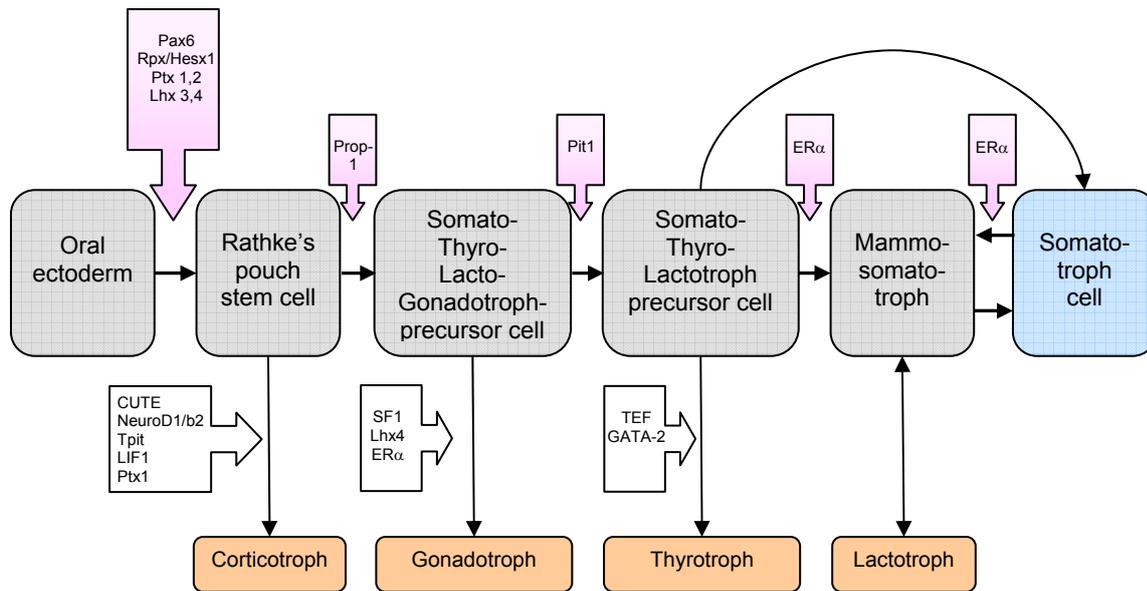


Figure 2. Transcription factors involved in adenohypophysis development. At early stages, the cells are multipotent, but sets of transcription factors contained within the nucleus determine the final cell type. Lhx3 positive cells acquiring the T-box transcription factor Tpit become the ventral corticotrophs. The Tpit interacts with other proteins like the corticotropin upstream transcription-binding element (CUTE), NeuroD1/ β 2 and pituitary homeobox factor 1 (Ptx1). Most of the pituitary cell types are derived from the cells that express the Prop-1 transcription factor. Those in the ventral part express the steroidogenic factor 1 (SF1) and GATA binding protein 2, which induces the differentiation to gonadotrophs. The dorsal cells of the Prop-1 lineage synthesize the Pit1 transcription factor and generate the somatotrophs, lactotrophs and thyrotrophs. Pit1 and thyrotroph embryonic factor (TEF) positive cells differentiate to thyrotroph while ER α positive cells differentiate to mammosomatotroph. Mammosomatotroph cells produce GH and PRL and can differentiate to somatotroph or lactotroph cells in a reversible process (adapted from Scully & Rosenfeld, 2002; Cohen, 2000; Asa et al., 1999).

1.4 Regulation of GH synthesis

In humans, genetic information for the synthesis of GH is localized on the long arm of chromosome 17, region q22-q24 (George et al., 1981). There are five genes encoding five different forms of GH. The most abundant form (more than 75% of the GH in circulation) is

the hGH-N synthesized by specialized pituitary cells; the rest are expressed principally in the placenta, they are hGH-V (variable) and hCS (chorionic somatomammotropin) types A, B and L (Seeburg, 1982). hGH-N, thereafter termed hGH, is a single chain peptide hormone of 191-amino acids.

GH expression is controlled by a pituitary-specific promoter that contains 2 binding sites for Pit1 (Nelson et al., 1988; Bodner et al., 1988; Ingraham et al., 1988; McCormick et al., 1990; Gaiddon et al., 1996; Cohen et al., 1999). Both sites are essential for GH promoter activity (Cohen et al., 1999). The dependence of GH transcription on Pit1 is evident by the fact that mice and humans carrying Pit1 mutations present with GH deficiency and dwarfism (Li et al., 1990; Radovick et al., 1992; Quentien et al., 2006).

The hGH promoter contains two additional core cAMP-response elements (CREs), one distal (located at -187/-183) and one proximal (located at -99/-95; Cohen et al., 1999), which consist of CGTCA motifs of the palindromic consensus sequence TGAC-GTCA. In contrast the rat GH promoter lacks CRE, responds to the cAMP/PKA signal through cAMP response element binding protein (CREB)-induced Pit1 (Tansey et al., 1993; Cohen et al., 1999). In addition rGH promoter has thyroid hormone responsive elements (TRE) and responds positively to thyroid hormone (T₃, 3,5,3'-L-triiodothyronine; Brent et al., 1988).

GH is positively and negatively regulated by factors originating from the hypothalamus and the periphery. The most important peptides involved in GH synthesis regulation (Giustina & Veldhuis, 1998) are listed in the following subitems.

1.4.1 GHRH

The main peptide regulating pituitary GH synthesis is the growth hormone-releasing hormone (GHRH). GHRH is found in the arcuate nucleus of the hypothalamus, in the anterior hypothalamic region and in the dorsomedial and ventromedial nuclei and is released from the nerve terminals and through the hypothalamo-hypophyseal portal system to the pituitary

(Bloch et al., 1983). GHRH is secreted in a pulsatile rhythm that reflects GH pulsatility (Cataldi et al., 1994).

GHRH mediates its effects by binding to the GHRH receptor (GHRHR) which belongs to the seven transmembrane domain G-protein coupled receptor superfamily. The intracellular part of the receptor associates with the stimulatory G-protein (Gs), which is composed of alpha (α), beta (β), and gamma (γ) subunits. Ligand binding induces a conformational change in the receptor, allowing the phosphorylation of guanine di-phosphate (GDP) to guanine tri-phosphate (GTP) on the G_α subunit. That induces the dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimer and the receptor. Both G_{α} -GTP and $G_{\beta\gamma}$ can then activate different signaling cascades, while the receptor is free to activate the next Gs protein. G_{α} subunit stimulates the membrane-bound adenylyl cyclase which converts adenosine-5'-triphosphate (ATP) to 3', 5'cyclic adenosine monophosphate (cAMP). cAMP binds to the regulatory subunits of protein kinase A (PKA), which becomes active allowing the free catalytic subunits to translocate into the nucleus. There they phosphorylate and activate the transcription factor CREB. In addition they phosphorylate and activate the transcriptional coactivator CREB binding protein (CBP). Activated CBP is recruited to the GH promoter through Pit1 homodimers (Cohen et al., 1999). CREB binds to the two CRE elements in the human GH promoter, which are located in close proximity to the distal Pit1 binding site at -123/-112. All these three sites are required for cAMP response (Cohen et al., 1999; Fig. 3). In the case of the rGH promoter which lacks CRE sites, GHRH-induced cAMP/PKA stimulates GH synthesis through CREB-induced Pit1 (Bodner et al., 1988; Ingraham et al., 1988; McCormick et al., 1990; Gaiddon et al., 1996).

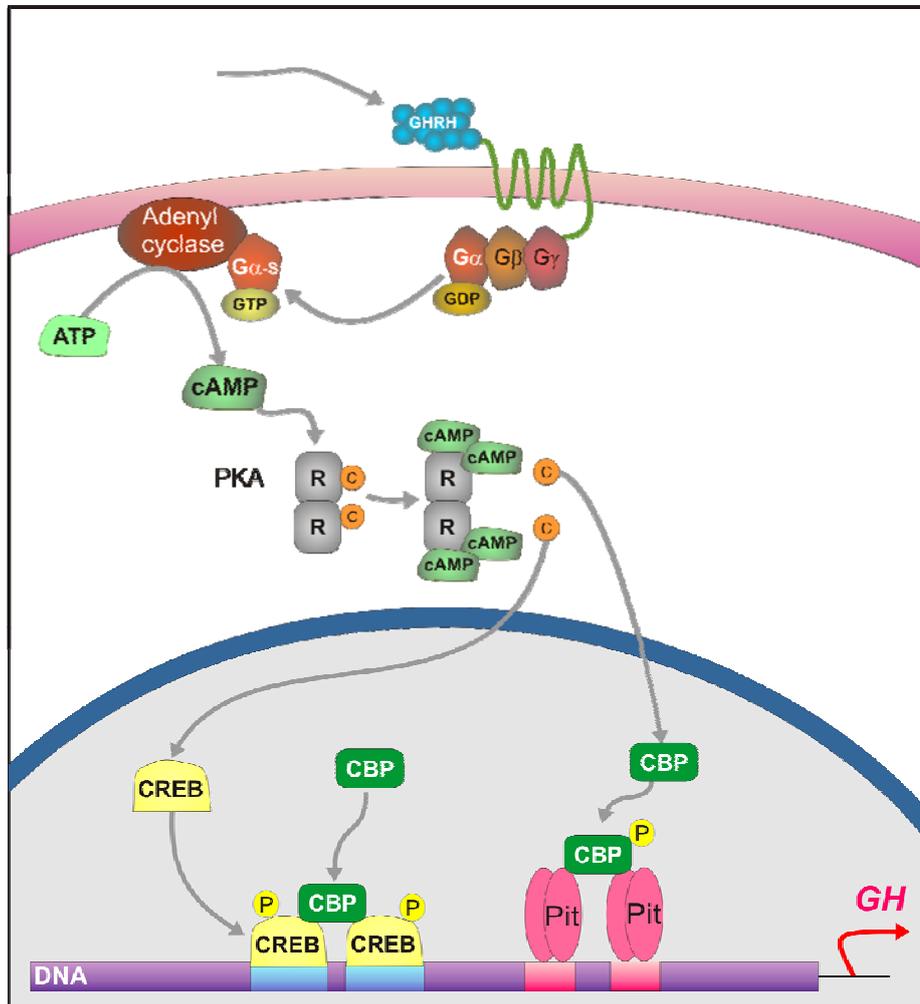


Figure 3. Basic scheme of regulation of hGH synthesis by GHRH in somatotroph cells. In brief, GHRH bound to its G protein coupled receptors triggers Gs and activates adenylate cyclase leading to rise in cAMP, which binds to the regulatory PKA subunit. The released catalytic subunit translocates to the nucleus where it phosphorylates CREB and CBP. Activated CBP is recruited to the hGH promoter by CREB and Pit1 bound to their respective DNA binding elements.

1.4.2 Somatostatin (SST)

SST or growth hormone-inhibiting hormone is a peptide hormone, which is synthesized in the hypothalamus by the neuroendocrine cells of the paraventricular nucleus (Reichlin, 1983). Additionally SST is synthesized in the delta cells of the pancreatic islets and in the gastric mucosa and small intestine (Goldsmith et al., 1975). There are two biologically active forms of SST, products of different cleavage of the same precursor protein; one is composed of 14 amino acids (SS-14) and the other of 28 amino acids (SS-28; Patel, 1999). SST acts through

five receptors that belong to the G-protein coupled receptor superfamily (SSTR1-5), four of which are present in the anterior pituitary (Viollet et al., 1995). SST inhibits GHRH-induced pituitary GH secretion (Tannenbaum et al., 1989).

1.4.3 Insulin-like growth factor I (IGF-I)

GH is under the negative feedback regulation from IGF-I directly at pituitary level and indirectly by the reduced GHRH release from the hypothalamus (Berelowitz et al., 1981). In addition IGF-I increases hypothalamic SST synthesis, which in turn suppresses pituitary GH secretion (Berelowitz et al., 1981; Bermann et al., 1994). IGF-I is a 70 amino acids protein similar in structure to insulin, which is produced primarily by the liver as an endocrine response to GH secretion (Daughaday & Rotwein, 1989). IGF-I mediates its effect by binding to the IGF-I receptor (IGF-IR; Jones & Clemmons, 1995).

1.4.4 Other regulators:

In addition to these main regulators, GH synthesis is also fine-tuned by other peptides, such as:

Ghrelin

Ghrelin is a 28 amino-acids peptide that was identified as the elusive ligand for the GH secretagogue receptor (GHS-R). GHS-R is a G-protein coupled receptor present in the pituitary gland and hypothalamus that was shown to mediate the potent GH rising action of synthetic GH secretagogues (Howard et al., 1996). Ghrelin is synthesized principally in specialized cells present in the fundus of the stomach, the epsilon cells of the pancreas and in the arcuate nucleus of the hypothalamus (Kojima et al., 1999). It is present in two forms, the unmodified des-octanoylated peptide and the octanoyl-ghrelin. Octanoyl-ghrelin is responsible for the GH secreting and the orexigenic effects of this hormone and induces adiposity (Kojima et al., 1999; Tschöp et al., 2000; Nakazato et al., 2001). The des-octanoylated peptide is also able to bind to several tissues and may have important role in the

cardiovascular system (Baldanzi et al., 2002) and adipogenesis (Thompson et al., 2004). Exogenous ghrelin administration strongly stimulates pituitary GH release through GHS-R present in the GHRH secreting neurons of the arcuate nucleus and pituitary gland (Takaya et al., 2000; Lucidi et al., 2005). However, the role of endogenous ghrelin on GH secretion is not well established and conflicting data exist about the role of physiological ghrelin on GH synthesis (Kamegai et al., 2004; Hataya et al., 2001).

Galanin

This 30 amino acids neuropeptide is strongly expressed in the median eminence in the hypothalamus, but is distributed widely in the mammalian brain, spinal cord and gut (Tatemoto et al., 1983; Skofitsch & Jacobowitz, 1985). Galanin was shown to enhance GH response to GHRH (Davis et al., 1987) possibly through an interaction with GHRH (Niimi et al., 1990).

Pituitary adenylate cyclase-activating peptide (PACAP)

This 38 amino acids peptide is present in the entire central nervous system but the highest concentrations are found in hypothalamus. Plasma extracted from the hypophyseal portal vessels of the pituitary stalk in rats, have revealed PACAP concentrations at much higher levels than in the plasma of the periphery (Arimura et al., 1991; Tamada et al., 1994), indicating a role of PACAP in the pituitary gland. PACAP treatment increases cAMP in rat pituitary cells and stimulates GH release (Miyata et al., 1989) independently of GHRH (Pisegna & Wank, 1993). However, PACAP's role in human pituitary physiology is still not clear.

Opioid peptides

GH secretion in humans was shown to be triggered by opioid peptides under stress conditions (e.g. extenuate exercise; Moretti et al., 1983).

Substance P

This 11 amino acid peptide is abundant in the central nervous system and has been related to the transmission of pain, anxiety, stress and mood disorders (De Felipe et al., 1998; Harrison & Geppetti, 2001). Substance P was found to increase both basal and GHRH stimulated GH levels (Coiro et al., 1992).

Melatonin

This hormone is secreted by the pineal gland and it has functions related to the circadian rhythm (Cassone et al., 1993). Oral melatonin administration increased GH levels (Valcavi et al., 1987), indicating that it may be regulated by endogenous melatonin. However, this was not confirmed in other studies (Wright et al., 1986) and no correlation was found between nocturnal melatonin and GH levels in men (Vaughan et al., 1978).

Thyrotropin-releasing hormone (TRH)

TRH is mainly synthesized in the paraventricular nucleus of the hypothalamus (Taylor et al., 1990) and stimulates the anterior pituitary gland to produce TSH and PRL. In rats TRH is potent GH secretagogue, but in humans it induces GH only in subjects suffering from acromegaly, diabetes, renal or hepatic failure, but not in healthy individuals (Czernichow et al., 1976; Scanlon et al., 1983; Valentini et al., 1989; Giustina et al., 1995).

Calcitonin

This 32 amino acids peptide, which is secreted by the C-cells of the thyroid gland, strongly regulates Ca^{2+} metabolism by decreasing renal clearance of calcium and phosphate and inhibiting osteoclastic (bone reabsorption) activity. Calcitonin decreases pituitary GH secretion (Cantalamesa et al., 1978), by modifying intra/extra cellular Ca^{2+} levels in pituitary cells (Borle, 1975; Thorner et al., 1988).

NPY

NPY is a peptide neurotransmitter produced mainly in the paraventricular nucleus of the hypothalamus that induces food intake and energy storage by the adipose tissue. It inhibits GH secretion directly acting at pituitary somatotroph level in humans (Adams et al., 1987) or indirectly by inducing hypothalamic SST release in rats (Rettori et al., 1990).

1.4.5 Energy availability & GH synthesis

In addition to its hormonal regulation, GH as major metabolic hormone is also expected to be regulated by energy availability. Caloric restriction was found to suppress GH transcription and secretion in rats (Armario et al., 1987; Oster et al., 1995; Sonntag et al., 1995; Han et al., 1998; Lopez-Varela et al., 2004; Chen, 2004), while in old rats it changes GH pulsatility similar to that seen in young animals (Sonntag et al., 1995). Furthermore plasma IGF-I is reduced in caloric restricted rodents (Breese et al., 1991).

In humans, there is not clear consensus on the effects of dietary restriction on the GH/IGF-I axis. Fasting in obese subjects was shown to facilitate GH response to GHRH (Kelijman & Frohman, 1988). Patients with anorexia nervosa, which are characterized by severe chronic caloric restriction, presented with increased GH but decreased IGF-I levels (Argente et al., 1997; Scacchi et al., 2003). Long term dietary restriction in nonobese subjects did not change their GH levels (Redman et al., 2010), while IGF-I levels were suppressed only when protein intake was reduced (Fontana et al., 2008).

1.5 Intra-cellular energy sensors

All living organisms need energy to carry out vital metabolic process, therefore the ability of the cells to sense their internal energy state is of fundamental importance. Specialized intracellular proteins detect changes in the concentration of molecules that are used as chemical energy transporters. ATP is the main molecule involved in energy transfer, and therefore its intracellular concentration is an accurate measure of the energy state of the cell.

In eukaryotic cells, ATP is mostly synthesized in the mitochondrion by oxidative phosphorylation via the electron transport chain where the oxidation of one NADH molecule results in the synthesis of three ATP molecules (Fig. 4).

The two most important proteins that sense changes in intracellular ATP levels are the 5' adenosine monophosphate-activated protein kinase (AMPK) and the sirtuin 1 (Sirt1).

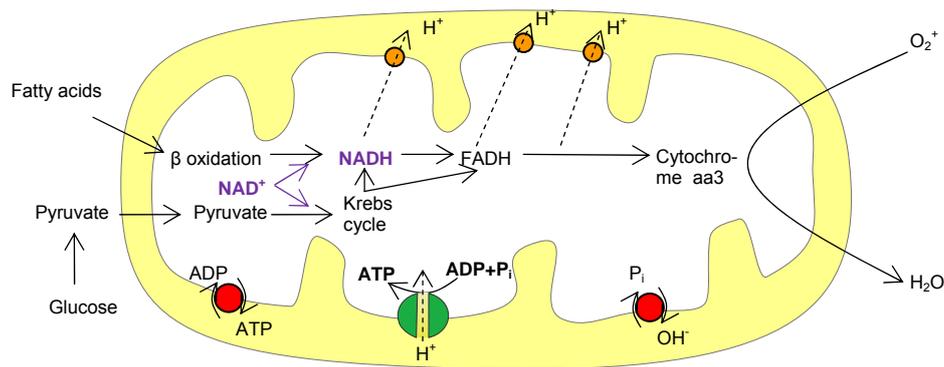


Figure 4. NAD⁺/NADH in energy metabolism taking place in mitochondria. Catabolism of glucose and fatty acids reduces NAD⁺ to NADH. Once NADH is incorporated to the respiratory chain, each molecule produces three H⁺, which at the end and through the ATPase (in green), result in the synthesis of three ATP molecules.

1.5.1 AMPK

AMPK is activated by low ATP and high AMP, i.e. low energy levels, to switch off systems which require energy (e.g. protein synthesis) and switch on systems that generate it (e.g. fatty acid oxidation; Carling, 2004; Hardie, 2007). AMPK is a complex serine/threonine kinase, composed by three different protein subunits: the catalytic subunit α and the regulatory subunits β and γ . The catalytic α subunit is activated when phosphorylated at threonine 172 by several kinases, including serine/threonine kinase 11 (STK11) or LKB1, transforming growth factor- β -activated kinase (TAK1), Ca⁺⁺/calmodulin-dependent protein kinase kinase β (CaMKK β) and, to a lesser extent, CaMKK α (Carling, 2004; Hawley et al., 2005). The β subunit unites and stabilizes the AMPK complex. When the β subunit is eliminated (e.g. by ubiquitination and subsequently degradation) AMPK levels decrease (Qi et al., 2008). The γ

subunit gives the enzyme the ability to sense the AMP:ATP ratio (Scott et al., 2004). High AMP concentration, indicative of low energy availability, cause allosteric modifications that protect the catalytic α subunit from serine/threonine protein phosphatases (e.g. PP2C α), which would otherwise suppress Thr¹⁷² phosphorylation and inactivate AMPK (Sanders et al., 2007). Similarly, AMP analogs, such as 5-aminoimidazole-4-carboxamide-1 beta-ribose (AICAR) are potent AMPK activators.

AMPK controls whole-body energy homeostasis, modulating multiple metabolic pathways in the brain and the periphery. AMPK expressed in hypothalamic neurons plays a fundamental role controlling food intake and regulating body weight. Low body energy state activates hypothalamic AMPK and triggers feeding, whereas food excess inhibits AMPK leading to suppressed appetite (Minokoshi et al., 2004). In liver, AMPK downregulates fatty acid and cholesterol synthesis, by inhibiting acetyl-CoA carboxylase (ACC) activity and fatty acid synthase (FAS) expression and inactivating hydroxymethyl-3-glutaryl-CoA (HMG-CoA) reductase. In addition liver and muscle AMPK increases fatty acid oxidation by activating mitochondrial fatty acid β oxidation (Kahn et al., 2005).

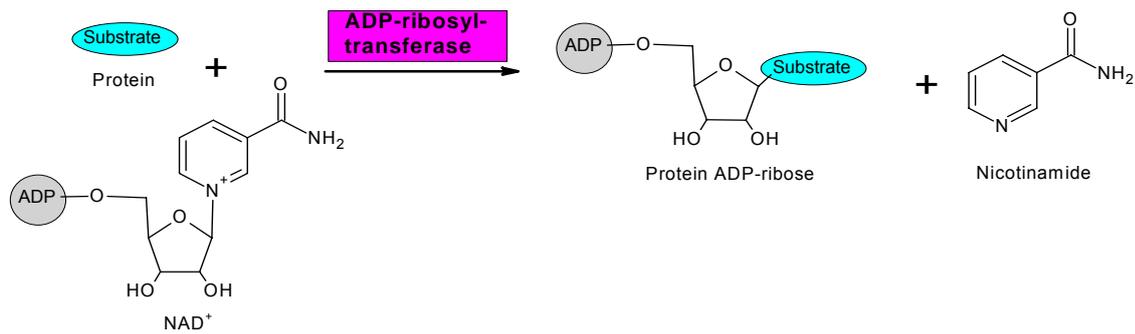
AMPK also has strong effects on glucose regulation. In fast-twitch muscles, AMPK activation increases cellular glucose uptake, by triggering glucose transporter 4 (GLUT4) expression and its translocation to the cell membrane, and glycolysis by stimulating hexokinase II expression (Holmes et al., 1999). In addition, AMPK suppresses muscle glycogen synthesis (Carling & Hardie, 1989) and hepatic gluconeogenesis (Lochhead et al., 2000). In fact, AMPK induces muscle mitochondrial biogenesis in response to diet induced chronic energy deprivation, helping the cell to maximize energy utilization under restrictive conditions (Zong et al., 2002). Interestingly, the widely used antidiabetic drug metformin was found to act, at least in part, by activating AMPK (Shaw et al., 2005).

1.5.2 SIRT1

Sirtuin 1 (Sirt1) activity is regulated by the relative concentration of oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD^+/NADH), which is involved in the redox reactions leading to ATP production (Fig. 4; Lin et al., 2004). In high energy intake, NAD^+ is reduced to NADH in pathways including glycolysis, Krebs cycle, and FFA oxidative phosphorylation. This leads to high NADH and low NAD^+ levels and consequently Sir2/Sirt1 inactivation. In contrast, when energy levels are low the organism consumes less NAD^+ , increasing its availability to Sir2/Sirt1.

Sirt1 is a protein highly conserved from bacteria to humans (Brachmann et al., 1995). Its homolog is the silent information regulator 2 (Sir2) which was discovered in *Saccharomyces cerevisiae* (Shore et al., 1984; Ivy et al., 1985; Rine & Herskowitz, 1987). Sir2 was identified because of a spontaneous mutation, which by relieving silencing at the mating-type loci HMR and HML resulted in a sterile *S. cerevisiae*. Sir2 was also found to silence genes near the telomeres and to be involved in cell cycle progress, genomic integrity and aging (Guarente, 1999). The Sir2 enzymatic activity was initially characterized as NAD^+ dependent mono-ADP-ribosyl transferase (Fig. 5A; Tsang et al., 1998). However, it was soon understood that its main action is as NAD^+ dependent protein deacetylase (Fig. 5B; Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Blander & Guarente, 2004; Imai & Guarente, 2010).

A.



B.

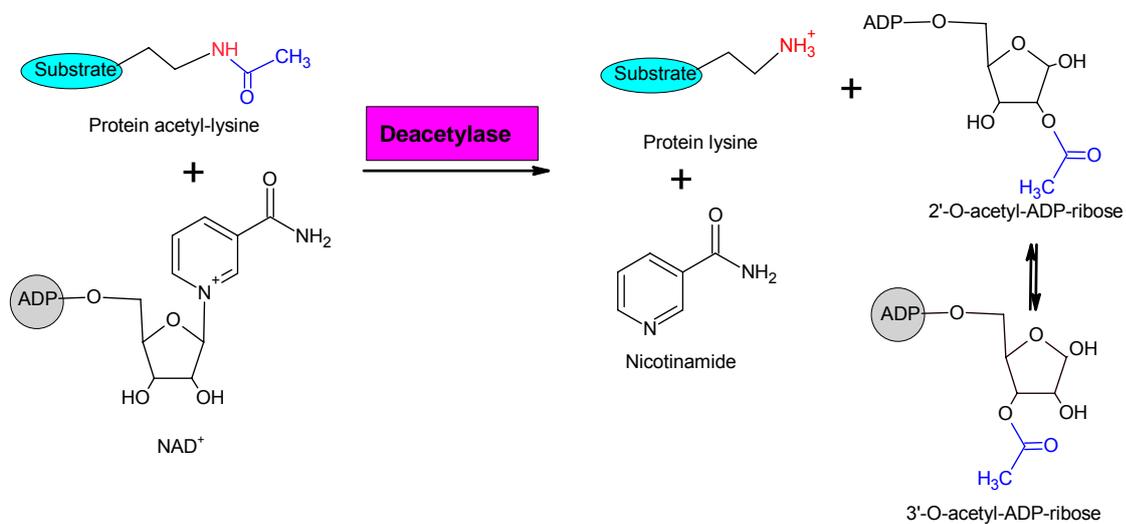


Figure 5. Generic chemical reactions of sirtuins, A. working as ADP-ribosyl-transferase and B. as protein deacetylase. It is evident that NAD⁺ is essential for the catalytic activity of the enzyme since it is substrate of the reaction. Nicotinamide is a product of the reaction, being at the same time a powerful inhibitor of the sirtuins sending a negative feedback to the enzyme.

1.5.2.1 Protein acetylation & deacetylation

The role of protein acetylation and deacetylation in regulating protein function is most widely studied in the case of histones. Histones are proteins found in eukaryotic cell nuclei, which package and order the DNA into structural units called nucleosomes and play a role in gene regulation. They are grouped into five major classes: H1/H5, H2A, H2B, H3, and H4 (Eickbush & Moudrianakis, 1978). Histones are acetylated by histone acetyltransferases (HAT), such as CBP/p300 (Chan & La Thangue, 2001) and deacetylated by histone deacetylases (HDAC). There are three different classes of HDAC, class I and II use Zn²⁺ as

cofactor and are inhibited by trichostatin A (TSA) and class III are NAD⁺-dependent and TSA insensitive (Thiagalingam et al., 2003). Sirt1 belongs to HDAC class III.

Histone acetylation is an important epigenetic modification. When acetylated by HATs, DNA is released from the histone and is accessible to specific enzymes and transcription factors that drive gene transcription. In contrast, HDAC mediated histone deacetylation compacts chromatin resulting in gene silencing (Fig. 6).

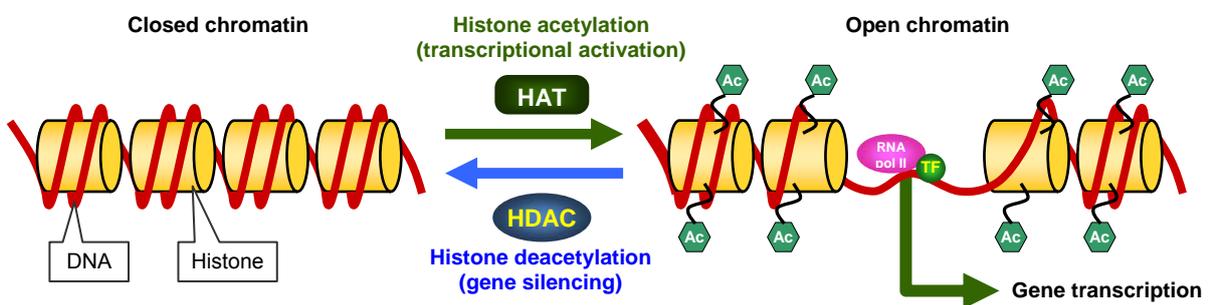


Figure 6 . Histone acetylation/deacetylation. DNA (red) tightly packed with histone proteins (yellow) as compact or closed chromatin, cannot be transcribed. HATs add acetyl groups (Ac) to the histones freeing the DNA and making it accessible to transcription factors (TF) and RNA polymerase (RNA pol II), which leads to gene transcription. In contrast, HDAC remove the acetyl groups from the histones packing the DNA up in a compact, transcriptionally silent form.

1.5.2.2 Mammalian sirtuins

Sirt1 belongs to the sirtuins, an ancient family of proteins conserved from bacteria to humans (Brachmann et al., 1995). In mammals, seven sirtuins are characterized, Sirt1 to Sirt7, which share a highly conserved catalytic core domain. **Sirt1** is the founding member of the family. It is a nuclear deacetylase that is expressed widely in mammalian tissues, and is particularly active in brain, fat, pancreas, muscle and liver. Apart from histones, Sirt1 also deacetylates an increasing number of proteins with ϵ -acetyl-lysine residues (Blander et al., 2005).

Sirt1 suppresses fat storage in white adipose tissue (WAT), by repressing peroxisome proliferator-activated receptor- γ (PPAR- γ) transcriptional activity and subsequently downregulating the transcription of the adipose tissue-specific fatty acid binding protein (aP2; Yang et al., 2006). This switches off the expression of genes involved in adipocyte

differentiation, insulin-induced fat storage and fatty acids mobilization. Similarly, Sirt1 suppresses muscle differentiation by deacetylating and inhibiting p300/CBP-associated factor (PCAF) acetyltransferase and the important for myocyte function MyoD, as well as the muscle specific transcription factor myocyte enhancer factor-2 (MEF2; Fulco et al., 2003). In liver Sirt1 activates gluconeogenesis and represses glycolysis by deacetylating and activating forkhead box protein O1 (FOXO1) and the peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC1- α), both of which are important for the transcription of gluconeogenic genes (e.g. phosphoenolpyruvate carboxykinase; Rodgers et al., 2005). In pancreatic β cells, Sirt1 increases insulin secretion by repressing uncoupled protein 2 (UCP2) gene expression. UCP2 diverts mitochondrial metabolism from ATP synthesis, therefore its downregulation results in more efficient use of glucose by the mitochondria and increase in intracellular ATP, closing ATP-dependent K^+ channels and opening voltage-gated Ca^{2+} which leads to insulin secretion. (Bordone et al., 2006). In the brain, where Sirt1 is abundantly expressed, it controls neurogenesis and protects from axonal degeneration (Araki et al., 2004; Prozorovski et al., 2008; Ramadori et al., 2008). Altogether Sirt1 affects cellular processes in organs important for the organism adaptation to energetic changes (Fig.7).

Mice overexpressing Sirt1 are lean and metabolically active, with reduced blood cholesterol, adipokines, insulin and fasting glucose and high glucose tolerance (Bordone et al., 2007). Sirt1 knockout mice show important developmental defects and only half of the embryos reach full term and are born. From these approximately 20% survive to adulthood, are smaller than their littermates and slow development characterized by perturbations in eyes and heart morphogenesis and sterility (Michan & Sinclair, 2007).

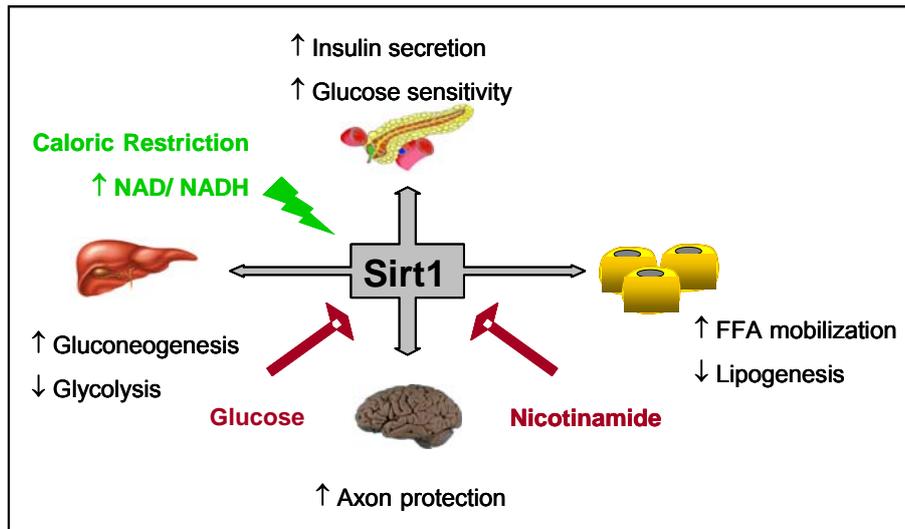


Figure 7. Sirtuins in mammals: Sirt1 is present in many tissues and organs like pancreas, liver, brain, adipose tissue and has an important function in them. Sirt1 is activated by caloric restriction translated by increased NAD^+/NADH ratio and inhibited by glucose, which decreases the ratio. Sirt1 is also inhibited by nicotinamide, which allosterically modifies the enzyme blocking the substrate access to the catalytic site.

Sirt2 is a deacetylase that shuttles between cytoplasm and nucleus where it associates with tubulin and histone H4 and affects cell cycle (Dryden et al., 2003; North et al., 2003; Michishita et al., 2005). In contrast **Sirt3**, **4** and **5** are mitochondrial proteins (Onyango et al., 2002; Schwer et al., 2002; Michishita et al., 2005); Sirt3 and 5 have deacetylase activity while Sirt4 is an ADP-ribosyl-transferase. Sirt3 is involved in thermogenesis and the Krebs cycle through the regulation of acetyl-CoA synthetase (Hallows et al., 2006; Schwer et al., 2006). Sirt4 in pancreas regulates amino acid induced insulin secretion (Haigis et al., 2006), whereas in liver it is speculated to facilitate gluconeogenesis during CR (Hagopian et al., 2003). **Sirt5** deacetylates and activates carbamoyl phosphate synthetase 1, which initiates the urea cycle, eliminating the ammonia produced during the starvation-induced amino acid utilization (Nakagawa et al., 2009). **Sirt6** is localized in the nucleus and is an ADP-ribosyltransferase playing an important role in DNA repair (North et al., 2003; Liszt et al., 2005; Michishita et al., 2005; Mostoslavsky et al., 2006). **Sirt7** is found in the nucleolus of mammals cells (Michishita et al., 2005; Ford et al., 2006), and although it lacks deacetylase and ADP-

ribosyltransferase activities, its interaction with RNA polymerase I is expected to play a role in rRNA transcription and protein synthesis and therefore in cellular growth and metabolism (North et al., 2003; Ford et al., 2006).

1.5.2.3 Sirtuin & caloric restriction

Low caloric diet or caloric restriction (CR) improves insulin sensitivity, decreases blood cholesterol, and is the most reproducible experimental condition increasing life span from yeast, worms, flies, crustaceans to mammals (Tuchweber & Salas, 1975). Indeed there is accumulating evidence that CR prevents the appearance of age related diseases, such as, cancer, cardiovascular and neurodegenerative diseases. CR is defined as suppression of total caloric intake by 25-50% compared to the ad libitum calorie consumption. Such diet is well equilibrated in nutrients and is not to be confused with malnutrition (Weindruch et al., 1986). In *S. cerevisiae* grown in medium with 0.5% glucose instead the standard 2% (w/v) glucose, which is defined as CR for yeast cells, the replicative lifespan is approx. 30% longer. Interestingly this effect was not observed in the absence of *SIR2*, suggesting that it may mediate some of the CR effects in the organism (Lin et al., 2000). Indeed Sir2 homologs were found to be essential for the metabolic improvement seen in several animal models under mild CR (Howitz et al., 2003; Cohen et al., 2004; Wood et al., 2004; Rogina & Helfand, 2004; Rodgers et al., 2005; Guarente & Picard, 2005; Haigis & Guarente, 2006; Michan & Sinclair, 2007; Cantó & Auwerx, 2009).

1.5.2.4 Sirtuin activating compounds

Sirt1 is under intense investigation for the development of pharmaceutical for the treatment of metabolic syndrome and age-related diseases. Screening for compounds that could activate the deacetylase action of Sirt1 led to the identification of several naturally occurring plant polyphenols, the most potent of which was found to be the 3,4',5 trihydroxystilbene ($C_{14}H_{12}O_3$) resveratrol (Resveratrol; Fig.8; Howitz et al., 2003). Resveratrol binding to Sirt1

promotes a conformational change that increases the affinity of the enzyme for the acetylated substrate (Borra et al., 2005).

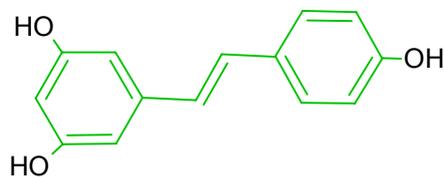


Figure 8 . Structure of resveratrol showing the stilbene core in green.

Resveratrol is synthesized in some plants as a defense mechanism against fungi attack or excessive UV light exposure (Langcake & Pryce, 1977; Schoeppner & Kindl, 1979). It is found in red grapes, peanuts, eucalyptus leaves and several berries (blueberry, bilberry and cranberry; Kimura et al., 1995; Burns et al., 2002). In dark grapes, resveratrol is concentrated mainly in the skin and seeds, from where it is extracted during the vinification process; as a result, red wines have high resveratrol concentration (up to 15 $\mu\text{g/ml}$) depending of the kind of grape and its origin. In fact, the presence of resveratrol in the red wine is believed to account in part for the so called ‘French paradox’, according to which French people suffer less by cardiovascular diseases despite their high fat diet compared to the US population (Sun et al., 2002; Das & Maulik, 2006; Lippi et al., 2010). Indeed in an experimental setting, resveratrol administration in mice fed with high fat diet improved insulin sensitivity, decreased plasma lipid levels and incidence of fatty liver and improved general health and survival (Baur et al., 2006). Resveratrol was also shown to have anti-platelet and anti-inflammatory (Renaud & de Lorgeril, 1992; Das & Das, 2007), chemopreventive (Jang et al., 1997), and neuroprotective action (Sun et al., 2010).

More potent synthetic Sirt1 activators, structurally related (SRT501; ResVidaTM) and unrelated (SRT1460, SRT2183, SRT1720, SRT2104, SRT2379) to resveratrol were identified. These substances are 1000-fold more potent than resveratrol and show an increased bioavailability (Milne et al., 2007), which is a big advantage since resveratrol is metabolically

unstable in the organism. SRT501, SRT2104 and SRT2379 are currently in clinical trials to test safety and efficacy in metabolic syndrome, cognitive function and cancer (clinicaltrials.gov).

2 AIM OF THE STUDY

GH as a major anabolic hormone is tightly regulated by energy availability. Caloric Restriction (CR) suppresses GH and IGF-I levels in several animal models. GH excess, as seen in patients with acromegaly-associated pituitary adenomas, leads to insulin resistance and is associated with increased mortality (Dekkers et al., 2008; Melmed, 2009). In contrast, genetically manipulating the GH/IGF-I axis improves insulin sensitivity and extends healthy life span (reviewed in Bartke & Brown-Borg, 2004; Holzenberger, 2004). Therefore, GH regulation by energy sensors may be one of the converge points where energy availability coordinates with hormonal stimuli to regulate organism homeostasis.

The aim of the study is to identify how intracellular energy sensors in the pituitary endocrine cells control the production of hormones pivotal in the regulation of organism growth and metabolism, such as GH. The putative role of AMPK and Sirt1 on the regulation of GH synthesis is investigated. Studies in the GHRKO mouse revealed that although moderate caloric restriction increases healthy life span in the wild type littermates, it does not further extend lifespan in this mouse (Bonkowski et al., 2006). Taking into consideration that GHRKO mice are GH resistant this finding indicates that caloric restriction needs an intact GH response in order to induce its beneficial effects. Subsequently, the study focuses on the role of Sirt1, as an intrapituitary energy sensor, in the regulation of GH synthesis.

3 MATERIALS & METHODS

3.1 Reagents

PRODUCT	COMPANY
ABC (Avidin-Biotin Complex) blocking kit	Vector laboratories (Burlingane, CA, USA)
Acetic acid	MERCK (Darmstadt, Germany)
Acridine orange	Sigma (St.Luis. MO, USA)
Acrylamide	Sigma (St.Luis. MO, USA)
5-Aminoimidazole-4-carboxamide ribotide (AICAR)	Calbiochem (Darmstadt, Germany)
Ammonium persulfate	Sigma (St.Luis. MO, USA)
Amphotericin B	Biochrom (Berlin, Germany)
Agar	Life Technologies (Paisley, Scotland, UK)
Agarose	Carl Roth (Karlsruhe, Germany)
β -mercaptoethanol	MERCK (Darmstadt, Germany)
Bovine serum albumin (BSA)	Invitrogen Corp.(Paisley, Scotland, UK)
Bradford protein assay	Biorad (Munich, Germany)
cAMP [125 I] determination RIA kit	Perkin Elmer (MA,USA)
Chloroform	Sigma (St.Luis. MO,)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
Diaminobenzidine (DAB)	Sigma (St.Luis. MO, USA)
Developer solution	Kodak (Stuttgart, Germany)
Diethyl-pyrocabonate (DEPC)	Sigma (St.Luis. MO, USA)
Dimethyl sulfoxide (DMSO)	Sigma (St.Luis. MO, USA)
DNase I	Invitrogen Corp.(Paisley, Scotland, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithuania)
Dulbecco's modified Eagle medium (DMEM)	Invitrogen Corp.(Paisley, Scotland, UK)
Dulbecco's modified Eagle medium (DMEM) with D-valine	Invitrogen Corp.(Paisley, Scotland, UK)
Entellan	MERCK (Darmstadt, Germany)
Ethanol 70%	Carl Roth (Karlsruhe, Germany)
Ethidium bromide	Sigma (St.Luis. MO, USA)
EZ ChIP Chromatin Immunoprecipitation Kit	Upstate (MA, USA)
Fetal calf serum	Gibco (Karlsruhe, Germany)
Fixer solution	Kodak (Stuttgart, Germany)
Formaldehyde 37%	Sigma (St.Luis. MO, USA)
Formamide	Sigma (St.Luis. MO, USA)
Forskolin	Sigma (St.Luis. MO, USA)
Glucose	MERCK (Darmstadt, Germany)

PRODUCT	COMPANY
Hepes	Sigma (St.Luis. MO, USA)
Hexanucleotide Mix	Roche (Mannheim, Germany)
HiSpeed Plasmid Purification System	Qiagen (Hilden, Germany)
Hyaluronidase	Sigma (St.Luis. MO, USA)
Hydrogen peroxide	Carl Roth (Karlsruhe, Germany)
Inhibitor 2 (I-2) specific for PP1	Millipore (MA, USA)
Insulin	Sigma (St.Luis. MO, USA)
Isoamylalcohol	MERCK (Darmstadt, Germany)
Isopropanol	Sigma (St.Luis. MO, USA)
L-Glutamine	Biochrom AG (Berlin,Germany)
Lumi-Light Western Blotting Substrate	Roche (Mannheim, Germany)
Luciferase kit dual-Luciferase Reporter Assay	Promega (Mannheim, Germany)
Magnesium chloride	MERCK (Darmstadt, Germany)
Marker 1kb Plus	Life Technologies (Paisley, Scotland, UK)
MEM-Vitamins	Biochrom AG (Berlin, Germany)
Metformin	Sigma (St.Luis. MO, USA)
Milk powder	Roth (Karlsruhe, Germany)
Nicotinamide	Calbiochem (Darmstadt, Germany)
Nitrocellulose membrane Hybond-ECL	Amersham Biosciences (Uppsala, Sweden)
Non-Essencial amino acids	Biochrom AG (Berlin, Germany)
Okadaic acid	Calbiochem (Darmstadt, Germany)
ONPG	Sigma (St.Luis. MO, USA)
Paraformaldehyde	Sigma (St.Luis. MO, USA)
Partricin	Biochrom AG (Berlin,Germany)
Penicillin+Streptomycin mix	Biochrom AG (Berlin, Germany)
Phenol	Roth (Karlsruhe, Germany)
Phosphatase inhibitor cocktail	Roche (Mannheim, Germany)
Phosphate based buffer (PBS)	Life Technologies (Paisley, Scotland, UK)
Polyacrylamide	Invitrogen Corp.(Paisley, Scotland, UK)
Potassium chloride	MERCK (Darmstadt, Germany)
Protease inhibitor cocktail	Sigma (St.Luis. MO, USA)
Protein A/G Agarose	Santa Cruz Biotech (CA, USA)
QuickChange II-Direct Mutagenesis Kit	Agilent Technologies, Inc. (CA, USA)
Reporter lysis buffer	Promega Corp. (Madison, WI, USA)
Resveratrol	Calbiochem (Darmstadt, Germany)
Reverse transcriptase (Super Script II)	Invitrogen Corp.(Paisley, Scotland, UK)
rGH [¹²⁵ I]	BIOTREND (Cologne, Germany)
rGH standard	National Institute of Diabetes & Digestive & Kidney Disease (CA, USA)

PRODUCT	COMPANY
rGHRH	Bachem (CA, USA)
RNAasin (RNase inhibitor)	Promega Corp. (Madison, WI, USA)
Rotiload	Roth (Karlsruhe, Germany)
Ser/Thr phosphatase Kit 1	Upstate (MA, USA)
Sirtinol	Calbiochem (Darmstadt, Germany)
Sodium chloride (NaCl)	Carl Roth (Karlsruhe, Germany)
Sodium hydrogen phosphate dehydrate	MERCK (Darmstadt, Germany)
SB-415286	Sigma (St.Luis. MO, USA)
Sodium Dodecyl Sulphate (SDS)	Carl Roth (Karlsruhe, Germany)
Sodium hydroxide (NaOH)	Carl Roth (Karlsruhe, Germany)
Sodium Selenate	Sigma (St.Luis. MO, USA)
Superfect	Qiagen (Hilden, Germany)
Taq DNA polymerase	MBI Fermentas (Vilnius, Lithuania)
TEMED	Sigma (St.Luis. MO, USA)
triiodothyronine T3	Sigma (St.Luis. MO, USA)
Toluidin Blue	Sigma (St. Louis, Mo, USA)
Transferrin	Sigma (St.Luis. MO, USA)
Trichloroacetic acid	Carl Roth (Karlsruhe, Germany)
Trypsin inhibitor	Sigma (St.Luis. MO, USA)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Triton X-100	Roth (Karlsruhe, Germany)
Trizol	Invitrogen Corp.(Paisley, Scotland, UK)
Trypsin	Sigma (St.Luis. MO, USA)
Tween 20	Sigma (St.Luis. MO, USA)
WST-1 assay	Roche (Mannheim, Germany)
Xylol	Carl Roth (Karlsruhe, Germany)

3.2 Solutions

Solution	Composition
Collagenase Mix 1000 U/ml	HDB ⁺ buffer : 100 ml Collagenase : 400 mg/ 100 ml solution Trypsin inhibitor : 10 mg/ 100 ml solution Hyaluronidase : 100 mg/ 100 ml solution BSA : 400 mg/ 100 ml solution DNase : 500 µl/ 100 ml solution
DNase Buffer 10X	Tris-HCL : 200 mM pH 7.2 MgCl ₂ : 50 mM DTT : 10 mM

MATERIALS & METHODS

Solution	Composition
HDB ⁺ buffer	Glucose : 10 mM NaCl : 137 mM KCl : 5 mM Na ₂ HPO ₄ : 0.7 mM HEPES : 25 mM Adjust pH 7.3 with NaOH Partricin : 500 µg/L Penicillin/Streptomycin : 10 ⁵ U/L
LB medium	Peptone : 10 g/L Yeast extract : 5 g/L NaCl : 5 g/L NaOH 1M : 2 ml/L Adjust to pH 7.0
Lower Tris-Base	Tris pH 8.8 : 182 g/L SDS : 4 g/L
Medium for tumor primary cell culture	DMEM Foetal Calf Serum (FCS) : 10% v/v Glutamine : 2.4 g/L Partricin : 500 µg/L Penicillin/Streptomycin : 10 ⁵ U/L MEM 1x Non-essential aminoacids (NEAA) 1x Insulin : 5 mg/L T3 : 60 pmol/L Transferrin : 5 mg/L Sodium Selenate : 20 µg/L
NP-40 lysis buffer for immunoprecipitation	Hepes pH 7.4 : 20 mM NaCl : 100 mM EDTA : 1 mM Nonidet P-40 : 1% v/v
ONPG buffer 2x	Na ₂ HPO ₄ 1M : 55.3 ml NaH ₂ PO ₄ 1M : 29.3 ml Water 1M : 339.2 ml MgCl ₂ -6H ₂ O : 154.5 mg ONPG : 500.0 mg 40 minutes stirring β-Mercaptoethanol 14M : 2.5 ml Freeze aliquots at -20 °C
Paraformaldehyde 4% (PFA)	paraformaldehyde : 4 g/100 ml Sodium phosphate buffer : 20 ml/100 ml solution Ampuwa water : 80 ml Add 1M NaOH to pH 7.4 Heat at 56°C to dissolve Filter and cool before usage Store at +4°C for maximum 2 days
Phosphate based buffer 1x (PBS)	NaCl : 8 g/L KCl : 0.2 g/L Na ₂ HPO ₄ ·2H ₂ O : 1.44 g/L KH ₂ PO ₄ : 0.2 g/L Adjust to pH 7.4
RIPA (RadioImmunoPrecipitation Assay) buffer	Tris HCl pH 8 : 50 mM NaCl : 150 mM NP-40 : 1% Sodium Deoxycholate : 0.5% SDS : 0.1%

Solution	Composition
Running Buffer for gel electrophoresis	Tris-base : 3.03 g/L Glycine : 14.42 g/L SDS : 1.00 g/L Adjust to pH 8.3
SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) running Gel 12%	Water (d) : 6.6 ml Acrylamide : 8 ml Lower Tris-Base pH 8.8 : 5 ml Ammonium persulfat 10%: 0.2 ml Temed : 0.008 ml
SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) stocking gel 5%	Water(d) : 4.1 ml Acrylamide : 1 ml Upper Tris Buffer pH 6.8 : 0.75 ml Ammonium Persulfat 10%: 0.06 ml Temed : 0.006 ml
Sodium acetate 2M	Sodium acetate trihydrate : 272 g/L DEPC : 200 µl /L Add acetic acid to pH 4.0 Leave at room temperature overnight
Sodium phosphate buffer 50 mM	Na ₂ HPO ₄ ·2H ₂ O : 7.06 g/L NaH ₂ PO ₄ ·H ₂ O : 1.32 g/L Adjust to pH 7.4
Stop mix	EDTA 0.5M : 16.5µl Tris-HCl 1M pH 8.5 : 150µl Water(d) sterile : 1.5 ml
Transfer Buffer for gel electrophoresis	Tris-base : 3.03g/L Gycine : 14.42 g/L SDS : 0.50 g/L Methanol : 200 ml/L Adjust to pH 8.3
Tris-acetic EDTA buffer (TAE) 50x	Glacial acetic acid : 57.1 ml/L EDTA 0,5M pH 8 : 100 ml/L Tris pure : 242 g/L Adjust to pH 8.0
Tris buffer 0.1M	Tris pure : 12.114 g/L Adjust to pH 7.6
Tris-Buffer Saline (TBS) 1x	Tris pure : 2.42 g/L NaCl : 8 g/L Adjust to pH 7.6
Tris- Buffer + Tween Saline (TBST)	Tris pure : 2.42 g/L NaCl : 8 g/L Tween 20 : 1 ml/L
Tris-HCl 1M	Tris pure : 121.14 g/L Add 25% HCl to a pH 8.2
Upper Tris Buffer 100ml	Tris-Base pH 6.8 : 60.5 g/L SDS : 4.0 g /L

3.3 Methods

3.3.1 Immunohistochemistry

Five frozen normal pituitary glands from autopsy cases of sudden death without any evidence for endocrine diseases taken 8-12 h after demise were cut in a cryostat (Leica CM3050 S), in 8 µm thick sections. The slides were fixed in 4% freshly prepared ice-cold paraformaldehyde, dehydrated and stored in 96% ethanol, at +4°C. At the time of the experiments, sections were incubated in TBS for 5 minutes, followed by 30 minutes blocking. Blocking was performed using serum from the animal species that gave rise to the secondary antibody; in our case the blocking solution consisted of goat serum diluted 1:20 in TBS. After blocking the endogenous peroxidase activity with 1% H₂O₂ diluted in TBS for 15 minutes, the sections were incubated overnight with the mouse monoclonal anti-Sirt1 primary antibody (Upstate, MA) at 4°C. Then, after washing three times with TBS (5 minutes each), the sections were incubated for 30 minutes with the secondary antibody at room temperature, and after 3 more washings, with the ABC complex for another 30 min. The ABC was prepared in saline-free tris-based buffer (Tris buffer) at least 30 minutes prior to usage in order to give time to form the complex. After washing three times in TBS the slides were immersed in freshly prepared DAB (1 mg/ml) supplemented with 0.01% hydrogen peroxide. Considering that DAB is light sensitive the reaction was carried on in semi-darkness, and the reagent was covered with aluminium foil during the experiment. The time of incubation in DAB varied and was determined for each primary antibody separately. The optimal time was the one giving the strongest expected signal with the lowest possible background.

After achieving a satisfactory signal level, the slides were washed three times in TBS and counterstained with toluidine-blue (which stains the cell nuclei pale blue) to allow an easy view of the tissue organization. Excess staining was removed by immersing the slides in 70% ethanol supplemented with acetic acid, and the sections were dehydrated, fixed in xylol and

coverslipped using Entellan. Evaluation of immunohistochemistry was done using the Axioscop II microscope (Zeiss).

3.3.2 Animals

The *in vivo* experiments were performed in agreement with the rules of laboratory animal care and international law on animal experimentation and the study was approved by the ethics committee of the University of Santiago de Compostela, where this part of the study was performed. In this experiment, adult male Sprague-Dawley rats (200-250 g) were used. The animals were housed in a temperature- and humidity-controlled room in a 12 hours light:12 hour darkness cycle. Five days before the experiment, they were anesthetized using ketamine-xylazine and intracardiac and chronic i.c.v. cannulae were implanted. After surgery, the animals were placed in isolation test camera and were fed with regular Purina rat chow and tap water (Seoane et al., 2004).

Animals (n=8-9) were injected i.v. with 10 µg/kg GHRH and intraperitoneally with 5 mg/kg vehicle or resveratrol for 1 hour and serial blood samples were withdrawn every 15 minutes for 6 hours (Seoane et al., 2004).

3.3.3 Cell culture

3.3.3.1 Cell lines

The immortalized cell line used in this study was the rat mammosomatotroph cell line GH3 (American Type Culture Collection). Cells were grown in DMEM supplemented with 10% FCS (Foetal Calf Serum), 2.4 g/L Glutamine, 500 µg/L Partricin, and 10⁵ U/L Penicillin/Streptomycin, and incubated at 37°C in humid atmosphere with 5% CO₂. When confluent, the cells were washed with PBS, trypsinized, centrifuged at 1200 x g for 4 minutes, and plated as needed for each experiment.

3.3.3.2 Rat pituitary primary cell culture

Pituitary primary cell culture was obtained from adult male Sprague-Dawley rats (180-250 g). The rats were maintained in the animal house for 5 days at a temperature of 21°C with light/darkness cycles of 12 hours, and fed Ad Libitum with standard food and water.

The animals were decapitated after narcosis with CO₂. The pituitary gland was extracted, washed with HDB⁺ buffer and sliced, and the fragments were digested and dispersed in a collagenase mix solution. The dispersed cells were centrifuged, washed and resuspended in growth media for cell culture.

The viability of the cells was determined by fluorescence microscopy, counting the cells in a Neubauer Chamber under UV light after staining them with ethidium bromide/acridine orange solution. Ethidium bromide dye is not able to pass through the intact membrane of the living cells, but it is able to do it through the damaged membrane of the dead cells, staining them with a red/orange fluorescence under UV light. Acridine orange passes through the membrane of healthy cells producing a green fluorescence under UV light.

The viability of the cells was higher than 95%. The cells were seeded into 96 well plates (1x10⁴ cells per well) and incubated at 37°C in a 5% CO₂ atmosphere for 48 hours before treating them as indicated in each experiment.

3.3.3.3 Human GH secreting pituitary adenomas

Thirty-eight human GH secreting pituitary adenomas or somatotrophinomas were investigated. This study was approved by the ethics committee of the Max Planck Institute and informed consent was obtained from each patient or their relatives. Tumors were diagnosed by clinical, radiological, surgical and biochemical findings. The patients were operated by the transsphenoidal microsurgical approach.

According to Hardy's classification (Hardy, 1979), pituitary adenomas are classified in four grades: Grade I for microadenomas (<10 mm in diameter), Grade II for macroadenomas (>10

mm in diameter) that may reach the suprasellar area but the surrounding bony structures are not affected. Grade III for adenomas that are locally invasive; and Grade IV refers to large invasive tumors that can affect the hypothalamus and the cavernous sinuses.

Tumors were put in DMEM immediately after surgical excision arriving to the laboratory within the same day or one day after the operation date. Samples were processed as following: After washing with HDB+ buffer, fibers and debris were removed and the tumor was divided in two pieces. One of them was snap-frozen with dry ice, for morphological analysis and the other was either frozen for RNA extraction or processed for cell culture as follows: after mechanical dispersion into small fragments and digestion with collagenase mix, the cells were processed and plated as described above for rat pituitary primary cell culture.

To exclude the contamination of normal anterior pituitary cells, RT-PCR (see 3.3.4) was performed using specific primers for the steroidogenic factor 1 (SF-1) transcription factor. SF-1 is expressed only in the gonadotroph lineage cells (Zafar et al., 1995; Fig. 2) therefore if detected in somatotrophinomas then this tissue has also normal pituitary cells. The SF-1 positive tumors were not taken into consideration for this study. Accordingly from the 31 somatotrophinoma RNAs, 25 were SF-1 negative and were therefore used for SIRT1 screening (Table 1).

Table 1. Diagnosis, age, gender and grade of severity of the pituitary adenomas involved in the study.

#	Diagnosis	Age	Sex	Grade	Assay
1	Acromegaly	32	M	III	RNA
2	Acromegaly	55	F	II	RNA
3	Acromegaly	42	F	III	RNA
4	Acromegaly	71	F	II	RNA
5	Acromegaly	28	M	III	RNA
6	Acromegaly	36	M	III	RNA
7	Acromegaly	23	M	II	RNA
8	Acromegaly	48	F	II	RNA
9	Acromegaly	58	F	II	RNA
10	Acromegaly	60	M	II	RNA
11	Acromegaly	54	M	II	RNA
12	Acromegaly	52	F	III	RNA
13	Acromegaly	49	M	II	RNA
14	Acromegaly	55	F	II	RNA
15	Acromegaly	36	F	III	RNA
16	Acromegaly	48	M	II	RNA
17	Acromegaly	52	F	III	RNA
18	Acromegaly	24	M	III	RNA
19	Acromegaly	51	F	II	RNA
20	Acromegaly	42	M	II	RNA
21	Acromegaly	50	F	II	RNA
22	Acromegaly	47	F	III	RNA
23	Acromegaly	24	F	II	RNA
24	Acromegaly	60	F	III	RNA
25	Acromegaly	27	M	III	RNA
26	Acromegaly	39	F	III	Cell culture
27	Acromegaly	54	F	II	Cell culture
28	Acromegaly	40	F	III	Cell culture
29	Acromegaly	42	M	III	Cell culture
30	Acromegaly	40	F	II	Cell culture
31	Acromegaly	54	F	II	Cell culture
32	Acromegaly	44	M	II	Cell culture
33	Acromegaly	38	F	III	Cell culture
34	Acromegaly	54	F	III	Cell culture
35	Acromegaly	37	F	III	Cell culture
36	Acromegaly	30	F	III	Cell culture
37	Acromegaly	51	M	III	Cell culture
38	Acromegaly	23	M	III	Cell culture

3.3.4 RNA extraction & reverse transcriptase-polymerase chain reaction (RT-PCR)

The experiment is based on the RNA isolation of treated and untreated cells, converting this RNA in its cDNA through reverse transcriptase (RT) reaction. Specific primers allow that only the cDNA obtained by the mRNA transcribed from the gene of interest is amplified by PCR. The product of the PCR is separated by electrophoresis in an agarose gel, and the bands of cDNA are visualized under UV light. The relative intensity of the bands reflects the relative quantity of cDNA, and therefore of source mRNA. This shows whether the treatment had some effect on the expression of the studied gene.

3.3.4.1 RNA extraction

Cells were plated in 6-well plates (3.5×10^5 cells/well) and treated according to the experiment. Once finished the treatment, the medium was removed, the cells were washed with cold PBS and 1 ml of Trizol was added to each well. The cells were homogenized and the homogenate was transferred to a 2 ml tube and incubated for 5 minutes at room temperature to allow the dissociation of nucleoprotein complexes; then 0.2 ml of chloroform was added to the homogenate, mixed for 15 seconds and incubated for 3 minutes at room temperature. The samples were centrifuged at $12000 \times g$ for 10 minutes at 4°C . At this stage, the mixture got separated into a lower red phenol-chloroform where the phenol-soluble proteins and cell remains can be found, an interphase containing the precipitated genomic DNA, and a colorless aqueous phase containing the RNA. The last phase was collected carefully, without disturbing the interphase, and was transferred to a new 2 ml Eppendorf tube. The RNA was precipitated from the aqueous phase by adding 500 μl of isopropanol. The samples were mixed and incubated 10 minutes at room temperature, followed by centrifugation at 4°C and $12000 \times g$. After 10 minutes of centrifugation, the supernatant was discarded, the pellet was washed with 1 ml of 70% ethanol, the sample was vortexed and the RNA was precipitated by

centrifugation at 7500 x g for 5 minutes at 4°C. Then the ethanol was carefully discarded, the RNA pellet was let to dry (air-dry) 5-10 minutes, and dissolved in 20-50 µl water supplemented with 0.1% (v/v) DEPC. DEPC inactivates RNases, protecting RNA against degradation.

When enough material was obtained each RNA sample was aliquoted, keeping one aliquot in -20°C for current use and the remaining at -80°C for long term storage.

To avoid using samples of RNA contaminated with genomic DNA for reverse transcriptase reaction, a polymerase chain reaction (PCR) was performed for a housekeeping gene (β -actin or GAPDH) using as template RNA sample that had not been reverse-transcribed (see PCR protocol).

DNase treatment protocol

In case of persistent DNA contamination the RNA sample was cleaned through DNase treatment.

For each RNA sample to be treated the following *DNase mix* was prepared

3.00 µl 10x DNase buffer

0.25 µl RNase free DNase I

0.25 µl RNasin

2.50 µl sterile water

Mix in the reaction tube

24 µl of DNA contaminated RNA (2-10 µg total)

6 µl DNase Mix

The DNA was digested incubating the tube for 30 minutes at 37°C.

After incubation, the reaction was finished by adding 33 µl of *stop mix*. Then 70 µl of Phenol-chloroform mix (1:1) was added and the tube was vortexed. To separate organic and aqueous phases, the tube was centrifuged for 5 minutes at 4°C and 12000 x g. The upper phase

(aqueous) was transferred to a new tube and sodium acetate 3 M pH 5.2 was added in a proportion 1/10. To precipitate the RNA again, 2.5 volumes of 100% ethanol were added and the mix was incubated at -20°C for 2 hours. After that, the sample was centrifuged at 4°C and 12000 x g. for 10 minutes. Supernatant was discarded, the pellet was washed with 1 ml of 70% ice-cold ethanol, and RNA was precipitated by centrifugation at 12000 x g for 10 minutes at 4°C. Then the ethanol was carefully discarded, the RNA pellet was let to dry (air-dry) 5-10 minutes, and finally it was dissolved in 10 µl water supplemented with 0.1% (v/v) DEPC.

Once RNA without genomic DNA was obtained, the concentration and quality of each RNA sample was determined using a spectrophotometer (Ultrospec II, Pharmacia). Absorbance (A) at wave lengths 260 and 280 nm was measured on a dilution of the RNA sample 1:60 (1 µl RNA solution + 59 µl DEPC water). Nucleic acids have a maximal absorption at 260 nm, while proteins have it at 280 nm. The ratio A₂₆₀/A₂₈₀ gives information about the quality of the RNA preparation. The optimal value of this ratio for a clean RNA solution should be between 1.9 and 2.0. The RNA concentration is calculated using the following formula: $A_{260} * 40 * 60 = x \text{ µg/ml}$, where 40 is the concentration in µg/ml of RNA when the A₂₆₀ is equal to 1, and 60 is the dilution in which the RNA sample was measured.

3.3.4.2 RT-PCR

Reverse transcriptase reaction was performed on the RNA samples. One µg of RNA diluted in DEPC water *quantum sufficiat* to 10 µl was added to a reaction mixture containing:

1 µl dNTP mix 2 mM

2 µl of random primers (Hexanucleotide Mix)

2 µl dithiothreitol (DTT) 10 mM

4 µl of 5x First Strand buffer

200 U reverse transcriptase (SuperScript II)

The mix was incubated at 45°C for 1 hour. The reaction was stopped by boiling at 95°C for 5 min. The obtained cDNA was stored at -20°C.

Polymerase Chain Reaction (PCR) Protocol

For the PCR reaction, 1 µl cDNA was mixed with a reaction mixture containing:

1.5 µl 10 x PCR buffer

0.9 µl 25 mM MgCl₂

1.5 µl 2 mM dNTP Mix

0.5 µl amplification primer 1 (10 pmol/µl)

0.5 µl amplification primer 2 (10 pmol/µl)

0.15 µl *Thermus aquaticus* (Taq) DNA polymerase 10x

8.95 µl autoclaved, distilled water

For the PCR reaction, the following program was used: denaturation at 95°C for 1 minute, annealing at 55-65°C for 1 minute, polymerization at 72°C for 1 minute, repeating these steps for 30-35 cycles.

After the PCR, the products were separated in agarose gel in a concentration depending on the size of the amplified product (1.2% for 400-1000 bp, 1.5% for 200-400 bp). The gel was prepared using TAE buffer and ethidium bromide (0.04%). Ethidium bromide is able to intercalate into the DNA producing fluorescence when visualized under UV light. Electrophoresis was run for 20-30 minutes at 90 V. The fragment size was determined using 1 Kb Plus DNA Ladder marker.

The primers used are listed in Table 2, with their sequences, melting temperature (T_m) and predicted product size. The sequence of each of them was checked using the NCBI BLAST program in order to exclude the possibility of a sequence similarity with genes other than the one under investigation. All primers were synthesized by Eurofins MWG Operon, were

reconstituted with autoclaved distilled water to reach a concentration of 100 μ M, and were stored at -20°C. The annealing temperature for each pair of primers was determined by PCR in a range of 55°C, 60°C, and 65°C using cDNA from normal human pituitary as template. The optimal temperature was the one yielding an intense signal with no secondary amplification fragments.

β -actin was quantified by digital analysis (Tina 4.0, Raytest, Munich, Germany). After subtraction of background signals, the relative gene expression levels were determined as the ratio of optical densities (OD) OD gene/OD β -actin. PCR reactions were performed in triplicates for each sample.

Table 2: Primers for PCR used in this study

Primers	Sequence (5'-3')	T _m	Fragment (bp)
β-actin-s human β-actin-a human	ACG GGG TCA CCC ACA CTG TGC CTA GAA GCA TTT GCG GTG GAC GAT G	60	660
GAPDH-s rat GAPDH-a rat	ATG GTG AAG GTC GGT GTG AAC G GTT GTC ATG GAT GAC CTT GGC	60	495
GH promoter-s rat GH promoter-s rat	GTG ACC ATT GCC CAT AAA CC TGC ATG CCC TTT TTA TAC CC	60	400
Pit1 promoter-s rat Pit1 promoter-a rat	TGA CGT CAA ATA AAG TTT CTG TTT T TGT TAA CCC GAA CTG TCT TTC TTA C	60	120
SF1-s human SF1-a human	GCA TCT TGG GCT GCC TGC AG CCT TGC CGT GCT GGA CCT GG	71	230
Sirt1-s human & rat Sirt1-a human & rat	TAA TGG TTT TCA TTC CTG TGA AAG T CAC AGT GTC ATA TCA TCC AAC TCA G	60	200

3.3.5 Western blot analysis

Through western blot analysis, one particular protein can be detected out of a mixture of many. A highly specific primary antibody for the protein of interest is used, and after that a

secondary one against the host of the first one. In this work the second antibody is conjugated with the enzyme horseradish peroxidase (HRP conjugated) which makes it possible to visualize the complex protein-antibodies through a chemiluminescent reaction. Information about the size and the relative quantity of the studied protein is obtained as well via this analysis.

3.3.5.1 Preparation of cell lysates for WB analysis from cells in culture

Cells were plated in 10 cm diameter petri (1.2×10^6 cells/petri), left 24 hours to recover and treated as indicated for 30 minutes, 1 hour, 3 hours, and 6 hours.

Once finished the treatment, the cells were washed twice with 10 ml of cold PBS, and then 1.5 ml of cold PBS was added. The cells were scraped using a cold plastic cell scraper, and the cell suspension was gently transferred to a pre-cooled 1.5 ml microcentrifuge tube and centrifuged at $1500 \times g$ at 4°C for 10 min. After the centrifugation, the supernatant was discarded and the pellet resuspended in 150 μL of RIPA Lysis Buffer containing protease inhibitor cocktail and phosphatase inhibitor. The cell suspension was passed several times through a 21 gauge syringe to lyse the cells.

3.3.5.2 Determination of the protein concentration

The protein concentration was measured using Bradford Protein Assay (Biorad, Munich, Germany). Bovine Serum Albumin (BSA) was used as protein standard.

The standard curve was determined for 25, 20, 10, 5 and 0 $\mu\text{g/ml}$ of BSA in double distilled water with RIPA in the same proportion as in the diluted samples. The Bradford reactive was diluted 1:1 in distilled water and the samples were diluted as much as needed so that the values of the protein concentration were into the range of the standard curve. 100 μl of each standard and each sample were put in transparent 96 well plate together with 50 μl of Bradford reactive. Absorbance was measured in Elisa reader M 5000 at λ 595 nm.

3.3.5.3 Sample preparation

Roti-Load Sample Buffer was added to 20 µgr of protein. The mix was denaturated by boiling 5 minutes.

3.3.5.4 Preparation of 12% SDS-polyacrylamide resolving gel

SDS-polyacrylamide resolving mini-gels were prepared using running gel and of stocking gel using the Mini-Protean 3 casting stand (Biorad).

3.3.5.5 Protein transfer

The equipment used was the Mini-Protean 3 Electrophoresis Cell and Mini Trans-Blot Cell of Biorad. The gels were put into the electrophoresis cell submerged in Running Buffer. Twenty µg of proteins were loaded into the wells of the gels. One well was always reserved for the marker in order to determine the size in kDa of the protein of interest. In this technique an electric field is applied to the electrophoresis cell so that the proteins (negatively charged) will migrate to the anode. The proteins are separated according to their sizes by the SDS-PAGE. Protein running was performed using constant 100 V. After the running the proteins were transferred into nitrocellulose membrane in the Trans-Blot Cell. In this case the negatively charged proteins are transferred into the special membrane that has a high affinity for proteins and it is positively charged. Blotting was performed overnight in cold room using 80 mA or two and a half hours using 500 mA, always keeping the transfer buffer at low temperature with ice into the chamber to preserve the gel from overheating. Finished the blotting the proteins and bands of the marker that were present in the gel were separated by size and transferred to the nitrocellulose membrane.

3.3.5.6 Membrane blocking and blotting

The nitrocellulose membrane was washed with TBST and blocked with 5% low fat milk powder dissolved in TBST (5% milk/TBST) during 1 hour.

Primary antibodies dissolved in 2.5% milk/TBST were incubated overnight (list of primary antibodies on Table 3). After incubation, the membranes were washed 3 times 5 minutes with TBST and then they were incubated 90 minutes with the secondary antibody HRP (also diluted in 2.5% milk/TBST) conjugated against the host of the primary one (list of secondary antibodies on Table 4). After incubation, the membranes were washed 3 times for 5 minutes with TBST and incubated with chemiluminescent substrate Lumi-Light for 5 minutes. The membranes were put in a cassette together with the films, trying different exposition times until the adequate for each particular experiment was found.

Table 3. List of primary antibodies used in western blot

Against	Company	Host	Dilution
Acetyl-lysine	New England Biolabs, Frankfurt, Germany	mouse	1:1000
Actin	Chemicon, Billerica, MA, USA	mouse	1:10000
AKT	New England Biolabs, Frankfurt, Germany	rabbit	1:1000
CREB	New England Biolabs, Frankfurt, Germany	mouse	1:1000
CREB	New England Biolabs, Frankfurt, Germany	rabbit	1:1000
GSK3 β	New England Biolabs, Frankfurt, Germany	rabbit	1:1000
I-2	R&D System, Minneapolis, MN, USA	mouse	1:500
Phospho-CREB (Ser ¹³³)	New England Biolabs, Frankfurt, Germany	rabbit	1:500
Phospho-GSK3 β (Ser ⁹)	New England Biolabs, Frankfurt, Germany	rabbit	1:500
Phospho-I-2 (Thr ⁷²)	Biozol, Eching, Germany	rabbit	1:500
Phospho-Threonine	New England Biolabs, Frankfurt, Germany	rabbit	1:500
Pit1 (X-7) sc442	Santa Cruz Biotech, Santa Cruz, CA, USA	rabbit	1:1000
PP1 α	Upstate, Billerica, MA, USA	rabbit	1:1000
Sirt1	Upstate, Billerica, MA, USA	mouse	1:2000

Table 4. List of secondary antibodies used in western blot Assay.

Against	Company	Conjugated	Host	Dilution
Rabbit	New England Biolabs, Frankfurt, Germany	Horseradish peroxidase	Goat	1:1000 - 1:2000
Mouse	New England Biolabs, Frankfurt, Germany	Horseradish peroxidase	Horse	1:1000 - 1:5000

3.3.6 Coimmunoprecipitation (Co-IP)

Through Co-IP it is possible to detect protein-protein interactions. The principle of this assay is the following:

One specific antibody against protein “X” which is part of the hypothetical complex is added to the cell lysate. The complex antibody-protein “X” is pulled down with help of protein A or G (which have a high affinity for antibodies) conjugated to a solid phase like agarose or sepharose. If one protein “Y” forms a complex with the protein “X”, then both will be pulled down together with the antibody. Other proteins that are not part of the complex are removed by washing the solid phase and, after releasing the complex from the matrix, the proteins “X” and “Y” can be detected for example through western blot analysis.

3.3.6.1 Preparation of cell lysates for Co-IP

Cells were plated in 10 cm diameter petri (1.2×10^6 cells/petri). Two petris were seeded per condition and 4 for Co-IP controls. They were left 24 hours to recover and treated with resveratrol 50 μ M for 60 minutes in 0% FCS medium. Finished the treatment, the petris were put on ice and the cells were washed twice with 10 ml cold PBS. Then 500 μ l of cold lysis buffer for immunoprecipitation NP-40 containing the protease inhibitor cocktail were added to the petris and the cells were scraped. The lysates of both petris with the same condition were collected in the same 1.5 ml Eppendorf tube and passed through a 21 gauge needle to improve the lysis of the cells.

The choice of the lysis buffer NP-40 to perform immunoprecipitation was made considering that its strength will preserve most of the protein-protein complex.

RIPA lysis buffer was not used because it has a higher stringency and could break the protein complex before being immunoprecipitated.

In order to preserve the integrity of the protein complex it is very important to keep always the lysate on ice. The lysates are centrifuged 10 minutes 12000 x g at 4°C the supernatants are transferred to new tubes.

3.3.6.2 Preparing Protein A/G Plus-Agarose

The commercial product Protein A/G Plus-Agarose of Santa Cruz was centrifuged at 4°C and 1000 x g for 10 min and washed (2x) with the same lysis buffer used above, resuspending the agarose without vortexing to avoid deformations of the solid matrix. After the washing the pellet agarose was resuspended in the same volume of lysis buffer to have 50% slurry. One hundred µl of the 50% slurry are needed for each tube. The mix has to be resuspended by flicking before use.

3.3.6.3 Pre-Clearing the cell lysates

To minimize unspecific binding of proteins to the agarose, the lysates have to be pre-cleared.

Fifty µl of the resuspended volume of the Protein A/G Agarose were added to each tube with the lysates and they were incubated on a rocker platform for 1 hour at 4°C.

The beads were pelleted by centrifugation at 1000 x g for 10 minutes and the supernatants were transferred to 1.5 ml Eppendorf tubes.

3.3.6.4 Adding the antibody

Because western blots of immunoprecipitated proteins have several unspecific bands, controls have to be incorporated and treated together with the samples from the beginning in order to run at the end in the same gel for western blot analysis.

Control tubes include:

- 1- Lysis buffer without cell lysate but with immunoprecipitating antibody.
- 2- Cell lysate without antibody but with unspecific IgG made in the same species than the immunoprecipitating antibody.
- 3- Positive control (complete cell lysate without IPP).

Antibodies were added to the respective tubes and incubated on a rocker platform at 4°C overnight. After the incubation, 50 µl of resuspended 50% slurry of Protein A/G Plus-Agarose were added to each tube, and they were incubated 3 hours on a rocker platform at 4°C. At this point the complex is bound to the agarose beads. The agarose was collected by centrifugation at 1000 x g for 10 minutes at 4°C and the supernatant was carefully aspirated and discarded. Pellets were washed 5 times with 1 ml NP-40 lysis buffer, each time repeating the centrifugation to eliminate the non-immunoprecipitated proteins. After the last wash the supernatant was discarded and the pellet was resuspended in 50 µl of Lysis Buffer with 1x Roti-Load Sample Buffer. Samples were boiled for 5 minutes to denaturize the proteins and release them from the agarose. After boiling tubes were centrifuged to pellet the solid matrix and the supernatants were analyzed using western blot assay following the protocol already described.

In the present work the following IPPs were performed (details of antibodies used on Table 5):

- CREB immunoprecipitation followed by western blot for acetyl-lysine, Sirt1 and CREB.
- Sirt1 immunoprecipitation followed by western blot for CREB and Sirt1
- I-2 immunoprecipitation followed by western blot for phospho-threonine and I-2.
- Acetyl-lysine immunoprecipitation followed by western blot for GSK3β.

Table 5. Antibodies used for protein Co-IP.

Antibodies	Company	Host	Dilution
Acetyl-Lysine (polyclonal)	New England Biolabs, Frankfurt, Germany	Rabbit	1:100
CREB (polyclonal)	New England Biolabs, Frankfurt, Germany	Rabbit	1:100
I-2 (monoclonal)	R&D Systems, MN, USA	Mouse	1:100
Sirt1 (monoclonal)	Upstate, MA, USA	Mouse	1:100

3.3.7 Chromatin immunoprecipitation (ChIP)

Through chromatin immunoprecipitation analysis (ChIP), it is possible to identify specific proteins that are associated with a region of the DNA (i.e. transcription factors) as well as regions of the DNA that are associated with specific proteins. The relative amount of linked protein-DNA in the cells can be determined for example by performing semiquantitative PCR.

3.3.7.1 Preparation of cells for ChIP analysis of GH promoter

GH3 cells were plated in two 10 cm diameter petri dishes (5×10^6 cells/petri) in 10 ml of growth media each, left 24 hours to recover. One of them was treated 1 hour with 50 μ M resveratrol and the other with vehicle. From that moment both petris were processed using the EZ ChIP Chromatin Immunoprecipitation Kit (Upstate) following the manufacturer's instructions.

Finished the previous treatment, the protein-DNA complexes were cross-linked with 270 μ l of formaldehyde 37%, and after 10 minutes at room temperature the excess formaldehyde was quenched with 1 ml 10x Glycine (kit provided). The plates were left at room temperature for 5 minutes to complete the reaction and then put on ice.

The medium was carefully aspirated to avoid losing cells. The cells were washed two times with cold PBS and after the last wash 1 ml of cold PBS containing 1x Protease Inhibitor Cocktail (kit provided) was added to each petri. The cells of each petri were scraped into 2

different microfuge tubes. Each tube (one control and one treated with resveratrol) was processed in identical way.

The cells were pelleted by centrifugation at 4°C and 700 x g for 5 minutes , the supernatant was removed and the pellet resuspended in 400 µl of SDS Lysis Buffer (kit provided) containing 1x Protease Inhibitor Cocktail.

The lysates were sonicated on ice to shear the cross-linked chromatin with a Sonifier cell disruptor B15 (6 output control, 80% duty cycle) applying pulses during 10 seconds. That was repeated 6 times, waiting 1 minute in between to allow heat dissipation. Different sonication conditions were tested to get the required DNA length from 200 to 1000 bp.

After sonication the lysates were centrifuged at 15000 x g at 4°C for 10 minutes and the supernatant of each tube was divided in three fractions of 100 µl.

Nine hundred µl of dilution buffer with Protease Inhibitor II (kit provided) were added to each of the three tubes containing 100 µl of sheared chromatin. Sixty microliter of Protein G Agarose 50% slurry (kit provided) were added to the tubes in order to remove proteins or DNA that might bind unspecifically to the Protein G Agarose (that is, to preclear the mix). The tubes were incubated 1 hour on a rotating platform and after incubation the agarose was pelleted by centrifugation at 5000 x g for 1 minute.

The supernatants were collected into 3 fresh 1.5 ml Eppendorf tubes and they were treated as follows:

- a) Five µg of antibody anti-acetyl-histone 3 (kit provided) were added to the first tube as a positive control and indirect indicator of Sirt1 histone deacetylase activity.
- b) In the second tube 5 µg of antibody against Pit1 (in the case of analysis of GH promoter) or CREB (in the case of analysis of Pit1 promoter) were added to immunoprecipitate the protein of interest.
- c) Five µg of Normal Rabbit IgG (included in the kit) were added to the third tube as a negative control.

The tubes were incubated overnight at 4°C on a rotating platform. The next day 60 µl of Protein G Agarose were added to each tube, and the incubation continued for 3 hours in the same conditions. The solid matrix captures the complex antibody-antigen/DNA. They were pulled by centrifugation at 5000 x g for 1 minute and the supernatant fraction was removed.

The pellet was washed with four different buffers, all of them provided by the kit. For each wash, the pellet was resuspended in 1 ml of the buffer, and then the tubes were rocked on the platform for 5 minutes and centrifuged at 4°C and 5000 x g for 1 minute. After that, the supernatant was carefully removed to avoid the lost of beads.

The washes were performed with the following buffers:

- 1- Low Salt Immune Complex Wash Buffer, one wash
- 2- High Salt Immune Complex Wash Buffer, one wash
- 3- LiCl Immune Complex Wash Buffer, one wash
- 4- TE Buffer, two washes

After washing, the complexes bound to the solid matrix were eluted with an elution buffer. This buffer was prepared using 10 µl 20% SDS, 20 µl 1M NaHCO₃, and 170 µl of sterile distillate water (total volume = 200 µl) for each tube. One hundred µl of elution buffer were added to each tube containing agarose, and they were mixed by flicking. After 15 minutes the agarose was pelleted by brief centrifugation and the supernatant was transferred into a new tube. The remaining 100 µl of elution buffer were added to the pellet and the elution steps were repeated. The respective elutes were pooled together.

Once eluted, all the samples were treated with 8 µl of NaCl 5M and incubated at 65°C overnight to reverse the cross-linking of DNA/Protein complexes. On the next day the samples were incubated with 1 µl of RNase A at 37°C during 30 minutes and after that 4 µl 0.5M EDTA and 8 µl Tris-HCL 1M and 1 µL of Proteinase K were added to each tube. They were digested for 2 hours at 45°C. At this point the free DNA that was before bound to the immunoprecipitated proteins was ready to be purified. Bind Reagent (kit provided) was added

to the 200 μ l of sample in a proportion 5:1 v/v to precipitate the DNA. Spin Columns (kit provided) are small columns with a special filter that retain the free DNA during centrifugation while the supernatant passes through and is discarded. The sample was put in the column, and was centrifuged for 30 seconds at 14000 x g. The DNA in the column was washed and eluted following the kit's instructions.

The purified DNA of the immunoprecipitates and their respective controls was analyzed using semi-quantitative PCR (protocol already described) using primers against the rat GH promoter (sense 5'-GTG ACC ATT GCC CAT AAA CC-3' and anti-sense 5'-TGC ATG CCC TTT TTA TAC CC -3') (Ezzat et al., 2005).

3.3.7.2 Preparation of cells for ChIP analysis of Pit1 promoter

To perform the ChIP on the Pit1 Promoter, two petri dishes with 5×10^6 GH3 cells each were pre-treated 30 minutes with 5 μ M forskolin in DMEM 0% FCS and then 1 hour with 50 μ M resveratrol or vehicle (DMSO). The cells were processed following the protocol already described, although this time antibody anti-CREB was used to immunoprecipitate the Pit1 promoter.

PCR assay was performed using primers for the rat Pit1 promoter (sense 5'-TGA CGT CAA ATA AAG TTT CTG TTT T-3' and antisense 5'-TGT TAA CCC GAA CTG TCT TTC TTA C-3'; see Table 1). Each ChIP was repeated twice.

3.3.8 Transfection

Cell transfection was performed with SuperFect (Qiagen, Hilden, Germany). Cells (3×10^5) were transfected for 3 hours with 1 μ g Flag-SIRT1 or Flag-SIRT1 H363Y plasmid, and 0.5 μ g GH-luc or CRE-luc reporter plasmids, and left in 2% FCS DMEM for 48 hours. The dual-Luciferase Reporter Assay (Promega) was used according to the manufacturer's instructions and luciferase activity was measured by the Berthold TriStar luminometer. Addition of 0.3 μ g

plasmid containing the Rous sarcoma virus promoter driving the β -Galactosidase gene ensured the proper control of the transfection efficiency.

β -galactosidase activity was measured as follows: 30 μ l distilled water were added to 20 μ l of the supernatant into a transparent 96-well plate, 50 μ l ONPG buffer (2x) were pipetted to the mix and the plate was incubated at 37°C in a light-protected box until the wells presented a light yellow color. β -galactosidase activity was measured in an ELISA plate reader at 420 nm. Data are expressed as the ratio of relative luciferase activity of plasmid to β -galactosidase activity.

3.3.8.1 RNA interference

Double-stranded small interfering RNA (siRNA) against rat Sirt1 (5'-GUA GAC CAA GCA ACA AAC ATT -3') was synthesized by Eurofins MWG Operon (Ebersberg, Germany). A scrambled siRNA (Scramble II; Eurofins MWG Operon) was used as a control. GH3 cells were transfected. Each experiment was performed in triplicates.

3.3.8.2 Plasmids

Competent bacteria Top 10 (Invitrogen, Paisley, UK) were transformed according to fabricant instructions with the desired plasmid (Table 6) and seeded on LB agar petri containing 50 μ g/ μ l ampicilin. The petri was incubated overnight at 37°C. One single colony was picked from the plate and put in 250ml LB medium containing 50 μ g/ μ l ampicilin and left to grow overnight at 37°C. The plasmid was purified using Qiagen HiSpeed plasmid purification system (Qiagen, Hilden, Germany).

The rGH-luc construct (kind gift of A. Gutierrez-Hartmann, University of Colorado, Denver, USA) has the proximal rat GH promoter upstream to the reporter gene luciferase. The CRE-luc construct (Mercury pathway profiling system, Clontech Laboratories, Inc., Palo Alto, CA) has the cAMP responsive element upstream to the TATA box of the herpes simplex virus thymidine kinase promoter and the reporter gene luciferase. Flag-SIRT1 (plasmid 1791) and

Flag-SIRT1 H363Y (plasmid 1792), this last one encoding an enzymatically inactive Sirt1, were both obtained in Addgene (Cambridge, MA, USA; deposited by M. Greenberg; Brunet et al., 2004). The -194pit1-luc, -92pit1-luc and the -231mutDCREpit1-luc and -231mutPCREpit1-luc bearing a mutation on the CRE binding element in the distal and proximal Pit1 promoter respectively were kind gift of C. Alvarez (Univ. of Santiago de Compostela; García et al., 2001).

Table 6. List of plasmids used in this study.

Plasmid	Source/kind gift of
-92pit1-luc	C. Alvarez, Univ. of Santiago de Compostela, Spain
-194pit1-luc	C. Alvarez, Univ. of Santiago de Compostela, Spain
-231mutDCREpit1-luc	C. Alvarez, Univ. of Santiago de Compostela, Spain
-231mutPCREpit1-luc	C. Alvarez, Univ. of Santiago de Compostela, Spain
β -Galactosidase	D. Spengler, MPI of Psychiatry, Germany
CRE-luc	Clontech Laboratories Inc., Palo Alto, CA, USA
Flag-SIRT1	Addgene. M. Greenberg; Brunet, Cambridge, MA, USA
Flag-SIRT1 H363Y	Addgene. M. Greenberg; Brunet, Cambridge, MA, USA
HA GSK3 β wt pcDNA3	Addgene. X. He et al.; Bethesda, Maryland, USA
rGH-luc	Gutierrez-Hartmann, University of Colorado, USA

3.3.9 cAMP intracellular radioimmunoassay

GH3 cells were seeded (320000 cells per well) in one 6 well plate. After 24 hour 3 wells were transfected with siRNA for Sirt1 and 3 wells were transfected with scramble non-specific RNA as control, as previously described. Next day the cells were removed from the plate using trypsin, the 3 wells with siSirt were pulled together, and the same was done with the 3 ones control. Then the cells were seeded in a 48 well plate. The next day they were treated for 1 hour with DMSO or resveratrol 50 μ M in DMEM medium without antibiotics, with

glutamine, and with 0.1% BSA and 0.5 mM IBMX. Forskolin 5 μ M was also used as control. After stimulation, the medium was carefully discarded taking care not to disturb the cells. Cold 5% trichloroacetic (TCA) acid (500 μ l) was added in the wells and incubated on ice overnight.

After incubation TCA was transferred from the wells into 10 ml glass tubes. TCA was extracted from the samples with 3 ml of diethyl ether saturated with water. The diethyl ether was aspirated and discarded. The extraction with the ether was done twice. Samples were frozen at -80°C overnight and next day they were lyophilized overnight in a P3K-S50 Lyophilizer.

cAMP determination was performed with a commercial RIA (Radio Immuno Assay) kit from Perkin Elmer. This assay is based on the specific binding between antibody and antigen. A competition takes place between radioactive cAMP [^{125}I] and non-radioactive cAMP (antigens) for the antibody. The system is incubated with a secondary antibody against the first one and the complex formed is precipitated through centrifugation to separate the bound from the free antigen. The radioactivity of the antigen-antibodies complex is measured to quantify the bound tracer. In this type of competitive analysis, the radioactivity measured is maximal when the amount of non-labeled cAMP is minimal and vice versa. A standard curve is constructed by performing the reaction with a constant concentration of labeled antigen and a variable concentration of standard cold cAMP. The unknown values of the samples were obtained by interpolation on this curve.

3.3.10 Measurement of cell proliferation

Cell proliferation was determined using the colorimetric WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany)) according to kit instructions. The cells were seeded in 96-well tissue culture plates (10.000 cells per well) and incubated at 37°C . After being left overnight to attach, cells were treated as each experiment demanded.

The WST-1 assay is based on a tetrazolium salt which is cleaved by the mitochondrial respiratory chain to a soluble formazan dye. The amount of dye obtained directly correlates to the number of viable cells in the culture. The reaction product was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm.

3.3.11 GH determination

In vitro: GH3 cells were seeded into 96 well plates (1×10^4 cells/well) in 10% FCS DMEM medium. After 24 hours the cells were treated with resveratrol or DMSO for 24 hours in 0% FCS DMEM medium. Once concluded the treatment, the supernatants were transferred and frozen for posterior analysis and WST-1 assay was performed on the cells to determine cell proliferation.

Human somatotrophinoma cells were seeded into 48 well plates (1×10^5 cells/well) in tumor medium. After 48 hours incubation, the cells were treated as needed for each experiment for 24 hours. The supernatants were transferred and frozen for posterior hGH analysis and WST-1 assay was performed on the cells to determine cell proliferation.

Human GH concentration in supernatant was determined with IMMULITE 2000 Growth Hormone which is a solid-phase, two-site chemiluminescent immunometric assay. In this assay a solid-phase (bead) which is coated with a monoclonal anti-hGH antibody binds to the hGH of the sample, a second antibody also against hGH but containing the enzyme alkaline phosphatase is added to the reaction mix and forms an antibody-sandwich complex with the beads and the hGH. After wash the excess of antibody through centrifugation, a chemiluminescent substrate is added to the reaction. The intensity of the signal is proportional to the hGH concentration.

In vivo: Rat GH (rGH) concentrations in supernatant and plasma were determined by RIA analysis. During this assay radioactive rGH [^{125}I] (BIOTREND, Cologne, Germany) and non-radioactive rGH (antigens) compete for the primary specific anti-rGH antibody (made in rabbit; NIDDK, CA, USA). The day after the samples were incubated with a secondary

antibody against rabbit made in goat (Chemicon, MA, USA) for 1.5 hours, the immunocomplex was precipitated with PEG 6%, the pellet was washed, and the radioactivity was measured. rGH concentrations were calculated by comparing them against a standard curve made using different concentrations of standard rGH (NIDKK, USA).

Characteristics of the GH secretory pattern (mean GH levels, area under the curve (AUC) and amplitude) were assessed by the ULTRA program (kindly supplied by E Van Cauter; Van Cauter, 1988).

3.3.12 Immunofluorescence assay

GH3 cells were split onto Falcon culture slides (BD bioscience), and stimulated for 3 hours with vehicle + 5 μ M forskolin or 5 μ M forskolin + 50 μ M resveratrol. After treatment, cells were fixed for 5 minutes in cold 4% paraformaldehyde, and then the slides were blocked in 5% goat serum with 0.1% (v/v) triton X-100 for 30 minutes at room temperature. Slides were incubated over night at 4°C with rabbit polyclonal pCREB-Ser¹³³ antibody (1:100, Enogene, USA). At the next day, the slides were washed 3 times with TBS and incubated 45 minutes at room temperature with Alexa Fluor 594 goat anti-rabbit antibody (Invitrogen, Paisley, UK). Prolong Gold antifade reagent with DAPI (Invitrogen, Paisley, UK) was used to visualize cell nucleus.

3.3.13 Protein Phosphatase 1 (PP1) activity

To determine PP1 activity, this phosphatase was immunoprecipitated from resveratrol or DMSO treated GH3 cells (8 million cells / condition). After three hours treatment cells were scraped in 1 ml NP-40 lysis buffer, sonicated. Lysates were centrifuged at 12000 x g for 10 minutes and the supernatants were transferred to a fresh tube and incubated overnight with 10 μ l of anti-PP1 α antibody (Santa Cruz Biotech, CA, USA) at 4°C. The immunocomplex was precipitated with 50 μ l of Agarose A/G Plus and washed 6 times with the lysis buffer.

Phosphatase activity was determined using the Ser/Thr phosphatase Kit 1 (Upstate, MA, USA), which is based in the malachite green phosphatase assay. Agarose pellets containing the immunoprecipitated PP1 were resuspended and incubated with 50 μ l of kit reaction buffer containing 500 μ M of phosphopeptide during 30 minutes at room temperature. The immunoprecipitated enzyme releases phosphate group from the peptide. After a short centrifugation, 25 μ l of the supernatant were transferred to a 96 well plate and 100 μ l of Malachite Green Solution was added to each well. After 15 minutes, color development absorbance was measured at 630 nm in a microtiter plate reader. Absorbance of blank (buffer containing enzyme immunoprecipitated in absence of phosphopeptide) was subtracted to absorbance of the wells with the enzyme and phosphopeptide. A standard curve was determined following kit instruction's, with quantities of standard phosphate in the range of 2000 pmol to 0 pmoles in 25 μ l. The amount of released phosphate by the enzyme was determined by comparing absorbance of the probes to the standard curve.

3.3.14 Site-Directed Mutagenesis

The site-directed mutagenesis technique enables to change a given gene sequence by introducing a point mutation and mutate a complete codon, but also by causing multiple amino acids insertions or deletions in the original DNA sequence. This technique can be used for characterizing relationships between protein structure and function, studying gene expression elements, and modifying vectors.

In the present study the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc.) was used. This method is based on the synthesis of both mutated plasmid strands using as template the original plasmid and specially design primers carrying the desired mutation (mutagenic primers). The primers, each complementary to opposite strands of the vector, bind the template during the annealing phase and are extended during temperature cycling by the enzyme PfuUltra high-fidelity (HF) DNA polymerase (kit provided). Extension of the oligonucleotide generates a mutated plasmid containing the

desired mutation. Following temperature cycling, the product is treated with the enzyme Dpn I (kit provided), which is specific for methylated and hemimethylated DNA and is used to digest the DNA template, preserving the synthesized DNA containing the mutation. That is possible because the DNA isolated from almost all *E. coli* strains, in this case competent *E. coli* Top 10 (Invitrogen, Paisley, UK), is methylated since these strands have the enzyme DNA adenine methyltransferase (Dam^+) while the new synthesized mutated plasmid is not methylated. After digestion, the mutant vector can be transformed into XL10-Gold ultracompetent cells (kit provided). The new plasmid was purified using Qiagen HiSpeed plasmid purification system (Qiagen, Hilden, Germany).

3.3.14.1 Synthesis of GSK3 β K205R plasmid

In this work, site-directed mutagenesis technique was used to change the adenine to guanine in the codon encoding for lysine at position 205 in human GSK3 β , resulting in a lysine to arginine substitution (K205R). The HA GSK3 β wt pcDNA3 (Addgene plasmid 14753, deposited by X. He) plasmid was used.

The mutagenic primers were designed following the kit's primer design guidelines and were: sense 5'-CTGTGACTTTGGAAGTGCAAGGCAGCTGGTCC-3', antisense 5'-GGACCAGCTGCCCTTGCACCTTCCAAAGTCACAG-3' with the mutated base shown in red (Eurofins MWG Operon, Ebersberg, Germany). The special PCR program was as follows:

5 μ l of 10 \times reaction buffer (kit provided)

20 ng of plasmid HA GSK3 β wt pcDNA3.

125 ng of sense primer

125 ng of antisense primer

1 μ l of dNTP mix (kit provided)

double distilled H₂O to a final volume of 50 μ l

1 μ l of PfuUltra HF DNA polymerase (2.5 U/ μ l) (kit provided)

The cycling parameters were: one single step at 95°C for 1 minute, and then denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, polymerization at 68°C for 7 minutes, repeating these 3 last steps for 12 cycles. After the thermal cycling, 1µl of the Dpn I restriction enzyme was added and incubated for 1 hour at 37°C. Competent cells were transformed by heat shock and plated in LB Agar with ampicillin. The mutated plasmid was prepared as described in the section **3.3.14.1**. Successful mutagenesis was confirmed by sequencing the area of interest (Sequiseve, Vaterstetten Germany).

3.3.15 Statistical Analysis

Differences were assessed by one-way ANOVA in combination with Scheffé's test. *P* values less than 0.05 were considered as significant.

4 RESULTS

4.1 AMPK on GH synthesis

4.1.1 Effect of AMPK activation on GH promoter activity

The effect of AMPK activation on GH was studied using a plasmid containing the luciferase gene downstreams to the rat GH promoter (GH-luc). The GH3 immortalized somatotrophinoma cells were transfected with the GH-luc plasmid and AMPK was activated using the two commonly used AMPK agonists AICAR and metformin. Both AICAR and metformin suppressed GH promoter activity (suppression as % of vehicle control: 21 ± 8 and 30 ± 3 respectively for AICAR 2 mM and metformin 1 mM; Fig. 9A-B).

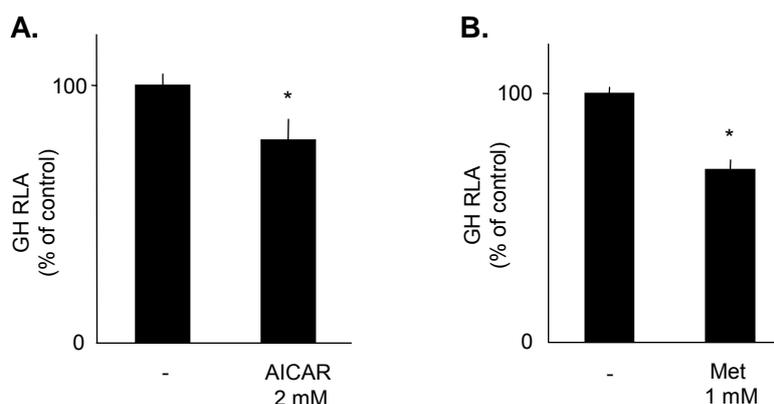


Figure 9. Effect of the AMPK activators A. AICAR and B. metformin on GH promoter activity. RLA: relative luciferase activity. Representative of three experiments is shown. * $P < 0.05$ to vehicle treated control.

4.1.2 Effect of AMPK activation on GH secretion *in vitro*

Treatment of rat anterior pituitary cells in primary cell culture with AICAR for 24 hours had a weak suppressive effect on GH secretion (suppression of GH secretion as % of control 17 ± 5 for 2 mM AICAR, $P = 0.013$; Fig. 10A). The effect of metformin was more potent than that of AICAR (% suppression: 31 ± 2 at the 1 mM and 18 ± 2 at the 0.5 mM dose, $P < 0.001$ and $P = 0.001$ respectively; Fig. 10B). Lower metformin doses at 0.15 mM had no significant effect on GH secretion.

To confirm metformin's suppressive effect on GH, ten human somatotrophinomas in primary cell culture were treated with 2 mM of the compound for 24 hours. Metformin suppressed GH

RESULTS

secretion from four out of ten cases (% suppression: 35 ± 16 ; Fig. 10C). However, the majority of cases did not respond to metformin by lowering GH secretion.

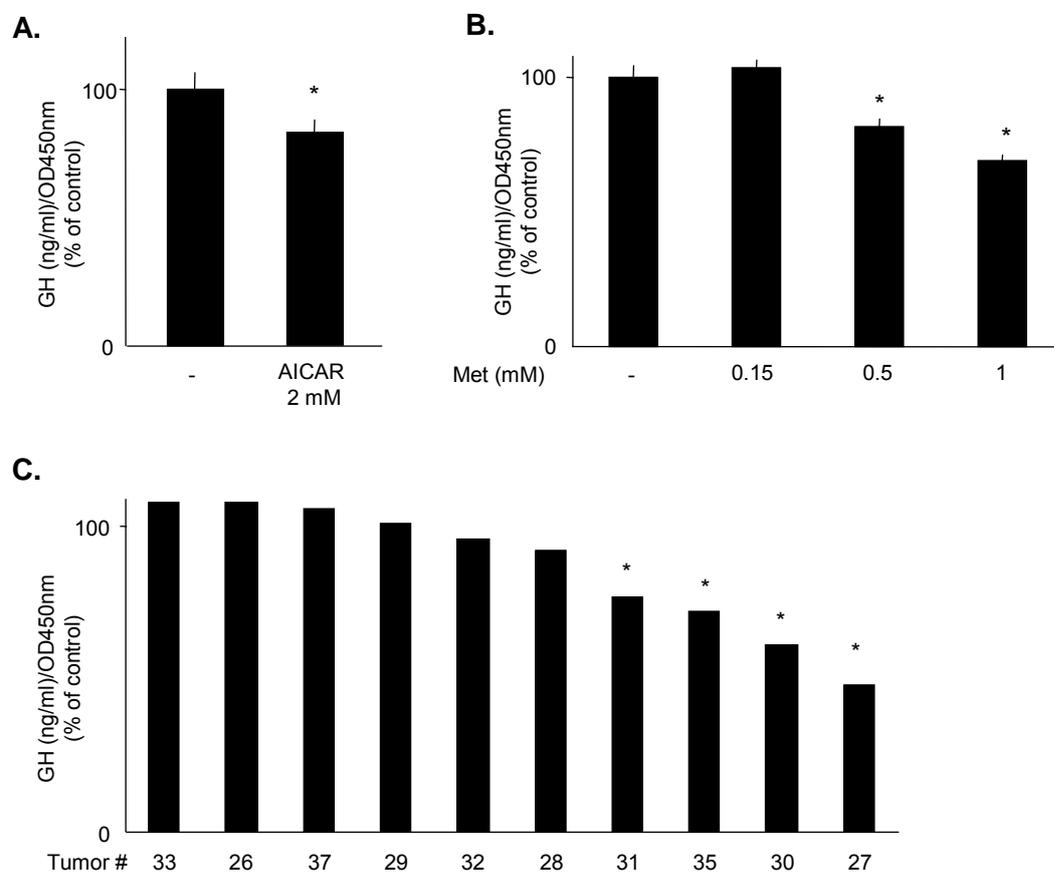


Figure 10. Effect of 24 hour treatment with A. 2 mM AICAR and B. 0.15, 0.5 and 1 mM metformin (Met) on GHRH (10 nM) induced GH secretion from rat GH-secreting GH3 cells. C. Metformin at 1 mM concentration suppressed GH secretion from four human somatotrophinomas in primary cell culture. All treatments were performed in serum free DMEM. For each condition, GH RIA values were divided to the cell viability counts as determined by WST-1 at OD 450nm. Final values are presented as percentage of vehicle control. * $P < 0.05$ to vehicle treated control.

Altogether the low, albeit significant, potency of the AMPK activators on GH synthesis indicates that AMPK may not be the primary energy sensor regulating GH synthesis in the pituitary.

4.2 Sirt1 on GH synthesis

4.2.1 The Sirt1 activator resveratrol reduces GH levels *in vivo*

To activate Sirt1 the natural polyphenol resveratrol was used (Howitz et al., 2003). To establish that resveratrol suppresses GH secretion, the compound was administered in adult male rats and serum GH levels were determined. I.p. administration of 5 mg/kg resveratrol, a treatment that improves insulin sensitivity (Baur & Sinclair, 2006), had a slight, but not significant, effect on basal GH levels (resveratrol treated 10 ± 2 ng/ml vs. vehicle treated 14 ± 10 ng/ml). I.v. GHRH injection raised GH levels after 5 minutes in both vehicle and resveratrol treated animals, but GH pulse amplitude was significantly lower in resveratrol treated rats (RSV: 492 ± 37 ng/ml vs. vehicle: 1125 ± 130 ng/ml; $P=0.005$, Fig. 11A). Furthermore, resveratrol suppressed total GHRH-induced GH secretion as determined by area under the curve (AUC; 1455 ± 107 vs. vehicle treated 5861 ± 2967 ; $P=0.007$, Fig. 11B), demonstrating that Sirt1 activation decreases circulating GH levels.

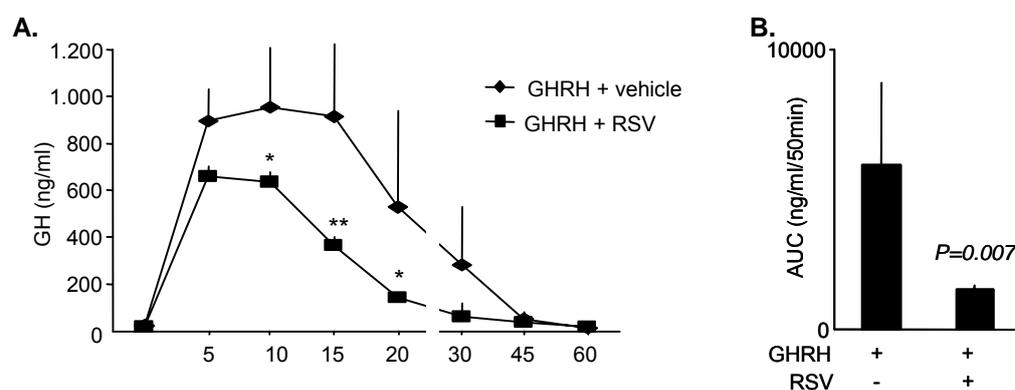


Figure 11. A. Mean plasma GH levels after administration of GHRH (10 μ g/kg i.v.) plus vehicle or resveratrol (RSV; 5 mg/kg i.p.) in male adult rats (n=8 per group). * $P<0.05$; ** $P<0.001$. Blood was taken every 5 minutes for the first 20 minutes and every 15 minutes for the remaining time and GH levels were determined by RIA. B. Mean AUC in male rats injected with vehicle or resveratrol in the presence of GHRH.

4.2.2 Resveratrol reduces GH secretion from pituitary somatotrophs *in vitro*

To identify if resveratrol suppresses GH secretion at pituitary level, rat anterior pituitary cells in primary cell culture were treated with 10 nM of GHRH (to induce GH) and 50 μ M resveratrol for 24 hours. Resveratrol suppressed GHRH-induced GH secretion in these isolated rat anterior pituitary cells demonstrating a direct pituitary action (Fig. 12A). It also suppressed GH secretion from the immortalized pituitary GH-secreting GH3 cells (Fig. 12B). These data demonstrate that resveratrol suppresses GH synthesis directly at pituitary level.

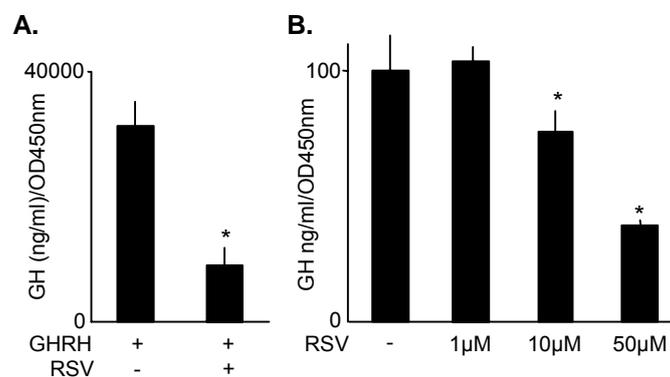


Figure 12. A. Inhibitory effect of 24 hour treatment with 50 μ M resveratrol on GHRH (10 nM) induced GH secretion on rat anterior pituitaries in primary cell culture. B. Resveratrol dose response (1 μ M, 10 μ M and 50 μ M) on GH secretion in rat GH-secreting GH3 cells. All treatments were performed in serum free DMEM. For each condition GH RIA values were divided to the cell viability counts as determined by WST-1 at OD 450nm. Final values are presented as percentage of vehicle control. * $P < 0.05$ to vehicle treated control

4.2.3 Sirt1 is expressed in the anterior pituitary

Intense Sirt1 immunoreactivity was found in the nuclei of all endocrine cells in human (Fig.13) and rat anterior pituitary.

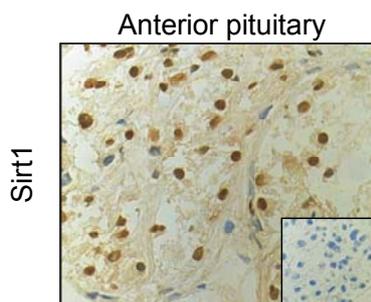


Figure 13. Sirt1 immunoreactivity on a human anterior pituitary. Sirt1 is present in the nuclei of all endocrine cells as shown by DAB immunostaining (brown). Inserts: negative control omitting the primary antibody. Nuclei were counterstained with toluidine blue. Magnification 200X.

4.2.4 Sirt1 activation reduces GH secretion from pituitary somatotrophs *in vitro*

To prove that resveratrol effect is mediated through Sirt1, GH3 cells were transfected with siRNA against Sirt1 or treated with the specific Sirt1 inhibitor sirtinol. Inhibiting Sirt1 with siRNA or sirtinol abolished the suppressive action of resveratrol on GH (Fig. 14A and B) demonstrating a Sirt1 specific effect. In addition, basal GH secretion was increased in cells in which Sirt1 was knocked down with siRNA (Fig. 14C) or inhibited with sirtinol (Fig. 14D) further demonstrating a suppressive action of Sirt1 on GH synthesis.

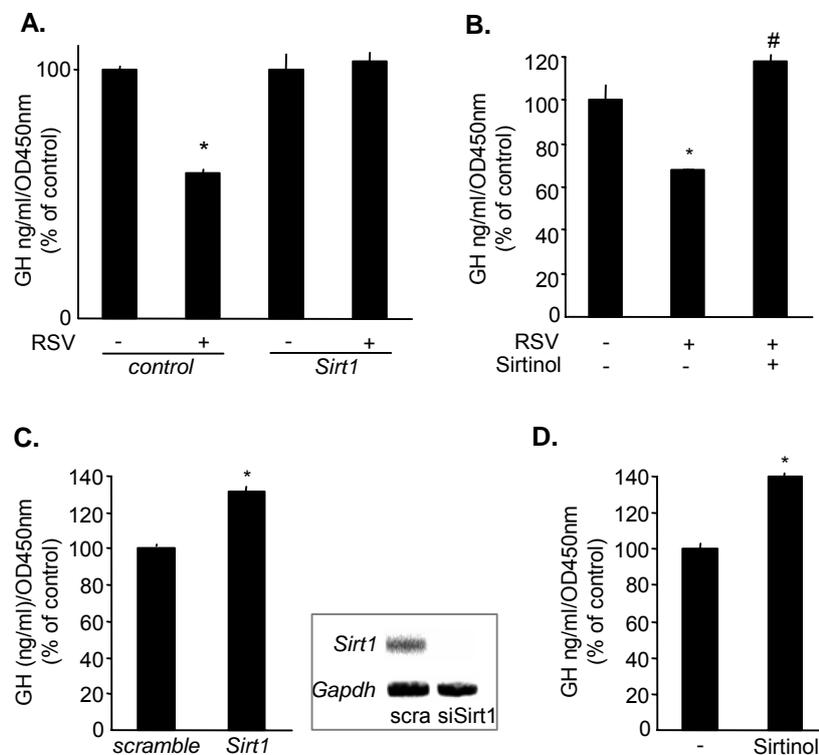


Figure 14. A. Effect of Sirt1 RNA interference on GH secretion. GH secretion was measured in GH3 cells transfected with 100 nM scramble (*control*) or Sirt1 (*Sirt1*) siRNA. Representatives of two independent transfection experiments are shown. B. Effect of the specific Sirt1 inhibitor sirtinol (10 μ M) on GH secretion. C. Effect of Sirt1 RNA interference on resveratrol's suppressive action on GH release. GH secretion was measured in GH3 cells transfected with 100 nM scramble or Sirt1 siRNA, and treated with resveratrol for 24 hours. D. Effect of sirtinol on resveratrol-suppressed GH secretion. All treatments were performed in serum free DMEM. For each condition GH RIA values were divided to the cell viability counts as determined by WST-1 at OD 450nm. Final values are presented as percentage of vehicle control. * $P < 0.05$ to vehicle treated control; # $P < 0.001$ to resveratrol treated.

4.2.5 Sirt1 activation reduces GH secretion from human somatotrophinomas *in vitro*

Resveratrol had effectively suppressed GH synthesis in the rat GH3 cells that are in fact GH-secreting pituitary tumor cells or somatotrophinomas (Fig. 12). These data indicated that it could also affect GH secretion from human somatotrophinomas from patients with acromegaly. Sirt1 transcript was readily detected in all 25 somatotrophinomas screened by RT-PCR (Fig. 15) albeit at varying levels.

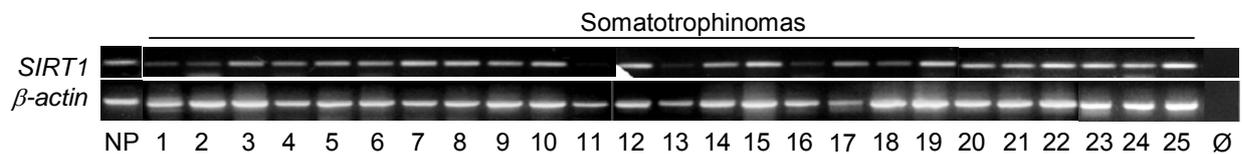


Figure 15. RT-PCR for SIRT1 and β -actin in one normal anterior pituitary (NP) and 25 somatotrophinomas (numbers indicate the tumors as shown in Table 1) from one representative experiment. \emptyset represents PCR reaction without template.

Treatment of 13 human somatotrophinomas in primary cell culture with 50 μ M resveratrol suppressed GH secretion more 20% in 8 out of 13 cases (% mean suppression 30 ± 7 ; Fig. 16A). Treatment with the Sirt1 inhibitor sirtinol in one case (#27) that provided enough cells for this experiment abolished the inhibitory effect of resveratrol on GH secretion (% mean suppression 28 ± 1.5 after resveratrol, $P=0.023$ vs. % mean increase 17 ± 1.1 after resveratrol+sirtinol, $P=0.025$ to resveratrol; Fig. 16B). This result confirms that the effect of resveratrol on GH secretion is through Sirt1 also in human somatotrophinoma cells.

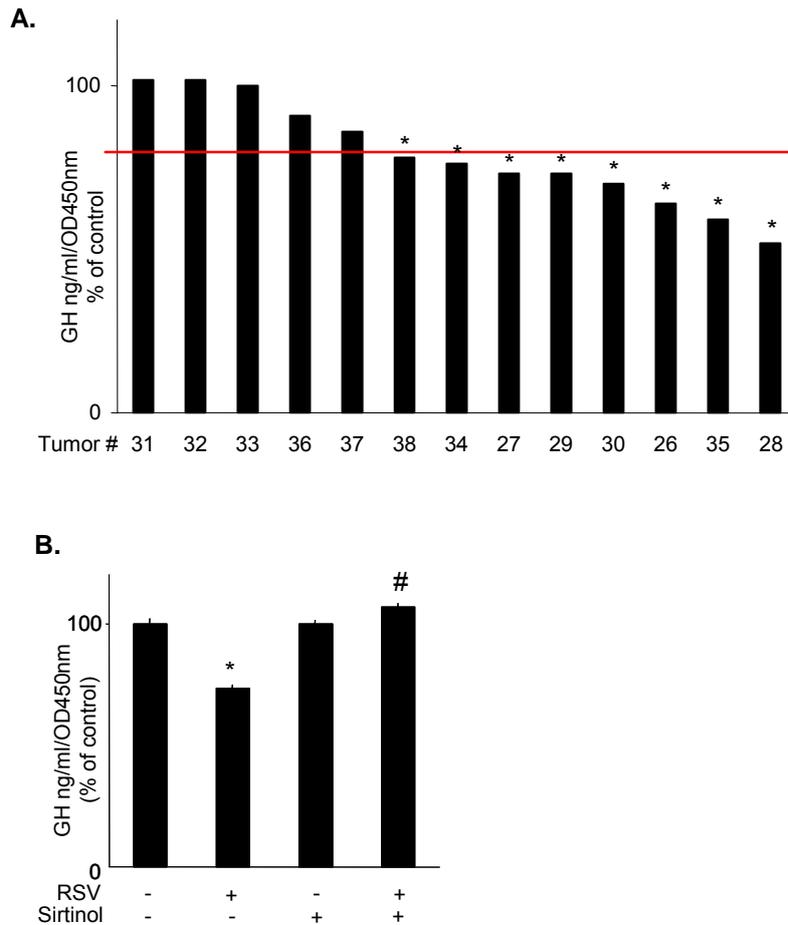


Figure 16. A. GH secretion from 13 human somatotrophinoma in primary cell culture (case # in Table) treated with 50 μ M resveratrol. B. Effect of the Sirt1 inhibitor sirtinol (1 μ M) on resveratrol (50 μ M) induced suppression in GH secretion in case #27. Treatments were performed in 10% FCS-DMEM for 24 hours. Each GH value was divided with the WST-1 colorimetric assay (determining cell viability) value obtained from the same sample. Data are presented as percentage of vehicle treated control. Each condition was done in quadruplicates. * P <0.05 to vehicle control; # P <0.05 to resveratrol.

Treatment in one case that provided enough cells for this experiment (#28) with 12, 25 and 50 μ M resveratrol for 24 hours suppressed GH secretion in one somatotrophinoma in primary cell culture (mean suppression of GH secretion as % of vehicle control: 48 \pm 1.3, 51 \pm 2, 62 \pm 2.8 respectively; all P <0.001; Fig. 17A). In contrast treating the same tumor with the Sirt1 inhibitor sirtinol significantly increased basal GH secretion by 20% (% mean increase of GH secretion 29 \pm 2.5 at 1 μ M, P =0.053; 20 \pm 2.2 at 100 nM, P =0.060; Fig. 17B).

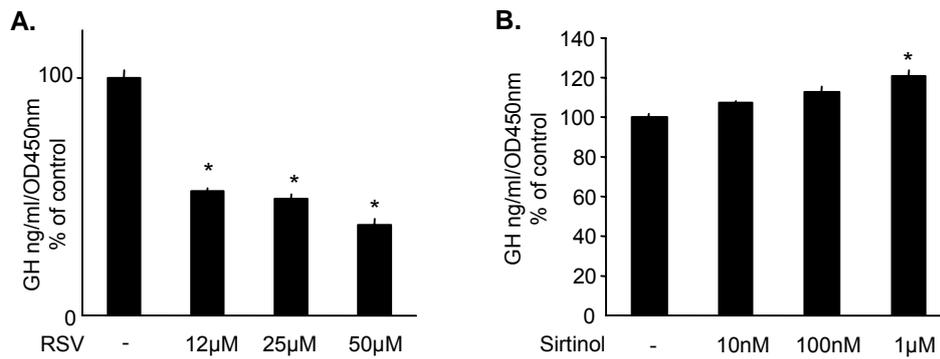


Figure 17. GH secretion from a human somatotrophinoma in primary cell culture (case #28 in Table) treated with A. resveratrol (* $P < 0.001$) and B. the Sirt1 inhibitor sirtinol (* $P = 0.043$). Treatments were performed in 10% FCS-DMEM for 24 hours. Each GH value was divided with the WST-1 colorimetric assay (determining cell viability) value obtained from the same sample. Data are presented as percentage of vehicle treated control. Each condition was done in quadruplicates.

These data indicate that Sirt1 activation decreases GH synthesis also in human somatotrophinomas.

4.2.6 Sirt1 reduces GH promoter activity

To elucidate the mechanism behind the inhibitory action of Sirt1 on GH synthesis, its action on the GH promoter was studied in GH3 cells. The cells were transfected using a plasmid containing the luciferase gene downstreams to the GH promoter (GH-luc).

Sirt1 overexpression suppressed GH promoter activity (Fig. 18A). In contrast inhibiting Sirt1 by siRNA (Fig. 18B), nicotinamide (NAM) or sirtinol increased GH promoter activity (Fig. 18C). These data demonstrate that Sirt1 affects GH levels by downregulating its transcription.

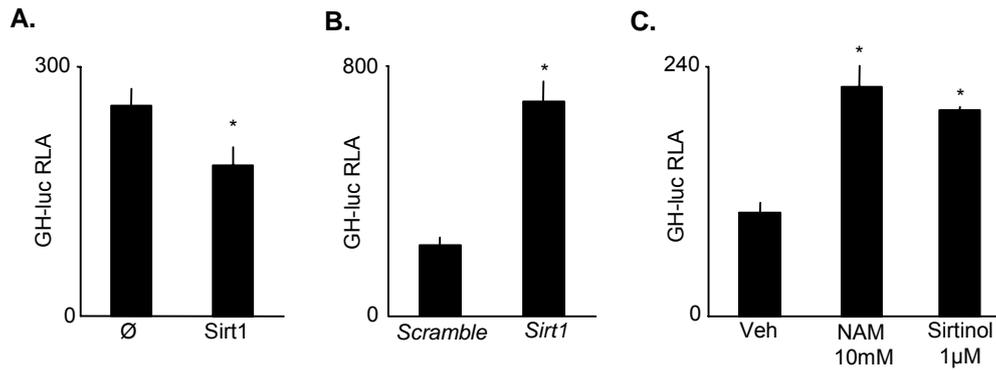


Figure 18. Effect of Sirt1 A. overexpression and B. knockdown on GH promoter activity in GH3 cells. C. Effect of the Sirt1 inhibitors NAM and sirtinol on GH promoter activity. Empty vector (Ø), scrambled sequence siRNA (Scramble) or Vehicle (Veh) were used as control. RLA: relative luciferase activity. Representative of three experiments is shown. $P < 0.05$.

4.2.7 Sirt1 activation reduces Pit1 binding to GH promoter by suppressing Pit1 transcription

To understand how Sirt1 inhibits GH promoter activity, chromatin immunoprecipitation (ChIP) experiments were performed in GH3 cells. Sirt1 did not directly bind to the rat GH promoter. No CREB binding was detected, which is in accordance with previous studies reporting absence of CRE on the rat GH promoter (Tansey et al., 1993; Cohen et al., 1999). In contrast, GHRH induced Pit1 binding was detected on the GH promoter, which was substantially decreased after 3 hours of 50 μ M resveratrol treatment (Fig. 19A). Resveratrol decreased Pit1 levels (Fig. 19B), and this is Sirt1 specific action since it was not observed in GH3 cells in which Sirt1 was knocked down by RNA interference (Fig. 19C). These data show that Sirt1 activation downregulates Pit1 transcription and limit its availability to the GH promoter.

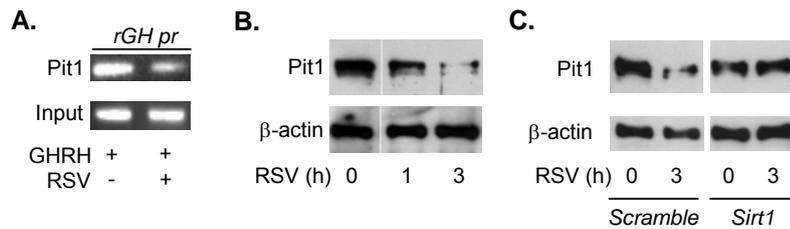


Figure 19. A. Effect of 3 hours treatment with 50 μ M resveratrol on Pit1 binding to the GH promoter in GH3 cells as determined by chromatin immunoprecipitation. Treatments were performed in serum free DMEM. Representative of two experiments is shown. B. Effect of resveratrol on Pit1 levels in GH3 nuclear extracts. C. Effect of Sirt1 RNA interference on resveratrol's suppressive action on Pit1. Representative of two experiments is shown.

4.2.8 Sirt1 suppresses CREB transcriptional activity

Pit1 transcription is primary governed by CREB and the Pit1 promoter has canonical CRE sites (McCormick et al., 1990). Eliminating Sirt1 by siRNA increased Pit1 promoter activity (Fig. 20A). However it did not affect Pit1 promoter constructs in which CRE sites were mutated (-231mutDpit1-luc and -231mutPpit1-luc) or progressively deleted (-194pit1-luc and -92pit1-luc; García et al., 2001; Fig. A) indicating that Sirt1 acts at CREB level.

ChIP for Pit1 promoter was performed on GH3 cells challenged with 5 μ M forskolin to increase CREB activity and treated with resveratrol. Resveratrol treatment decreased forskolin-induced CREB binding to the Pit1 promoter (Fig. 20B). Sirt1 activation with resveratrol did not affect CREB transcription and CREB protein levels (Fig. 20C). Instead, its overexpression suppressed CRE transcriptional activity (Fig. 20D) while the opposite was observed after inhibiting Sirt1 with siRNA, nicotinamide or sirtinol (Fig. 20E & 20F). These data indicate that Sirt1 suppresses CREB transcriptional activation.

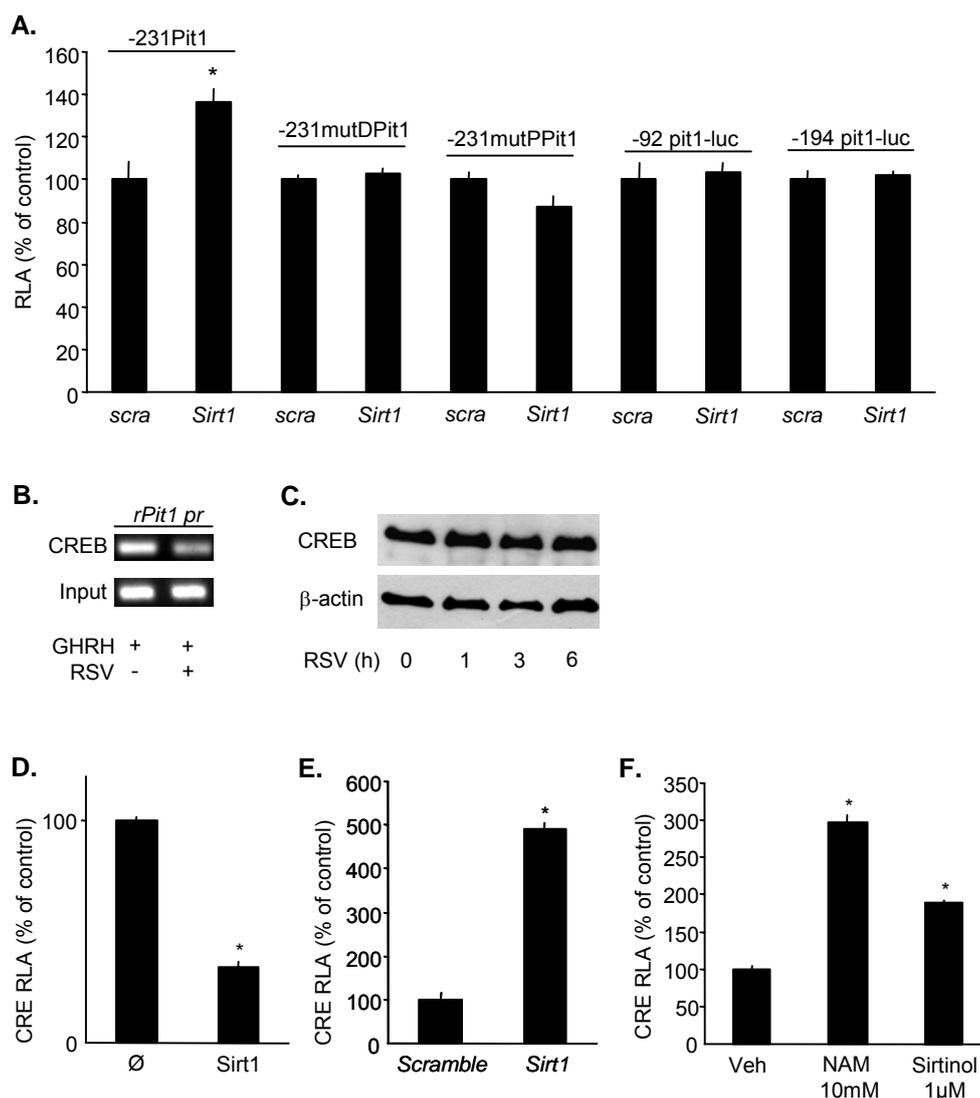


Figure 20. A. Effect of Sirt1 knockdown (*Sirt1*) on intact Pit1 promoter (-231Pit1) activity and on constructs lacking both or one of the two CRE (-92pit1-luc and -194pit1-luc), or having mutation in the CRE (-231mutDPit1 and -231mutPPit1). Results are shown as RLA: β -galactosidase ratio. Data are expressed as percentage of individual scrambled controls. * $P < 0.05$. B. Effect of 2 hours 50 μ M resveratrol treatment on CREB binding to the Pit1 promoter in GH3 cells as determined by chromatin immunoprecipitation. Cells were pretreated with 5 mM forskolin for 30 minutes. Representative of two experiments is shown. C. Total CREB levels in GH3 cells treated with 50 μ M resveratrol as determined by western blot. Effect of D. Sirt1 overexpression, E. Sirt1 siRNA and F. Sirt1 inhibition by 10 mM NAM or 10 μ M sirtinol on CRE transcriptional activity in GH3 cells. Empty vector (\emptyset), scrambled sequence siRNA (Scramble) or Vehicle (Veh) were used as control. Treatments were performed in serum free DMEM. Results are shown as RLA: β -galactosidase ratio and data are expressed as percentage of control. Each experiment was repeated twice.

4.2.9 Sirt1 suppresses CREB phosphorylation

GHRH and cAMP-raising agents, such as forskolin, activate CREB transcriptional activity by inducing its phosphorylation at Ser¹³³, resulting in increased CREB-driven transcription. Sirt1 activation with resveratrol or inhibition by RNA interference did not affect basal or forskolin

RESULTS

induced intracellular cAMP levels (Fig. 21A), indicating that its suppressive effect on CREB phosphorylation is not due to decreased intracellular cAMP levels. Resveratrol treatment suppressed forskolin induced CREB-Ser¹³³ phosphorylation (Fig. 21B), but not in GH3 cells in which Sirt1 was knocked down demonstrating a Sirt1 specific effect (Fig. 21C). In addition, GH3 cells overexpressing Sirt1 displayed lower levels of forskolin-induced pCREB-Ser¹³³ compared to the empty vector control (Fig. 21D).

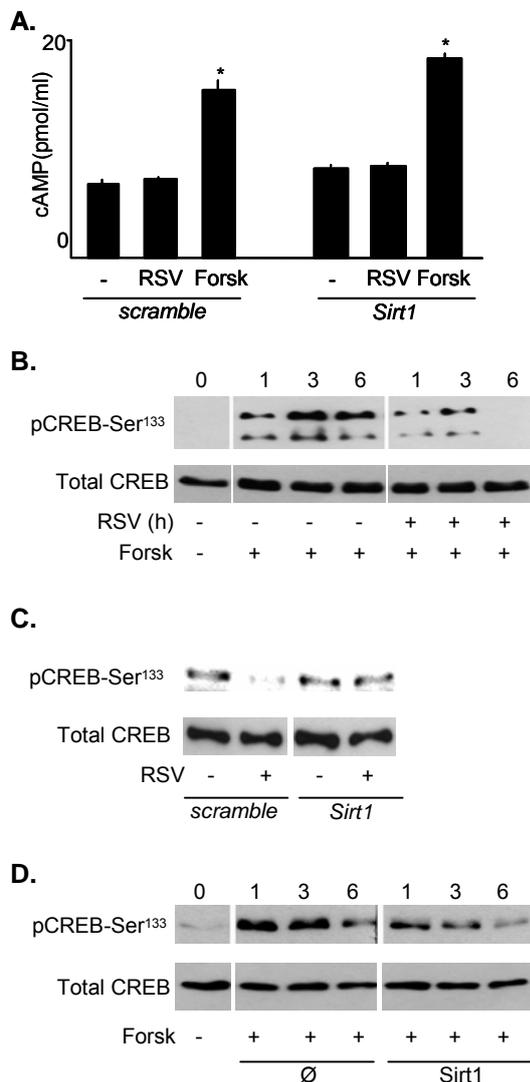


Figure 21. A. Intracellular cAMP release was determined after 1 hour treatment with 50 μM resveratrol in GH3 cells transfected with 100 nM scramble or Sirt1 siRNA. For control, cells were treated with 5 μM forskolin. Data are presented as percentage of each control. *: P<0.001. Representatives of two independent transfection experiments are shown. B. GH3 cell lysates treated with 50 μM vehicle or resveratrol for 1, 3 and 6 hours in the absence or presence of 5 μM forskolin analyzed by western blot using anti-pCREB-Ser¹³³ and -CREB. C. Effect of Sirt1 RNA interference (*Sirt1*) on pCREB-Ser¹³³ levels in GH3 cells treated with 50 μM vehicle or resveratrol for 1 hour. D. pCREB-Ser¹³³ levels after forskolin treatment for 1, 3 and 6 hours in GH3 cells transfected with a control (∅) or Sirt1 overexpressing plasmid as determined by western blot. Representatives of three experiments are shown.

The suppressive effect of Sirt1 on forskolin-induced CREB phosphorylation at Ser¹³³ was confirmed by immunofluorescence in GH3 cells treated with resveratrol (Fig. 22). These data further demonstrate an inhibitory action of Sirt1 on CREB phosphorylation.

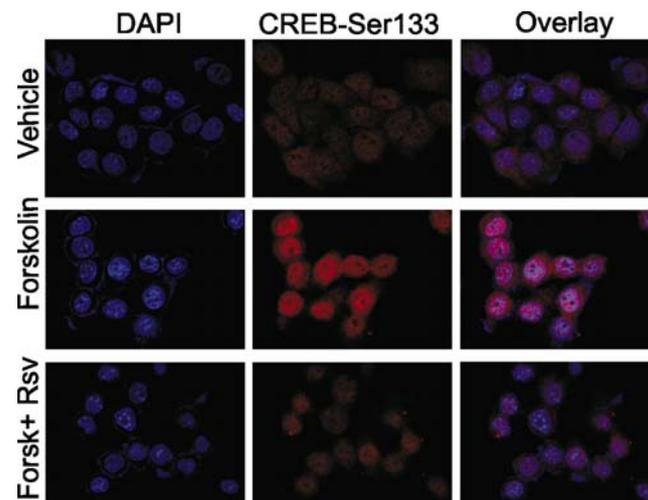


Figure 22. Effect of resveratrol on forskolin induced pCREB-Ser¹³³ immunofluorescence.

4.2.10 Sirt1 acts through the protein phosphatase 1 (PP1)

CREB is primarily dephosphorylated by the protein phosphatase PP1 (Alberts et al., 1994). PP1 exists as an oligomeric complex composed of the catalytic subunit PP1c and other regulatory subunits, including the inhibitor I-2. Inhibiting PP1 by introducing I-2 in GH3 cells abolished the suppressive effect of resveratrol on CREB phosphorylation (Fig. 23B). The Sirt1-induced decrease in pCREB-Ser¹³³ phosphorylation levels indicates the involvement of a protein phosphatase. Indeed pretreatment with the serine/threonine phosphatase inhibitor okadaic acid abolished resveratrol's suppressive action on pCREB-Ser¹³³ levels (Fig. 23A). Sirt1 overexpression increased PP1 activity (Fig. 23C). In contrast, inhibiting Sirt1 with RNA interference suppressed PP1 activity (Fig. 23D), indicating a role for Sirt1 on PP1 activation.

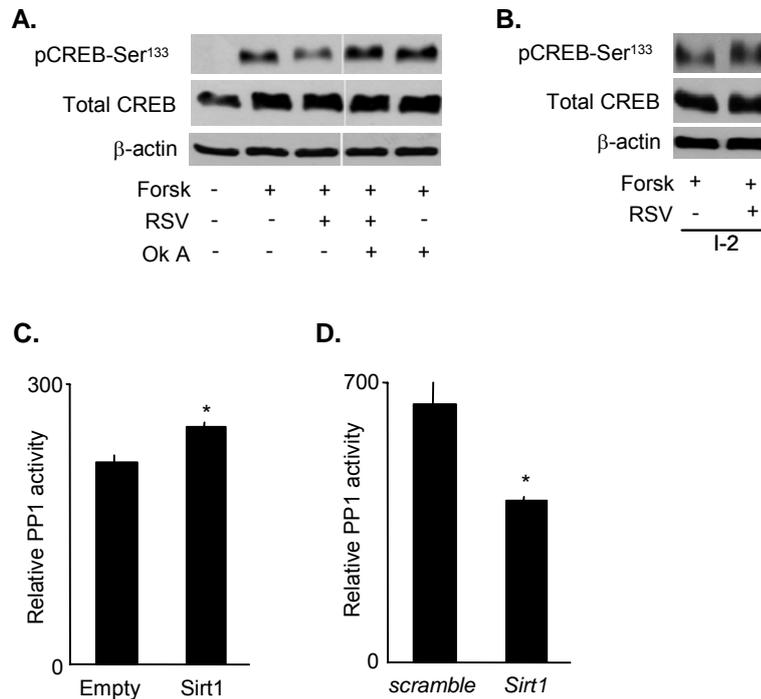


Figure 23. Effect of 50 μ M resveratrol (RSV) on forskolin-induced pCREB-Ser¹³³ in cells A. pretreated with 10 nM okadaic acid (OkA) for 2 hours or B. co-treated with I-2, as determined by western blot. C-E. Relative PP1 activity as determined in OD 630 in GH3 cells transfected with empty vector or Sirt1; scramble control or Sirt1 siRNA. Representative of two experiments are shown.

4.2.11 Sirt1 acts through GSK3 β

PP1 is activated when I-2 is phosphorylated at threonine residues and released from the complex. Sirt1 activation with resveratrol increased the immunoprecipitated I-2 levels detected with a phospho-threonine antibody (Fig. 24A). I-2 is primarily phosphorylated by GSK3 β at Thr⁷² (Hemmings et al., 1982). Resveratrol treatment increased I-2-Thr⁷² phosphorylation levels indicating an effect through GSK3 β (Fig. 24B). Inhibiting GSK3 β with SB-415286 abolished resveratrol's suppressive action on phosphorylated pI-2-Thr⁷² levels (Fig. 24B). Furthermore, it abolished its suppressive action on forskolin-induced CREB phosphorylation levels (Fig. 24C), demonstrating an important role for GSK3 β in Sirt1 dependent CREB dephosphorylation.

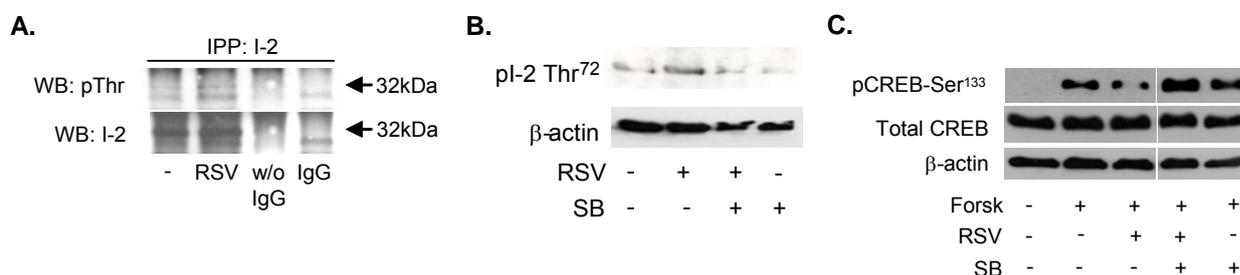


Figure 24. A. GH3 cells were treated with 50 μ M resveratrol for 2 hours and cell lysates were immunoprecipitated with agarose A/G and anti-I-2 or a control rabbit IgG. The immunoprecipitated fractions were analyzed by western blot using anti-phosphothreonine (pThr) or anti-I-2. B. western blot for I-2 phosphorylated at Thr⁷² in GH3 cells treated with vehicle control, resveratrol. In the last two lanes cells were pretreated with 14 μ M of the GSK3 β inhibitor SB-415286 (SB). C. Forskolin induced pCREB-Ser¹³³ levels in GH3 cells treated with 50 μ M resveratrol (RSV) alone or plus 14 μ M SB-415286 (SB).

Interestingly, SB-415286 treatment potentiated basal CREB transcriptional activity and CREB-Ser¹³³ phosphorylation, indicating a general suppressive role for GSK3 β on CREB activity (Fig. 25).

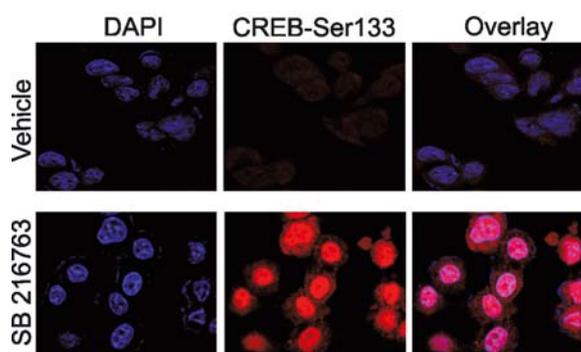


Figure 25. Effect of the GSK3 β inhibitor SB-415286 on basal pCREB-Ser¹³³ immunofluorescence.

4.2.12 Sirt1 affects GSK3 β phosphorylation and activates GSK3 β

GSK3 β is inhibited upon serine phosphorylation. Sirt1 activation with resveratrol suppressed the inhibitory GSK3 β -Ser⁹ phosphorylation (Fig. 26A). Cells in which Sirt1 was inhibited by RNA interference displayed high pGSK3 β -Ser⁹ levels (Fig. 26B). Neither intervention affected basal GSK3 β levels. Active GSK3 β phosphorylates glycogen synthase (GS) at Ser⁶⁴¹.

Sirt1 activation increased GS -Ser⁶⁴¹ levels and this was abolished by inhibiting GSK3 β with the specific inhibitor SB-415286, confirming its stimulating effect on the kinase (Fig. 26C).

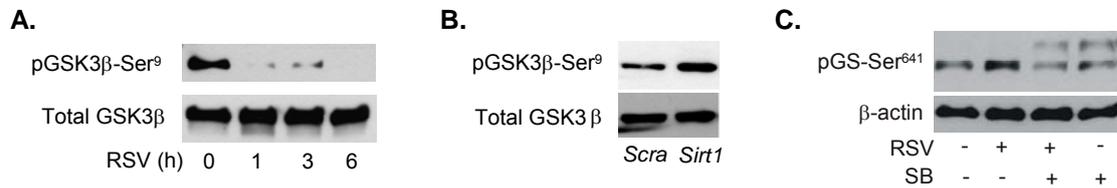


Figure 26. A. GH3 cell lysates treated with 50 μ M resveratrol for 1, 3 and 6 hours analyzed by western blot using anti-pGSK3 β -Ser⁹ and -GSK3 β . B. GH3 cells transfected with 100 nM scramble or Sirt1 siRNA and analyzed by western blot using anti-pGSK3 β -Ser⁹ and -GSK3 β . C. GH3 cells treated with 50 μ M resveratrol for one hour alone or with the 14 μ M GSK3 β inhibitor SB-415286 and analyzed by western blot for pGS-Ser⁶⁴¹. Representatives of three experiments are shown.

4.2.13 The deacetylase activity is essential for Sirt1's action

Sirt1 is a deacetylase so a mutant Sirt1 bearing a point mutation in the deacetylase domain (histidine at codon 363 substituted by tyrosine) was employed in order to test whether this enzymatic activity is needed for its effects on GH promoter activity, CRE activation and phosphorylation. Transfection with the deacetylase-defective Sirt1 H363Y did not suppress pCREB-Ser¹³³ levels (Fig. 27A). In contrast, although forskolin-induced CREB phosphorylation declined after three hours in mock transfected cells, pCREB-Ser¹³³ levels remained high even after 6 hours in cells transfected with the non-deacetylating SIRT1 H363Y, demonstrating that the deacetylase activity of Sirt1 is important for CREB dephosphorylation. This was also corroborated by the observation that contrary to the wild type SIRT1, SIRT1 H363Y could not suppress CREB transcriptional activity (Fig. 27B). In addition, no suppression of rat GH promoter activity was observed in cells transfected with the catalytically inactive Sirt1 (Fig. 27C). These data show that Sirt1 suppresses CREB phosphorylation and its deacetylase activity is needed for a physiological CREB dephosphorylation.

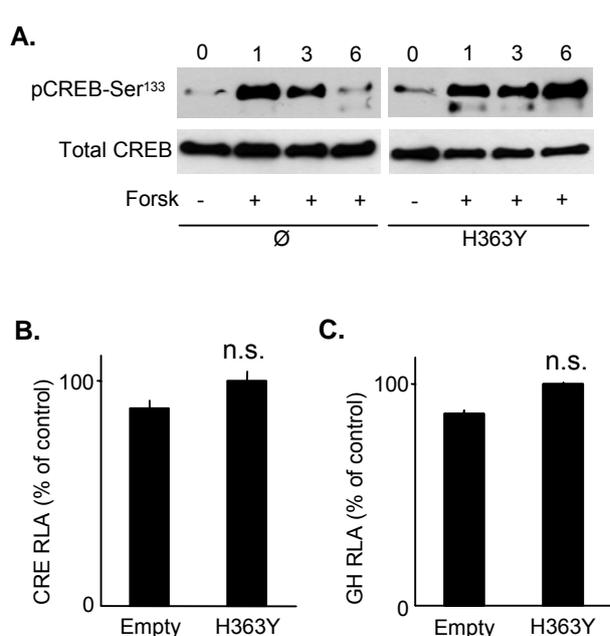


Figure 27. A. pCREB-Ser¹³³ levels after forskolin treatment for 1, 3 and 6 hours in GH3 cells transfected with a control or a plasmid overexpressing the deacetylase dead SIRT1 H363Y as determined by western blot. Empty vector (Ø) was used as control. Each experiment was repeated twice. Effect of the enzymatically inactive SIRT1 H363Y on B. CRE transcriptional activity and C. GH promoter activity. RLA: relative luciferase activity. Luciferase was measured 48 hours after transfection. Results are shown as luciferase:β-galactosidase ratio. Each experiment was repeated twice. *P<0.001.

4.2.14 Sirt1 deacetylates CREB

CREB was previously reported to be acetylated, an event that leads to enhanced CREB-dependent transcription (Lu et al., 2003). In GH3 cells, Sirt1 activation with resveratrol decreased the levels of acetylated CREB detected with an acetyl-lysine specific antibody (Fig. 28B), while co-immunoprecipitation experiments revealed a physical association between Sirt1 and CREB (Fig. 28A). Therefore, Sirt1 activation could suppress CREB transcriptional activity by physically associating with and deacetylating CREB.

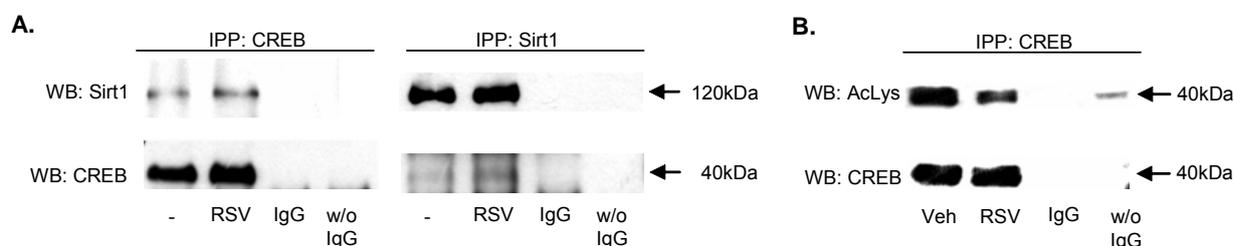


Figure 28. A. Cell lysates from GH3 cells treated with 50 μM resveratrol for 2 hours were immunoprecipitated with agarose A/G and anti-CREB or a control rabbit IgG or anti-Sirt1 or a control mouse IgG. The immunoprecipitated fractions and the whole lysates were analyzed by western blot using anti-Sirt1 or anti-CREB. B. Similarly cell lysates were immunoprecipitated with agarose A/G and anti-CREB or a control rabbit IgG and the immunoprecipitated fractions and the whole lysates were analyzed by western blot using anti-acetyl-lysine (Ac-Lys). Representatives of two experiments are shown.

4.2.15 Sirt1 affects GSK3 β acetylation and activity

The data until now revealed that a deacetylase intact Sirt1 is needed for its action on CREB dephosphorylation. In fact, transfection with the deacetylase dead SIRT1 H363Y suppressed PP1 activity and increased basal GSK3 β -Ser⁹ phosphorylation indicating the importance of a deacetylation event (Fig. 29A-B). In addition, the suppressive action of GSK3 β on CREB transcriptional activity depends on Sirt1 since transfection with the deacetylase dead Sirt1 mutant H363Y abolished this effect (Fig. 29C).

Endogenous GSK3 β was readily detectable in acetyl-lysine immunoprecipitates (Fig. 29D), which were decreased after Sirt1 activation by resveratrol (Fig. 29E). Co-immunoprecipitation studies revealed a physical association between endogenous Sirt1 and GSK3 β (Fig. 29D).

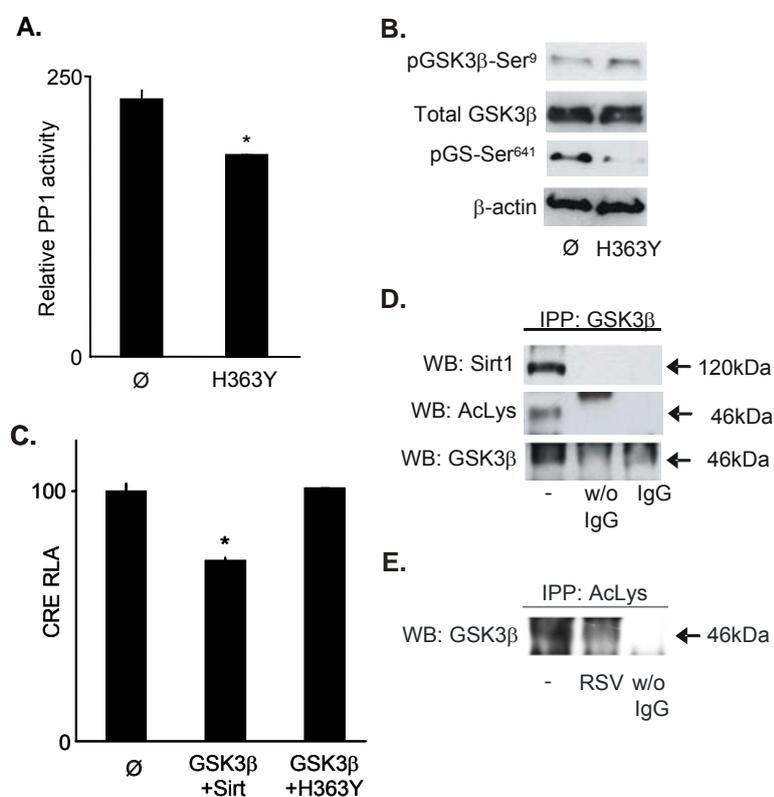


Figure 29. A. Relative PP1 activity as determined in OD 630 in GH3 cells transfected with empty vector (\emptyset) or deacetylase dead SIRT1 H363Y. B. GH3 cells transfected with empty vector (\emptyset) or SIRT1 H363Y analyzed by western blot using anti-pGSK3 β -Ser⁹, -GSK3 β , and -pGS-Ser⁶⁴¹. C. CRE transcriptional activity in GH3 cells transfected with GSK3 β and Sirt1 or mutated Sirt1 H363Y. RLA: relative luciferase activity. Luciferase was measured 24 hours after transfection. Results are shown as luciferase: β -galactosidase ratio. Each experiment was repeated twice. *P<0.001 to empty plasmid control D. Cell lysate from GH3 cells immunoprecipitated with GSK3 β antibody with no antibody or rabbit IgG used as negative controls. Immunoprecipitated fractions were analyzed by western blot using anti-Sirt1, acetyl-lysine, and GSK β . E. GH3 cells treated with vehicle or 50 μ M resveratrol for 2 hours, its lysates were immunoprecipitated with anti-acetyl-lysine. Immunoprecipitated fractions were analyzed by western

blot using anti-GSK3 β . Representatives of three experiments are shown.

These data show that GSK3 β is acetylated and imply a regulatory association between acetylation and its phosphorylation at Ser⁹ and subsequently the kinase activity. A web-based tool (PHOSIDA database; Choudhary et al., 2009) predicted two putative acetylation sites, one of which (K205) resides in the priming pocket that regulates the kinase activity. To test the putative role of this residue in GSK3 β activity, a mutant GSK3 β was created by changing lysine to arginine (K205R). Arginine is a basic amino acid similar to lysine, but cannot be acetylated; therefore changing lysine to arginine maintains the positive charge while producing a nonacetyl mutant. Transfection with GSK3 β K205R abolished Sirt1 suppression on CRE transcriptional activity (Fig. 30A), indicating that Lys²⁰⁵ deacetylation is an important step in Sirt1's action on CREB transcriptional regulation.

Mutation of lysine to arginine imitates a hypoacetylated state, and transfection with GSK3 β K205R suppressed basal CRE transcriptional activity and CREB-Ser¹³³ phosphorylation levels (Fig. 30B-C) and increased GS-Ser⁶⁴¹ phosphorylation levels (Fig. 30C), showing that GSK3 β is activated when hypoacetylated.

GSK3 β is autoregulated through PP1, which dephosphorylates GSK3 β at Ser⁹ (Zhang et al., 2003). Indeed, pretreatment with okadaic acid abolished resveratrol's suppressive effect on GSK3 β -Ser⁹ phosphorylation (Fig. 30D).

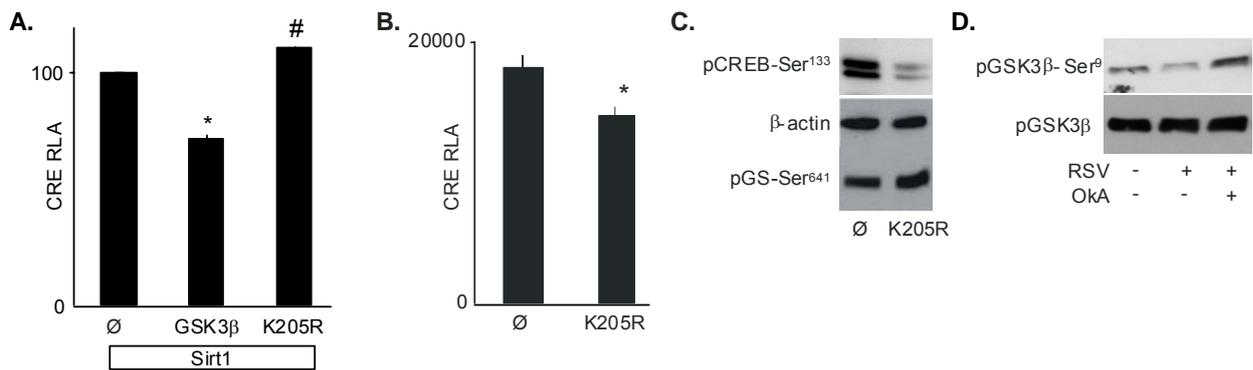


Figure 30. A. CRE transcriptional activity in GH3 cells transfected with Sirt1 in the presence of GSK3 β and mutated GSK3 β K205R. B. CRE transcriptional activity in GH3 cells transfected with mutant GSK3 β K205R. RLA: relative luciferase activity. Luciferase was measured 24 hours after transfection. Results are shown as luciferase: β -galactosidase ratio. Each experiment was repeated twice. * $P < 0.001$ to empty plasmid control (\emptyset); # $P < 0.05$ to empty plasmid control. C. pCREB-Ser¹³³ and pGS-Ser⁶⁴¹ levels in GH3 cells transfected with GSK3 β K205R. The same membrane was used and reblotted after sequential strippings. D. Effect of 2 hour pretreatment with 10nM okadaic acid (OkA) on resveratrol's suppressive effect on GSK3 β -Ser⁹. Immunoblots were repeated twice.

Altogether, these data reveal a novel role for Sirt1 in the regulation of CREB phosphorylation and transcriptional activation. Sirt1 by activating GSK3 β and PP1 promotes CREB dephosphorylation inhibiting its transcriptional activity and the final consequence of these events in pituitary somatotrophs is decreased Pit1 and GH gene transcription (Fig. 31A). It is possible that Sirt1 acts as a scaffold binding to and bringing in close proximity CREB, PP1 and GSK3 β when it is activated (Fig. 31B).

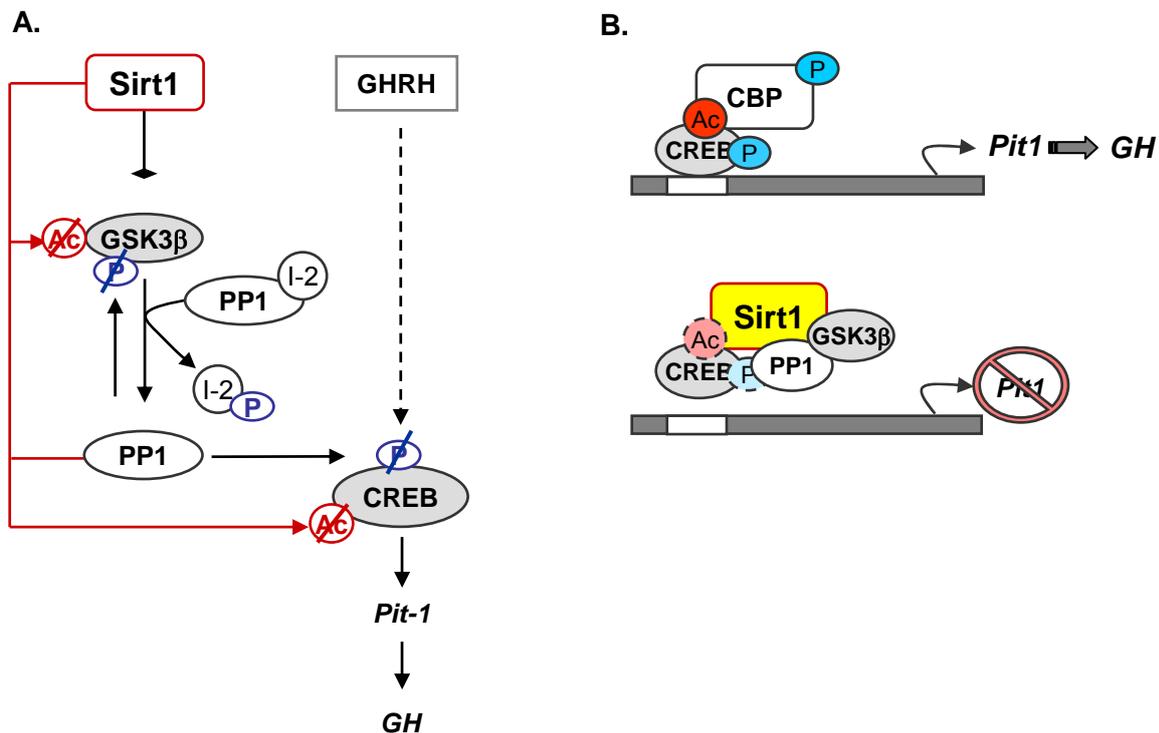


Figure 31. A. Schematic presentation of the signaling events taking place after Sirt1 activation, according to the findings of the present work. Sirt1 intercepts the cAMP pathway downstream to GHRH (dotted line) at CREB level. Sirt1 physically associates with GSK3 β , PP1 and CREB (red line). Sirt1 activation deacetylates and activates GSK3 β . The activated GSK3 β phosphorylates the PP1 inhibitor I-2, releasing it from the PP1 complex. This leads to the suppression of the inhibitory Ser⁹ phosphorylation and further activation of GSK3 β via an autoregulatory loop. The free active PP1 associates with CREB and dephosphorylates it, while Sirt1 deacetylates it. The de-phosphorylated and de-acetylated CREB loses its transcriptional activity so there is less Pit1 transcription, which compromises the sufficient GH synthesis.

B. GHRH-activated PKA pathway phosphorylates (shown in blue circle) the transcription factor CREB and the transcriptional coactivator, acetyltransferase CBP. Phosphorylated CREB is acetylated (shown in red circle) by the activated CBP, an event that enhances its binding to CRE sites on the Pit1 promoter, resulting in Pit1 transcription. Pit1 binds to the rat GH promoter where it recruits the activated CBP, driving GH transcription. When Sirt1 is activated, it binds to the acetylated CREB and deacetylates it. At the same time it brings CREB in close proximity to the GSK3 β -activated PP1, which dephosphorylates CREB. These events result in decreased DNA binding and suppression of Pit1 transcription.

5 DISCUSSION

Changes in energy intake have been repeatedly reported to affect endocrine and metabolic processes. Since the identification of intracellular energy sensors activated by low energy levels like Sirt1, there has been a growing interest in their impact on the regulation of metabolism. However, their role on hormone synthesis in endocrine cells remains an open question. The findings presented here show that Sirt1 inhibits the GH/IGF-I axis, the most important neuroendocrine mechanism in the regulation of growth, metabolism and lifespan, at pituitary level by suppressing GH synthesis.

GH is a major anabolic hormone and regulator of diverse metabolic functions, whose synthesis is tightly regulated by metabolic signals (Davidson, 1987; Dieguez & Casanueva, 1995). Two important intracellular energy sensors were studied, AMPK and Sirt1. Activation of AMPK with AICAR and metformin suppressed GH promoter activity and secretion. However this effect, that was also reported in an independent study (Tulipano et al., 2011), was not potent enough to warrant further investigation. Subsequently, the present study focused on the NAD⁺-dependent Sirt1, which was detected in all endocrine cells of the anterior pituitary, suggesting that it may act as a local energy sensor to regulate not only GH but also the other pituitary hormones. Indeed, Sirt1 was recently described to regulate TSH secretion from pituitary thyrotrophs (Akieda-Asai et al., 2010).

The plant polyphenol resveratrol was used to pharmacologically activate Sirt1 (Howitz et al., 2003; Wood et al., 2004). Although previous reports have questioned resveratrol's specificity on Sirt1 (Jang et al., 1997; Gehm et al., 1997), recent studies *in vivo* established that it acts through Sirt1 (Price et al., 2012). Indeed, knocking down Sirt1 abolished the inhibitory effect of resveratrol at 50 μ M concentration on GH secretion, Pit1 transcription and CREB phosphorylation, ensuring a Sirt1 specific effect in GH3 cells. An interesting finding was that Sirt1 inhibition in somatotrophs increased basal GH levels and GH promoter activity, similar

to what was observed in the Sirt1-null mouse, which displays normal circulating GH levels despite the small pituitary size indicative of increased GH synthesis rate (Lemieux et al., 2005). Sirt1 was also highly expressed in human anterior pituitaries and in most somatotrophinomas derived from acromegalic patients, where resveratrol treatment was also able to suppress GH secretion. The fact that pituitary cells in primary cell culture are difficult to get transfected prevented experiments with Sirt1 RNA interference. Nevertheless, pharmacological Sirt1 inhibition abolished resveratrol's effect and increased GH secretion from human somatotrophinomas, similar to what was observed in rat GH3 cells. Altogether, these data demonstrate a suppressive role for Sirt1 on GH synthesis.

Sirt1 affected not only endogenous GH and Pit1 synthesis but also their extrachromosomal promoters upstreams to luciferase gene indicating that its action is not exclusively due to its histone deacetylating activity. Human GH transcription is governed by the pituitary specific transcription factor Pit1 and CREB (Shepard et al., 1994). In contrast, rat GH promoter lacks the CRE consensus sequence and relies on Pit1 to convey the cAMP/PKA stimulatory signal (Tansey et al., 1993). In the rat somatotroph GH3 cells, Sirt1 modulated rat GH and Pit1 transcription, but no direct binding was detected to either promoters. In contrast, Sirt1's effect on Pit1 was dependent on CREB. The cAMP cascade activates CREB and resveratrol was recently shown to increase cAMP levels in muscle and adipose tissue by blocking cAMP phosphodiesterases (Park et al., 2012). However, Sirt1 did not affect cAMP levels in pituitary cells, but it suppressed CREB activity.

CREB is activated when phosphorylated at the Ser¹³³ residue as a response to cAMP increase (Johannessen et al., 2004). This initial burst is then followed by a gradual CREB-Ser¹³³ dephosphorylation, the so called attenuation phase, which is mediated by the serine/threonine phosphatases PP1 and PP2A (Hagiwara et al., 1992; Alberts et al., 1994; Wadzinski et al., 1993). This process is dependent on HDAC1, which recruits PP1 to CREB (Canettieri et al., 2003; Lu et al., 2003). In fact, CREB acetylation by CBP is tightly linked to its

phosphorylation and transcriptional activation (Lu et al., 2003). Resveratrol treatment suppressed CREB acetylation and although Sirt1 was described to deacetylate and inhibit the closely related to CBP p300 (Bouras et al., 2005), the physical association of CREB with Sirt1 suggested a direct effect. Indeed, Sirt1 was recently shown to deacetylate CREB directly and not through CBP (Qiang et al., 2011).

Interestingly, Sirt1 activates PP1 in contrast to HDAC1 that does not affect its phosphatase activity (Michael et al., 2000; Chen et al., 2005). PP1 is a holoenzyme highly conserved among eukaryotes that exists as a combination of several regulatory subunits (Cohen, 2002). It is inactive when bound to I-2 and activated when the phosphorylated I-2 dissociates from its catalytic subunit (Aggen et al., 2000). Several kinases were shown to phosphorylate I-2 at several sites, however the most important kinase is GSK3 β that phosphorylates I-2 at Thr⁷² (Hemmings et al., 1982; Aitken et al., 1984). GSK3 β was pivotal for Sirt1's inhibitory action on CREB. Sirt1 did not affect basal GSK3 β levels, contrary to HDAC1 (Jin et al., 2009), but it activated it instead. All Sirt1's effects on GH synthesis required its intact deacetylase function, as transfection with a deacetylase dead Sirt1 inhibited GSK3 β and phosphatase activity, prolonged forskolin induced CREB phosphorylation (suggestive of loss of the attenuation phase) and induced basal CREB transcriptional activity. The present study shows for the first time that GSK3 β is acetylated and that Sirt1 activation with resveratrol decreases its acetylation levels. GSK3 β acetylation status affected Sirt1's action on CREB. In addition, hypoacetylated GSK3 β had higher activity and suppressed CREB phosphorylation and transcriptional activity. In contrast, GSK3 β inhibition activated CREB, similar to what was previously reported (Grimes & Jope, 2001; Götschel et al., 2008), indicating an important role for GSK3 β on basal CREB regulation.

GSK3 β regulation is quite complex with phosphorylation events taking place before the final inhibitory phosphorylation at Ser⁹ (Jope & Johnson, 2004). However, the suppressive Sirt1's

effect on GSK3 β -Ser⁹ was abolished using protein phosphatase inhibitors, indicating that it is downstream to PP1. GSK3 β induces PP1 activity, but is also activated when the phosphatase dephosphorylates Ser⁹ in an autoregulatory loop that intensifies phosphatase activity and CREB dephosphorylation, reinforcing the inhibitory role for GSK3 β on CREB (Alao et al., 2006; Zhang et al; 2003; Grimes & Jope, 2001; Szatmari et al., 2005). Sirt1 was found to physically associate with both GSK3 β and PP1, suggesting that it may act as a scaffold bringing the complex together. Therefore, the data presented herein suggest that Sirt1 deacetylates GSK3 β , which accesses, activates and becomes targeted by PP1.

Altogether, the present study shows that Sirt1 is expressed in pituitary somatotroph cells, where it suppresses GH synthesis. GH has lipolytic and anabolic actions, which are counterbalanced by disrupted glucose metabolism (Møller et al., 2009). Therefore, the suppressive action of resveratrol on GH secretion may mediate some of its beneficial effects on metabolic homeostasis observed in humans (Timmers et al., 2011). Indeed, resveratrol was found to decrease circulating IGF-I levels in healthy volunteers contributing to its chemopreventive action (Brown et al., 2010). Finally, the study presents with a novel mechanism through which CREB acetylation controls its phosphorylation. CREB integrates diverse stimuli to drive the expression of hormones pivotal in organism growth and metabolism (Sassone-Corsi, 1998; Mayr & Montminy, 2001), therefore this new pathway is expected to be involved in cell types and processes outside the anterior pituitary.

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CURRICULUM VITAE

José Luis Monteserín García

PERSONAL DATA

Name: José Luis Monteserín García

Birth: May 9th, 1972, Buenos Aires, Argentina

Academic degree: M.Sc. from the University of Morón, Morón, Argentina

Address: Hanauer str. 35, 80992, Munich, Germany

WORK ADDRESS

Max Planck Institute of Psychiatry

Kraepelinstr. 10

D-80804, Munich

Germany

Tel: +49 89 30622272

Email: monteserin@mpipsykl.mpg.de

LANGUAGES

English (fluent in oral and written communication)

German (Oberstufe I)

Spanish (native speaker)

APPOINTMENTS

2009-Today Laboratory assistant. Max Planck Institute of Psychiatry, Department of Endocrinology, Munich, Germany.

2006-2009 Ph.D. Research Fellow at the Max Planck Institute of Psychiatry, Department of Endocrinology, Munich, Germany.

2005-2006 Guest Scientist at the Max Planck Institute of Psychiatry, Department of Endocrinology, Munich, Germany.

2004-2005 Time dedicated for the study of German language. Volkshochschule München, Munich, Germany. Graduated with Oberstufe I.

2000-2003 Laboratory Assistant. Alcorta Clinic, Haedo Provincia de Buenos Aires, Argentina

- 1999-2000 Undergraduate Research Fellow. Posadas Hospital, Haedo Provincia de Buenos Aires, Argentina
- 1998 Undergraduate Research Fellow. Morón Hospital, Morón Provincia de Buenos Aires, Argentina

EDUCATION

- 2002 Graduation as Biochemist from University of Morón, Morón Provincia de Buenos Aires, Argentina

Thesis: The AD7c-NTP protein is a new marker for Alzheimer disease. Qualified as very good (9 out of 10).

- 1991 Graduation as Chemical Technical Assistant from the High School ENET N°27 “Hipólito Yrigoyen”, Buenos Aires, Argentina.

Courses

Theoretical and practical course in high performance liquid chromatography (HPLC); Morón, Nov. 1997.

Theoretical and practical course in gas chromatography (GC); Morón, Nov. 1997.

PUBLICATIONS

1. Correa-de-Santana E, Fröhlich B, Labeur M, Páez-Pereda M, Theodoropoulou M, Monteserin JL, Renner U, Stalla GK. NOD2 receptors in adenopituitary folliculostellate cells: expression and function. *J Endocrinol.* 2009 Oct; 203(1):111-22.
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Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen bzw. einer Prüfungskommission vorzulegen, noch eine Doktorprüfung durchzuführen.

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José Luis Monteserín García