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THE MECHANISM OF ACTION OF SOCS2 AND ITS ROLE IN METABOLISM AND GROWTH

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Till Alexander,
och till minne av hans farfar

ABSTRACT

A well-known function of Growth Hormone (GH) is the regulation of postnatal longitudinal growth but it also affects other biological processes, for instance metabolism and inflammation. Actions of GH are tightly regulated at several levels and by several different factors and are initiated by GH binding to membrane bound GH receptors (GHR). The intracellular signaling of GH and other related hormones and cytokines is predominately mediated by the JAK-STAT pathway. This pathway is regulated in a negative feedback manner by the Suppressors of Cytokine Signaling (SOCS) family of proteins. One of the family members, SOCS2, is intimately tied to GH by virtue of the phenotype that results from its absence. SOCS2$^{-/-}$ mice are 40% larger than wildtype littermates due to increased GH sensitivity.

Here, the molecular mechanism behind SOCS2s negative regulation of GH signaling, and its effects on metabolism and inflammation are described. We demonstrate that SOCS2 assembles a canonical E3 ubiquitin ligase complex with Elongin B, Elongin C, Cullin 5 and Rbx2 and that this complex has intrinsic E3 ligase activity *in vitro*. Overexpression of SOCS2 and its complex members leads to ubiquitination and proteasomal degradation of the GHR. We also outline the importance of the different domains of SOCS2, and demonstrate the necessity of the SOCS-box for proper SOCS2 activity. In a follow up study the claim that the naturally occurring Ser52Asn polymorphism of SOCS2 affects its activity and may contribute to acromegaly in humans was investigated. The Ser52Asn mutant was however found to be as efficient at regulating GH signaling as the wildtype and we conclude that it is unlikely to contribute to increased GH sensitivity. In Paper III the phenotype of SOCS2$^{-/-}$ mice under conditions of dietary stress is described. We report that SOCS2 deletion protects against high fat diet (HFD) induced hepatic steatosis but simultaneously leads to decreased insulin sensitivity. SOCS2$^{-/-}$ mice were found to have increased triglyceride output from the liver but also increased plasma levels of proinflammatory cytokines without apparent macrophage infiltration. *In vitro* examination of macrophages revealed increased phagocytic activity and cytokine production in the absence of SOCS2 and suggests a direct role for SOCS2 in the regulation of TLR4 signaling. Finally, the results of a screening effort to identify SOCS2-modulating, drug-like molecules are included. We have identified a prospective hit that binds to and inhibits SOCS2 activity *in vitro*. In summary, SOCS forms an E3 ligase complex which targets the GHR for degradation. This forms the molecular basis of its physiological actions. SOCS2$^{-/-}$ mice are protected from HFD induced hepatic steatosis but suffer from deteriorated insulin sensitivity related to increased inflammation.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. **Vesterlund M**, Zadjali F, Persson T, Nielsen M.L, Kessler B.M, Norstedt G and Flores-Morales A. The SOCS2 Ubiquitin Ligase Complex Regulates Growth Hormone Receptor Levels.
PLoS One. 2011 6(9): e25358.

II. **Vesterlund M**, Al-Kharusi A, Brandt P, Norstedt G and Flores-Morales A. The effect of the SOCS2 S52N mutation on GH signaling.
Manuscript.

III. Zadjali F, Santa-Farre R, **Vesterlund M**, Carow B, Mirecki-Garrido M, Hernandez-Hernandez I, Flodström-Tullberg M, Parini P, Rottenberg M, Norstedt G, Fernandez-Perez L and Flores-Morales A. SOCS2 inactivation protects against hepatic steatosis but worsens insulin resistance in high fat diet fed mice.
FASEB J. 2012. 26(8):3282-3291.

RELATED PUBLICATION

I. Zadjali F, Pike A.C, **Vesterlund M**, Sun J, Wu C, Li S.S, Rönnstrand L, Knapp S, Bullock A.N and Flores-Morales A. Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase. J Biol Chem. 2011. 286(1):480-490.

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LIST OF ABBREVIATIONS

cAMP	Cyclic Adenosine Mono-Phosphate
ASB	Ankyrin repeat SOCS-box protein
ATP	Adenosine Tri-Phosphate
Bim	Bcl-2-Interacting Mediator of cell death protein
BMDM	Bone Marrow Derived Macrophage
BSA	Bovine Serum Albumin
BTB	Broad complex, Tramtrack, Bric-a-brac domain
CAND	Cullin-Associated and Neddylation-Dissociated protein
CIS	Cytokine-Inducible SH2-containing protein
CRL	Cullin-RING Ligase
CS	Chorionic Somatomammotropin
Cul	Cullin
Da	Dalton
DAG	Diacylglycerol
DC	Dendritic Cell
DCN	Defective in Cullin Neddylation protein
DDB	DNA-Damage-Binding protein
E_2	17β-estradiol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
ECS	Elongin C-Cullin-SOCS-box complex
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-Regulated Kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
G-CSF	Granulocyte-Colony Stimulating Factor
GH	Growth Hormone
hGH	Human Growth Hormone
GHBP	Growth Hormone Binding Protein
GHD	Growth Hormone Deficiency
GHS-R	Growth Hormone Secretagogue Receptor
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GHIH	Growth Hormone Inhibiting Hormone
GLUT4	Glucose Transporter type 4
GR	Glucocorticoid Receptor
Grb	Growth factor receptor bound
HA	Hemaglutinin
HECT	Homologous to the E6-AP Carboxyl Terminus
HFD	High Fat Diet
Hif	Hypoxia Inducible Factor
HOMA-IR	Homeostatic Model of Insulin Resistance

IAP	Inhibitor of Apoptosis Protein
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IFN	Interferon
IκB	Inhibitor of NFκB.
IKK	IκB kinase
IL	Interleukin
IP3	Inositol trisphosphate
ipGTT	Intraperitoneal Glucose Tolerance Test
ipITT	Intraperitoneal Insulin Tolerance Test
IRAK	IL-1 Receptor Associated Kinase
IRF	Interferon Regulatory Factor
IRS	Insulin Receptor Substrate
ITC	Iso-Thermal Calorimetry
JAK	Janus Kinase
JNK	c-Jun amino terminal Kinase
KIR	Kinase Inhibitory Region
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats
MAPK	Mitogen-Activated Protein Kinase
Mal	MyD88 adaptor-like protein
MyD88	Myeloid Differentiation factor 88
NEDD8	Neural precursor cell-Expressed Developmentally Downregulated protein 8
NFκB	Nuclear Factor κB
NK	Natural Killer cell
PI	Phosphatidylinositide
PI3K	Phosphoinositide 3-Kinase
PIAS	Protein Inhibitor of Activated STATs
PL	Placental Lactogen
PLC	Protein Lipase C
PKC	Protein Kinase C
PRL	Prolactin
PRLR	Prolactin Receptor
PTP	Protein Tyrosine Phosphatases
PVDF	Polyvinylidene Fluoride
Pyk2	Proline-rich tyrosine Kinase 2
Rbx	RING-box protein
RING	Really Interesting New Gene
SCF	Skp-Cullin-F-box complex
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SH2	Src Homology domain 2
Shc	SH2 domain-containing transforming protein
SHP	SH2 domain-containing PTP
SIRP	Signal-Regulatory Protein
Skp	S-phase-Kinase-associated Protein
SOCS	Suppressor Of Cytokine Signaling
SOS	Sons Of Sevenless

SPRY	SPlA and Ryanodine receptor domain
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-like Modifier
TAB	TAK1 binding protein
TAG	Triacylglyceride
TAK	TGF-β Activated Kinase
Th	T helper cell
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor Associated Factor
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-β
βTrCP	β-Transducin repeat-Containing Protein
Ub	Ubiquitin
UBD	Ubiquitin Binding Domain
UbE	Ubiquitin-dependent Endocytosis motif
UBL	Ubiquitin-Like protein
VHL	von Hippel-Lindau protein

1 INTRODUCTION

Higher organisms are composed of a multitude of cells with diverse functions that are secluded into specific organs in the body. Essential for the survival and proper functioning of the organism is the existence of a reliable system for communication between the cells, both within and between various organs. One method for intercellular communication is the secretion of molecules from one cell that migrate to and trigger a response in a target cell. Hormones constitute one class of such molecules. They are secreted from specialized organs within the body, travel through the bloodstream to their target tissue where they elicit a response by binding to their respective receptors. Signaling substances that are secreted locally within one organ or tissue are usually referred to as cytokines. Hormones and cytokines regulate a plethora of bodily functions including reproduction, metabolism, immune responses, growth and development.

The importance of well-functioning intercellular and endocrine signaling is highlighted by the association between perturbations of hormonal signaling and endocrine disorders. Several of these conditions are caused by or associated with a lack or excess of a particular hormone but of equal importance is that the target cells have an adequate response to the stimuli. Aberrations in intracellular signaling cascades can produce syndromes similar to lack of hormones e.g. type 2 diabetes and Laron syndrome or to excess of hormones e.g. polycythemia vera.

To ensure proper responses to external stimuli cells have developed several mechanisms for regulation and propagation of intracellular signaling. Some are broad and cover many pathways while some are more specific in their actions. Modes of regulation include degradation of receptors and signaling intermediates, de-activating events such as de-phosphorylation, competitive binding and seclusion of signaling components into intracellular organelles. An interesting family of intracellular regulators is the Suppressors of Cytokine Signaling (SOCS). SOCS proteins regulate the signaling of several hormones and cytokines. This thesis is concerned with the actions of SOCS2, a known regulator of the actions of Growth Hormone (GH). The role of GH in biological processes and pathological conditions has been investigated for almost a century. The pleiotropic actions that have been described underscore its importance and motivate further studies of the regulators of GH and their actions in the body and on the cellular level.

1.1   GROWTH HORMONE

The existence of growth promoting substances produced by the anterior pituitary gland was shown in 1921 by Evans and Long when they managed to induce gigantism by repeatedly injecting rats with an emulsion of the gland intraperitoneally [1]. Almost a quarter of a century later, in 1944, Evans and Li isolated growth hormone from bovine pituitaries for the first time [2]. Human GH (hGH) was isolated a decade later [3] and the first successful treatment of a patient with short stature using human GH was done by Maurice Raben in the late 1950's [4,5].

Growth hormone, also known as somatotropin, somatropin or somatotrope hormone, belongs to the same family of hormones as the prolactins (PRL) and placental lactogens (PL, also known as chorionic somatomammotropin (CS)) [6]. GH is a polypeptide hormone and in its most prevalent form it has a size of 22 kDa and contains 191 amino acids in humans. The protein structure was solved in 1987 and it showed that the GH protein molecule consists of four α-helices that are arranged in an up-up-down-down fashion and that the helices are further joined by two disulfide bridges [7]. Two sites for GH interaction with its cellular receptor have been identified. Site 1 consists of residues 54 to 74 located in a loop region between the first and second helix, and the C-terminal part which contains parts of the forth helix [8]. Site 2 is located on the opposite side of the GH molecule and consists of the N-terminal part, corresponding to the start of the first helix, and the latter part of the third helix [9].

Replacement of hGH residue Gly120 with an arginine creates a GH antagonist since the residue is located on the third helix in site 2 and replacement with a bulky residue abrogates the binding to a tryptophan on the Growth Hormone Receptor (GHR). Since site 1 is left intact the antagonist still interacts with the GHR but fails to initiate further downstream signaling and thus acts as a competitive antagonist [10,11]. This receptor antagonist has been further modified by pegylation to increase its half-life in the body, and by additional mutations in site 1 that increase its affinity for the GHR, and is now used clinically under the name Pegvisomant®, to treat an un-common form of acromegaly [12].

The human GH gene is part of a gene cluster of five similar and related genes that have evolved from duplications of the same ancestral gene that occurred over 350 million years ago. The five genes in the cluster are the GH-normal (GH-N) gene, the GH-variant (GH-V) gene, the CS-like (CS-L) gene and the CS-A and CS-B genes [13,14]. The GH-N gene encodes the predominant form that is secreted from the pituitary while GH-V encodes a variant of the hormone that is expressed by the human placenta [15].

1.2   THE GROWTH HORMONE AXIS

The somatotropic cells of the anterior pituitary gland are the primary producers of GH in the body. The production of GH by the somatotrophs is mainly regulated by the pituitary transcription factor Pit-1. Translated GH is then stored in secretory granules and increased concentrations of cAMP or Ca^{2+} induces the release of GH from the somatotrophs [16-18]. The release and transcription of GH is controlled by two peptides secreted by the neuroendocrine cells of the neurosecretory nuclei of the

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hypothalamus that bind to specific G-protein coupled receptors on the somatotrophs. GH production and release is stimulated by Growth Hormone Releasing Hormone (GHRH), also known as somatocrinin, and the release is inhibited by Growth Hormone Inhibiting Hormone (GHIH), also known as somatostatin. The release of GH from the pituitary is largely regulated by the balance of GHRH and GHIH [14,18,19].

GH secretion in humans is pulsatile and plasma levels can vary between 1 and as high as 72 ng/ml. GH secretion occurs primarily during sleep but peaks can be observed in plasma during the day [20]. The secretion is sexually dimorphic; males have higher nocturnal pulses and small pulses during daytime. Women on the other hand have a more continuous secretion of GH and more frequent pulses with similar amplitude [21]. Sex-different GH secretion patterns seem important for the regulation of sex-specific hepatic gene transcription in rodents [22,23] and most likely in humans as well [24]. Sex-differences in GH secretory patterns can at least partly explain differences in growth between males and females.

Once GH is released into the bloodstream it travels to target organs where it binds to the GHR. GHR is ubiquitously present with the highest levels found in the cells of the liver. GHR signaling triggers the expression and release if Insulin-like Growth Factor-1 (IGF-1), also known as somatomedin C, which is a major mediator of somatic GH action [25-27].

GH release and production is also negatively regulated by several factors. Both IGF-1 and GH increase the actions of GHIH and diminish the actions of GHRH and thus act in a negative feedback loop that leads to a decreased release of GH [28-30]. Ghrelin, a peptide produced by the intestine, stimulates GH release via Growth Hormone Secretagogue Receptors (GHS-R) present on the surface of the somatotropic cells [31,32]. Other factors that regulate GH release include glycemic levels, exercise, sex hormones, glucocorticoids and thyroid hormones [30,33-37].

GH actions are also influenced by a factor known as Growth Hormone Binding Protein (GHBP) which is identical to the extracellular domain of the GHR. GHBP is encoded by the same gene as the GHR and is produced by proteolytic cleavage of the extracellular part of GHR but may also be produced by alternative splicing of the mRNA transcript [38,39]. GHBP decreases the availability of the GHR and also competes with the receptor for binding to the hormone, this negatively influences GH action. However, GHBP also prevents renal clearance of GH, thus prolonging its presence in plasma which means it may have both inhibitory and enhancing effects on GH actions [40].

1.3   SOMATIC EFFECTS OF GROWTH HORMONE

Growth hormone is mitogenic hormone and its primary somatic effect is the promotion of longitudinal growth [14,41]. GH promotes the proportional growth of several organs in the body including liver, muscle, bone and adipose tissue and exerts effects on cellular differentiation, metabolism and nutrient uptake [42-46]. It is also involved in the regulation of immune cells and hematopoiesis [47,48] and can act on the brain to

influence emotion, behavior and other cognitive responses [49,50]. GH actions are pleiotropic and influenced by factors such as age, gender and secretory pattern [51-53].

1.3.1 Growth

Somatic growth is, as mentioned above, brought about by a proportional increase in the weight and size of several organs. Elongation of the long bones in the body is an important step in the longitudinal growth of the body. This is achieved by proliferation and hypertrophy of the chondrocytes situated in the epiphyseal growth plates at both ends of the bone [54]. In humans, increased levels of estrogen causes apoptosis and ossification of the growth plate towards the end of puberty leading to a halt of longitudinal growth [55].

The important role of GH in longitudinal growth is evident from the phenotypes produced in genetic and surgical animal models. $GHR^{-/-}$ mice are proportionally smaller than wild type mice and exhibit a 50% decrease in body weight [56] and $GHRH^{-/-}$ mice reach an adult size of 60% of wild type littermates [57,58]. Analogously, hypophysectomized animals also show markedly decreased post-natal growth [59,60].

In 1957, Salmon and Daughaday proposed the somatomedin hypothesis which stated that GH does not exert its growth promoting effects directly on target tissues, but rather, through an intermediate signaling substance [26]. This unknown substance was later dubbed somatomedin [61] and eventually identified as IGF-1 [62]. Circulating IGF-1 is primarily produced by the liver in response to GH and is often used as a clinical marker for GH secretion since the serum IGF-1 levels are more stable over time as compared to GH levels [63,64]. The importance of IGF-1 for longitudinal growth is evident from knockout studies in mice; $IGF-1^{-/-}$ mice exhibit a 60% reduction in growth compared to wild type littermates [65].

However, the original somatomedin hypothesis has been subject to revision in recent years as is has become evident that not all the growth promoting effects of GH are mediated by hepatic IGF-1. GH does exert direct effects on muscle, bone and adipose tissue and local production of IGF-1 has been shown to be of importance for growth [66]. Interestingly, tissue specific hepatic $IGF-1^{-/-}$ mice do not show a growth reduction phenotype, but serum levels of IGF-1 are decreased by 75% [67]. Since a large portion of serum IGF-1 is bound to IGF binding proteins (IGFBP) that are also produced by the liver, it has been suggested that the extrahepatic IGF-1 might be present as free IGF-1 at a higher rate than hepatic IGF-1, thus allowing for unchanged levels of bioavailable IGF-1 [68]. Based on mice knockout experiments where $GHR^{-/-}$ and $IGF-1^{-/-}$ mice were crossed it has been estimated that 17% of the postnatal growth rate can be attributed to processes unrelated to GH and IGF-1, 35% of the growth is directly associated with GH-independent effects of IGF-1 and 14% to IGF-1-independent actions of GH. The remaining 34% is dependent on the actions of GH mediated by IGF-1 [66,69]. An updated version of the somatomedin hypothesis is depicted in Figure 1.1.

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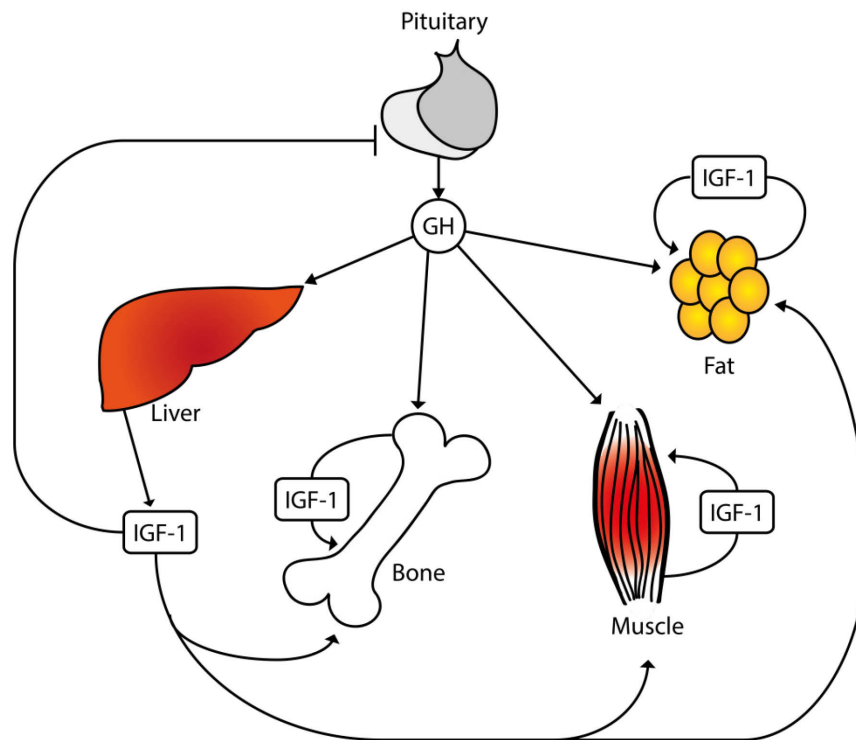
Figure 1.1 The updated somatomedin hypothesis. GH promotes systemic production of IGF-1 by the liver which negatively regulates GH secretion. However, GH also exerts direct effects on muscle, bone and adipose tissue. Local production of IGF-1 further mediates GH actions in these tissues.

1.3.2 Metabolism

The growth promoting actions of GH also involve complex regulatory action on metabolism. GH anabolic actions in muscle and bone involve the stimulation of protein synthesis while promoting catabolism of fatty acids instead of glucose. Consequently, GH actions leads to hyperglycemia and decreased insulin sensitivity and GH is generally considered to have diabetogenic properties [66,70]. Increasing fatty acid supply to muscle tissue involves distinct actions in adipose tissue and liver. GH promotes lipolysis in adipose tissue and blocks the uptake of fatty acids through inhibition of lipoprotein lipase which leads to increased circulating levels of free-fatty acids (FFA) and glycerol [53,71,72]. This is highlighted by the GH transgenic mice that have decreased body fat but develop severe insulin resistance [73].

In the liver, GH promotes gluconeogenesis and glycogenolysis and reduces the uptake of glucose. It also promotes lipogenesis, inhibits lipolysis and increases lipid output by the liver [74]. Mice with a liver specific deletion of the GHR exhibit decreased hepatic triglyceride secretion, insulin resistance and spontaneous hepatic steatosis [75]. In muscle, GH promotes amino acid uptake and protein synthesis without increasing proteolysis [76]. Interestingly, muscle specific deletion of GHR seems to protect from high-fat diet (HFD) induced insulin resistance. The exact mechanisms behind this phenotype are not clear but might be attributable to decreased diabetogenic GH action on the muscle [77]. In adipose tissue GH promotes lipolysis and targeted deletion of

GHR in fat leads to a doubling of the fat mass, but has no effect on glucose homeostasis [78]. GH also seems to play a role in insulin secretion from the β cells in the pancreas. Mice with a GHR deletion in the β cells exhibit decreased glucose stimulated insulin release and decreased β cell hyperplasia in response to HFD [79].

In contrast to the diabetogenic effects of GH, IGF-1 mimics the actions of insulin and promotes glucose uptake, inhibits gluconeogenesis and enhances adipogenesis [80,81]. Due to the similarities in somatic effects between IGF-1 and insulin, IGF-1 has even been used as an adjuvant therapy to treat diabetes in insulin resistant patients [82]. However, IGF-1 has effects distinct from insulin since it increases protein synthesis and inhibits proteolysis, while insulin mainly promotes the latter [83]. In summary, IGF-1 acts as both a mediator of the growth promoting actions of GH and at the same time counters its deleterious diabetogenic action.

Another interesting somatic effect of GH is its effect on aging. GHR$^{-/-}$ mice have a dramatically increased lifespan, while GH transgenic mice exhibit decreased longevity [84]. The GHR$^{-/-}$ mouse has the "world record" in longevity amongst transgenic animals and interestingly, humans with GHR mutations (Laron dwarfs) seem to have long lifespan too. The effect seems to be independent of IGF-1 actions and is likely linked to the perturbations of the carbohydrate metabolism and the reduced oxidative stress observed in dwarf mice [85].

1.3.3 Inflammation and immunity

Besides its actions on postnatal growth and metabolism GH also plays a role in the regulation of the immune system. Several different immune cells have been shown to express both the GHR, which suggests that GH signaling directly affects immune functions, and GH, which implies that paracrine and autocrine hormone secretion might play a role in immunity [86]. GH treatment has been shown to promote monocyte migration [87] and enhance T cell [88] and B cell development [89].

In mice, transgenic overexpression of bovine GH alters T cell function and decreases cytokine production [90]. GHR$^{-/-}$ mice have increased plasma levels of anti-inflammatory cytokines and decreased levels of proinflammatory cytokines. Since inflammatory processes are linked to aging, obesity and insulin resistance it is possible that the effects on GH on these somatic processes could be mediated, at least in part, by its actions on the immune system and inflammation [91].

GH is able to directly affect cytokine production by immune cells, but the mechanisms and effects are unclear. GH have been shown to promote the production of proinflammatory cytokines such as Interleukin (IL) -1α, IL-6 and Tumor Necrosis Factor α (TNF-α) in immune cells both *in vitro* [92] and *in vivo* [93]. In a Finnish study, GH treatment of critically ill patients was found to increase both morbidity and mortality, likely through modulation of immune function [94], and high levels of GH and low levels of IGF-1 are also linked to septic shock and are associated with poor outcome [95,96]. However, other studies have found GH treatment both *in vivo* and *in vitro* to have a reducing effect on proinflammatory cytokine production [97,98]. While

it is clear that GH modulates immune functions, the distinct effects and underlying mechanisms remain to be elucidated.

1.3.4 Pathological conditions associated with GH

Due to its pleiotropic functions GH is associated with several pathophysiological conditions. In humans and animals, deficiencies in the GH axis or in the GH signaling pathway lead to dwarfism while excessive amounts of GH lead to gigantism and acromegaly. These conditions are characterized by their growth phenotypes but they encompass more than altered stature and have provided important insight into the metabolic actions of GH.

1.3.4.1 Dwarfism

There are more than 200 distinct medical conditions that lead to dwarfism and some of these affect the GH axis. GH deficiency (GHD) may lead to dwarfism if left untreated and can in some cases be related to mutations that affect the secretion and function of GH. GHD in children often responds well to GH treatment, and a near normal final height is often achieved [99].

Another cause for dwarfism is Laron Syndrome, which is characterized by insensitivity to GH. This condition was first described by Laron et al. in 1966 [100]. Laron syndrome is normally caused by mutations in the extracellular domain of the GHR. Patients with Laron syndrome have high levels of circulating GH and low IGF-1 levels that do not increase in response to GH treatment [101]. These patients can be considered the human equivalent of the GHR$^{-/-}$ mouse, which is sometimes referred to as the Laron mouse. In line with the physiological traits of the GHR$^{-/-}$ mice, Laron syndrome seems to lead to a reduction in pro-aging signaling and protect against diabetes and cancer [102]. Clinical treatment of Laron syndrome consists of IGF-1 therapy before puberty. However, it is not as effective as GH replacement therapy in GHD patients. The reason is believed to be the reduced hepatic production of IGFBP which leads to a faster clearance of IGF-1 and the fact that the IGF-1 independent effects of GH on growth are as of yet unattainable in Laron syndrome patients [103].

1.3.4.2 Acromegaly

Excessive GH secretion in children leads to accelerated growth and a significant increase in height and is referred to as gigantism. If the increase in GH levels occurs or persists after the fusing of the epiphyseal plate at puberty, the condition is termed acromegaly [104]. The term acromegaly was coined by Pierre Marie who described two cases in 1886 [105,106].

The most prevalent cause of acromegaly is excessive GH secretion from pituitary adenomas that derive from the somatotropic cells. In some cases increased GH or GHRH secretion from tumors in other parts of the body lead to the condition. Common visible features are enlargement of the hands and feet, prognathism and macroglossia [107]. However, the increased plasma levels of GH seen in these patients also cause comorbidities and the patients are at an increased risk for developing diabetes and

cardiovascular disease [108]. Again this is in line with the phenotype seen in the GH transgenic mice described above.

Treatment for acromegaly is either, surgical removal of the GH or GHRH producing tumor, radiation therapy of the tumor, treatment with somatostatin analogues to decrease the release of GH, Pegvisomant treatment to antagonize GH action directly or a combination of these. Symptomatic treatment of comorbidities may also be called for [108].

1.4   INTRACELLULAR GROWTH HORMONE SIGNALING

When GH reaches a target organ it binds to dimerized GHR that span the cellular plasma membrane. The two separate binding sites on the GH molecule each interact with one GHR protein molecule. Previously, GH was thought to cause receptor dimerization [9] but recent studies have shown that the GHR is already present as a dimer in the membrane [109] and that GH induces a conformational change in the receptor that affects the intracellular part of the GHR and triggers downstream signaling [110].

In humans the GHR consist of 620 amino acids and has an approximate size of about 100 kDa. Following receptor translation the receptor is heavily glycosylated in the endoplasmic reticulum and Golgi and its final size when present in the plasma membrane increases to around 130 kDa [111-113]. The GHR belongs to the class I cytokine receptor family and is closely related to the PRL receptor (PRLR) and the erythropoietin receptor [114]. Cytokine receptors are able to initiate signaling through several different signaling pathways. Among them, the JAK-STAT pathway is of key importance.

1.4.1   JAK-STAT Pathway

The members of the class I cytokine receptor family do not have intrinsic kinase activity; instead, each receptor molecule associates with an intracellular tyrosine kinase. Four different tyrosine kinases belonging to the same family have been shown to associate with cytokine receptors; Janus Kinase (JAK) 1-3 and Tyk2 [115]. The GHR was the first receptor of this family to be identified and it is associated with JAK2 [116]. Upon GH binding to the dimerized GHR the two JAK2 proteins come in close proximity of each other and cross-phosphorylate. Following JAK2 cross-phosphorylation the two kinases phosphorylate the GHR [117]. This promotes the binding of the Signal Transducer and Activator of Transcription (STAT) protein to the GHR [118]. STATs bind the phosphorylated tyrosines on GHR through its Src Homology 2 (SH2) domain [119], and, once bound it in turn becomes phosphorylated by JAK2 [120]. Phosphorylated STAT dissociates from the receptor, dimerizes through the SH2 domain and migrates into the nucleus of the cell where it acts as a transcription factor and initiates the transcription of GH responsive genes [121-123].

A total of seven different STAT proteins have been identified in mammals; STAT1-4, STAT5a and b and STAT6 [115]. GHR signaling is primarily mediated by the two

STAT5 isoforms; however, studies have shown that GH is also able to trigger transcription through STAT1 and STAT3 activation [124,125]. STAT5a and b are encoded by separate genes but their amino acid sequence is 96% homologous [126]. The divergence between the two isoforms is primarily found in their C-terminal transcription activation domains [127] and the two isoforms exhibit differences in their transcriptional activity [128]. The downstream effects overlap to a large degree but each isoform also has separate effects on transcription [129]. Furthermore, the tissue specific distribution varies for the isoforms, for instance STAT5b is the predominant isoform in the liver [130,131].

Further clues on the roles of the different STAT proteins come from knockout studies in mice. STAT1$^{-/-}$ mice have decreased resistance to viral infections and do not respond to interferons (IFN) [132,133]. STAT2$^{-/-}$ mice have a similar phenotype [134], while STAT3$^{-/-}$ is embryonic lethal [135]. However, tissue specific knockouts of STAT3 reveals that it plays an important role in mediating T cell proliferation and anti-inflammatory signaling in macrophages through IL-2 and IL-10, respectively [136]. Disruption of STAT4 and STAT6 causes defects in T cell differentiation; STAT4$^{-/-}$ mice have impaired T helper (Th) 1 cell differentiation and STAT6$^{-/-}$ have impaired Th2 cell differentiation [137]. Silencing of the two STAT5 isoforms creates distinct phenotypes that unveil their respective roles. The generation of STAT5a$^{-/-}$ mice revealed the importance of STAT5a for PRL signaling. The knockouts have impaired mammary gland development and fail to lactate after parturition [138]. The phenotype of the STAT5b$^{-/-}$ mice highlights its importance for GH signaling; postnatal growth is reduced in the males and the expression of female predominant genes in the liver is upregulated [139]. Disruption of the STAT5b gene feminizes the male mice since it abolishes their ability to convert the male specific pulsatile GH secretion into pulsatile GHR signaling and the signaling pattern becomes more continuous and similar to the secretion pattern seen in females [140]. In humans, inactivating mutations in the STAT5b gene cause a syndrome similar to Laron type dwarfism coupled with autoimmunity likely due to low levels of T regulatory cells [141]. The condition is rare however and so far only seven subjects have been identified [142].

STAT5b triggers the hepatic expression of several important GH responsive genes notably, IGFBP3, IGF-1, SOCS1-3, and CIS [143-145]. STAT5b also induces the expression of the transcription factor hepatic nuclear factor 6, which in turn promotes further activation of GH responsive genes [146]. It has also been demonstrated that the growth promoting actions of STAT5b in the liver are influenced and dependent on the glucocorticoid receptor (GR). GR binds to STAT5b and helps promote gene transcription and hepatocyte-specific deletion of GR leads to stunted growth [147].

1.4.2 Mitogen-Activated Protein Kinase Pathway

Another well-known pathway that is activated by the GHR is the Mitogen-Activated Protein Kinase (MAPK) or extracellular signal-regulated kinase (ERK) pathway [148]. The SH2 domain-containing transforming protein (Shc) binds to the phosphorylated GHR and is phosphorylated by JAK2. This activates Shc and it in turn, binds to and activates the Growth factor receptor bound 2 (Grb2) protein [149]. Grb2 interacts with

Sons Of Sevenless (SOS) and this triggers further signaling by the downstream signaling proteins Ras, Raf and MEK [150] which leads to the activation of the MAP kinases ERK1 and 2 (also known as p44/42) [151,152]. GH activation of MAPK signaling is, unlike the STAT signaling triggered by GH, not necessarily dependent on JAK2. Deletion of the JAK2 binding site on the GHR leads to a complete abrogation of STAT signaling, but the ERK pathway can still be activated [153], likely through GHR association to the Src family kinase Lyn [154].

The activation of the ERK1/2 kinase signaling cascade results in serine phosphorylation of STATs [155], activation of ribosomal S6 kinases p90^{RSK} and p70^{RSK} [156,157], and activation of phospholipase A2 [158] and the transcription factor c-jun [53]. GH is also able to activate other MAPK superfamily members, namely p38 [159] and the c-Jun amino terminal Kinase (JNK) [160].

1.4.3 IRS and PI 3-Kinase Pathway

Canonical activators of the Insulin Receptor Substrates (IRS) are insulin and IGFs. However, GH signaling is also able to promote activation of IRS1-3 by JAK2 phosphorylation [161-163]. Phosphorylation of IRS-1 has been shown to augment and amplify GH activation of ERK1/2 [164]. Phosphorylated IRS proteins also constitute a binding site for the 85 kDa regulatory subunit (p85) of the Phosphoinositide 3-Kinase (PI3K). Activated PI3K phosphorylate phosphatidylinositide (PI) lipids on the 3' position of the inositol ring, and the phosphorylated PIs mediated the downstream effects of PI3K [165]. PI3K activation is known to regulate glucose uptake by translocation of the glucose transporter type 4 (GLUT4) to the cell membrane [166]. It is also involved in DNA synthesis, cell cycle regulation, cell proliferation and regulation of apoptosis [167-169].

The activation of the IRS-PI3K pathway by GH, and the subsequent translocation of GLUT4, is believed to explain the acute insulin-like effects of GH observed *in vitro* [166,170]. The anti-lipolytic and lipogenic effects of GH are also mediated by PI3K activation [163]. In addition to this, GH effects on cell proliferation, metabolism, survival and cell cycle regulation have been shown to involve PI3K [165]. Interestingly, induction of the p85 regulatory subunit of the PI3K has been proposed to explain the anti-insulinic actions of GH [171].

1.4.4 Other Signaling Pathways

In addition to the pathways described above, GH has been shown to activate several other pathways. There is evidence for ample crosstalk between the various signaling cascades triggered by GH and the responses to GH are sometimes cell-type and tissue specific.

It has been demonstrated that Protein Lipase C (PLC) activity is increased in response to GH and PLC-γ has been shown to be interact with GHR and JAK2 and become phosphorylated in response to GH. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate which produces two second messenger molecules; diacylglycerol (DAG)

and inositol trisphosphate (IP3). DAG interacts with and activates Protein Kinase C (PKC) while IP3 transiently increases free Ca^{2+} concentration in the cell. The increase in Ca^{2+} also serves to activate PKC. PLC/PKC mediated GH actions include activation of the ERK1/2 pathway [172], chemotaxis [173], induction of c-fos expression [174] and increasing intracellular Ca^{2+} concentrations [175].

The Focal Adhesion Kinase (FAK), which is primarily involved in integrin signaling [176], can be phosphorylated in response to GH in a JAK2 dependent fashion [177]. FAK is also involved in the regulation of cell motility and morphology [178] and it has been suggested that GH effects on actin rearrangement and chemotaxis might be mediated by FAK [165] in a process that requires p38 MAPK activation [159].

GH has also been reported to activate Nuclear Factor-κB (NFκB) and that this might in part be dependent on PI3K activation of Akt [179]. GH initiated NFκB activation in chondrocytes has been shown to be of importance for chondrogenesis and metatarsal growth and dependent on STAT5b [180]. The effects of GH on cytokine production in immune cells also seem to be mediated by NFκB activation [181].

The SH2-domain containing protein SH2Bβ is another regulator of GH signaling events. SH2Bβ augments the activity of JAK2 and increase its kinase activity in response to GH [182].
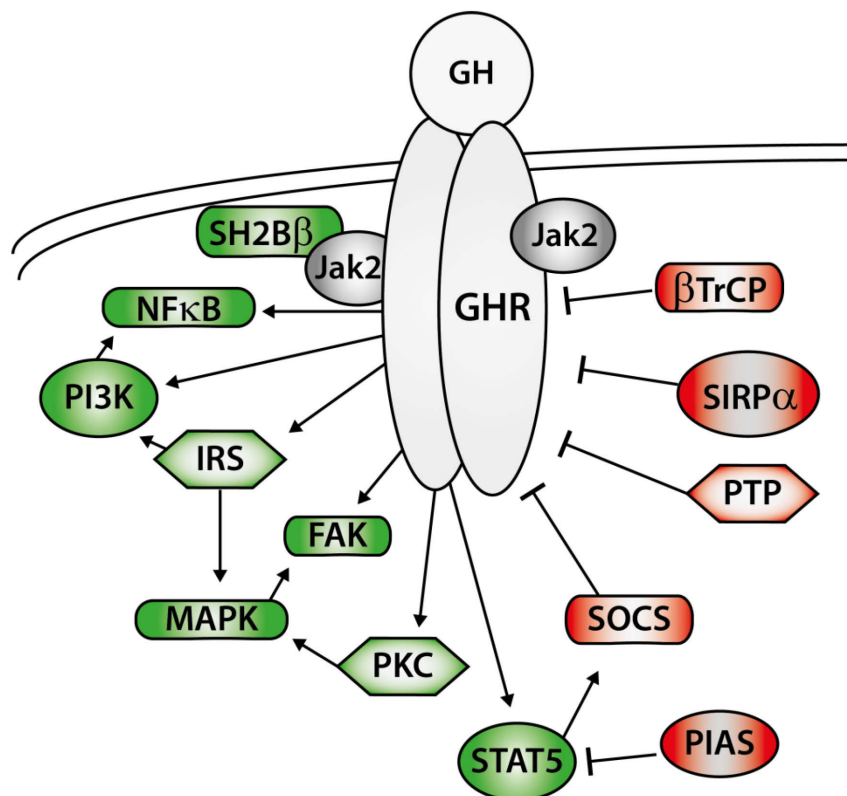

Figure 1.2 Signaling pathways activated by GH and the negative regulation of GH signaling. GH signals mainly through the JAK-STAT pathway but can also activate IRS-PI3K and MAPK signaling among others. The negative regulation of GHR signaling involves dephosphorylation by phosphatases, inhibition of JAK and endocytosis and degradation of GHR. Abbreviations are explained in the text.

1.5 NEGATIVE REGULATION OF GH SIGNALING

As mentioned earlier, intracellular signaling cascades need to be regulated to ensure adequate responses and avoid unintended outcomes. Besides the regulation of GH secretion described above, intracellular GH signaling is regulated by a number of different factors that influence the actions it elicits.

1.5.1 Protein Inhibitors of Activated STATs

One way by which the JAK-STAT pathway can be suppressed is by the Protein Inhibitor of Activated STATs (PIAS) family of proteins. PIAS proteins bind to activated dimerized STATs and prevent them from binding to DNA and initiating the expression of their target genes. PIAS proteins also exhibit E3 ligase activity and are able to modify other proteins by covalently attaching the Small Ubiquitin-like Modifier protein (SUMO) to them [183]. While PIAS proteins were originally discovered as STAT inhibitors it has become clear that they can repress and modify the actions of several other transcription factors, for instance p53 [184] and NFκB [185]. PIAS1 is known to inhibit STAT1 while PIAS3 has been shown to inhibit STAT3 and STAT5. PIASx and PIASy are known to interact with STAT4 and STAT1, respectively, but their functional mechanism remains to be elucidated [186]. PIAS inhibition of STATs does not require SUMOylation of the STAT-proteins but the E3 ligase activity may matter in some instances, suggesting that the inhibition might involve the SUMOylation of other targets [183].

The expression of PIAS proteins is constitutive and it has been suggested that PIAS proteins act in a buffering capacity by maintaining the concentration of activated STATs at a certain level [186]. Furthermore, there is no evidence that PIAS proteins influence GH signaling directly but given their role in the regulation of STAT mediated transcription it cannot be excluded that they have an impact on the actions of GH.

1.5.2 Phosphatases

GH signaling propagation is mainly achieved by phosphorylation of signaling proteins. Dephosphorylation by phosphatases is another method by which GH signaling is negatively regulated. GHR is dephosphorylated by the protein tyrosine phosphatases (PTP), PTP1B and PTPH1. Two additional PTPs have been implicated in the regulation of GHR phosphorylation, TC-PTP and SAP1, but it remains to be elucidated if these two really do dephosphorylate the GHR *in vivo* [187,188]. PTP1B$^{-/-}$ mice exhibit increased STAT5b and JAK2 phosphorylation in response to GH [188] and it has been demonstrated that PLCγ1 is required for the negative regulation of GH signaling by PTP1B since it acts as a bridge between PTP1B and JAK2 and brings the two proteins into close proximity of one another [189].

Another class of phosphatases that regulate GH signaling is the SH2 domain-containing PTPs (SHP), SHP1 and SHP2. GH is able to activate SHP1, which enables the phosphatase to bind to and dephosphorylate JAK2 [190]. SHP1 has also been shown to interact with STAT5 and decrease its activity [191]. Deletion of SHP1 leads to an

increased duration of JAK2 and STAT5b phosphorylation and demonstrates its role as a negative regulator of GH signaling [190]. For the related SHP2 on the other hand, both inhibiting and signaling promoting activity has been demonstrated. SHP2 interacts with GHR, JAK2 and signal-regulatory protein (SIRP) α and GH stimulation leads to SHP2 phosphorylation and induces interaction with Grb2. Over-expression of catalytically inactive SHP-2 leads to decreased GH-induced gene expression suggesting that it is a positive regulator of GH signals [192]. SHP2 has been shown to interact with phosphorylated tyrosine 595 and tyrosine 487 on the GHR and mutation of these residues leads to an increase in GH signaling duration suggesting that SHP2 might act as a negative regulator of GH signaling [193]. It is worth noting that other negative regulators of GH signaling also interact with these residues and that the effect seen might be attributable to their activity rather than that of SHP2 [194,195].

SHP2's interaction partner SIRPα is also known to negatively regulate GH signaling. SIRPα is a transmembrane glycoprotein that becomes phosphorylated in response to GH. Phosphorylated SIRPα inhibits GH induced JAK2, STAT5 and ERK phosphorylation but the mechanism for its actions is unclear [196,197].

1.5.3 GHR endocytosis

The availability of GHR in the membrane of a target cell naturally influences downstream signaling. Absence of receptors renders a cell resistant to the actions of GH while an abundance of receptors sets the stage for a substantial downstream signaling cascade. As mentioned previously, the GHR is present as a homodimer in the cell membrane where it either binds to GH or is proteolytically cleaved to create GHBP. Both cleaved and intact receptors are endocytosed via clathrin-coated pits in the membrane [198], and possibly by the caveolae [199], although this is a subject of debate [200]. GHR internalization is constitutive [201], however, GH stimulation is known to amplify the process [202]. Once internalized, the GHR is degraded at either the lysosome or the proteasome [196].

Extensive studies in Chinese hamster ovary cells by Strous et al. have provided a lot of insight into the mechanisms of GHR internalization and processing. Internalization of the GHR requires a functional ubiquitin-proteasome system, but does not require ubiquitination of the receptor itself [200]. GHR internalization depends on two small motifs located in the juxtamembranous region of the intracellular part of the GHR: the ubiquitin-dependent endocytosis (UbE) motif (DSWVEFIELD), which is required for endocytosis under both induced and basal conditions, and a small degron motif (DSGRTS), which contributes to endocytosis under basal conditions [203]. Mutation of the phenylalanine in the UbE motif effectively blocks GHR internalization and prolongs GH signaling [204]. It has been shown that the WD40 domain of the ubiquitin ligase β-Transducin repeat-Containing Protein (βTrCP) interacts with the UbE and degron motifs and promotes the endocytosis and ubiquitination of the GHR [203,205].

1.5.4 SOCS proteins

An important family of negative regulators of hormone, cytokine and growth factor signaling are the SOCS proteins. The SOCS family contains 8 members; Cytokine-Inducible SH2-containing protein (CIS) and SOCS1-7. SOCS proteins are characterized by a C-terminal SOCS-box, a central SH2 domain and an N-terminal domain of variable size; SOCS1 and 3 also contain a Kinase Inhibitory Region (KIR) situated in the N-terminus [206]. Their domain organization is summarized below in Figure 1.5.

Four of the SOCS-family members have been implicated in the regulation of GHR signaling: SOCS1-3 and CIS [207]. They act in a negative feedback loop, where GH triggers their expression and once translated into proteins they terminate the signal. The kinetics for the expression differs somewhat, with an immediate, transient induction of SOCS1 and 3 and a slower increase of CIS and SOCS2 expression following GH stimulation [208,209]. Different mechanisms for the actions of SOCS proteins have been elucidated: they can directly inhibit kinase activity by the KIR domain (SOCS1 and 3), compete with other signaling molecules for binding to phosphorylated tyrosines and act as ubiquitin ligases that mediate the proteasomal degradation of their targets [210]. The actions of SOCS proteins will be described in greater detail in the following chapters but it is interesting to note that SOCS2$^{-/-}$ mice display increased GH sensitivity and growth.

1.6   THE UBIQUITIN-PROTEASOME SYSTEM

In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for the discovery of ubiquitin mediated protein degradation. In the late 1970's they discovered how covalent attachment of a small polypeptide to other proteins mediated their degradation [211]. The small polypeptide they identified was eventually named ubiquitin (Ub). Since the initial discovery several structurally similar proteins that are also used as post-translational modifications have been described. Of note are the SUMO proteins [212] and the Neural precursor cell-Expressed Developmentally Downregulated (NEDD8) protein [213]. The field of ubiquitin research has expanded significantly and ubiquitin is now known to have other effects besides degradation and to affect a multitude of biological processes. For instance, therapies that block the system are employed clinically to influence the growth of cancer cells [214].

The ubiquitin protein consists of 76 amino acids of which 7 are lysines [215,216]. The process of ubiquitination is the covalent attachment of the C-terminal end of ubiquitin, on usually a free lysine, on a target protein [216]. The process requires three enzymes: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase enzyme (E3). First, the C-terminus of ubiquitin is covalently bound by a thioester linkage to a serine on the E1 in an ATP dependent reaction. Next, the ubiquitin moiety is transferred to a serine on the E2. In the final part of the process, the E2 interacts with the E3 which in turns interacts with the target protein. The ubiquitin is transferred from the E2 and linked through an isopeptide bond to a lysine on the target protein (sometimes via the E3) [217].   Figure 1.3 schematically depicts this process.

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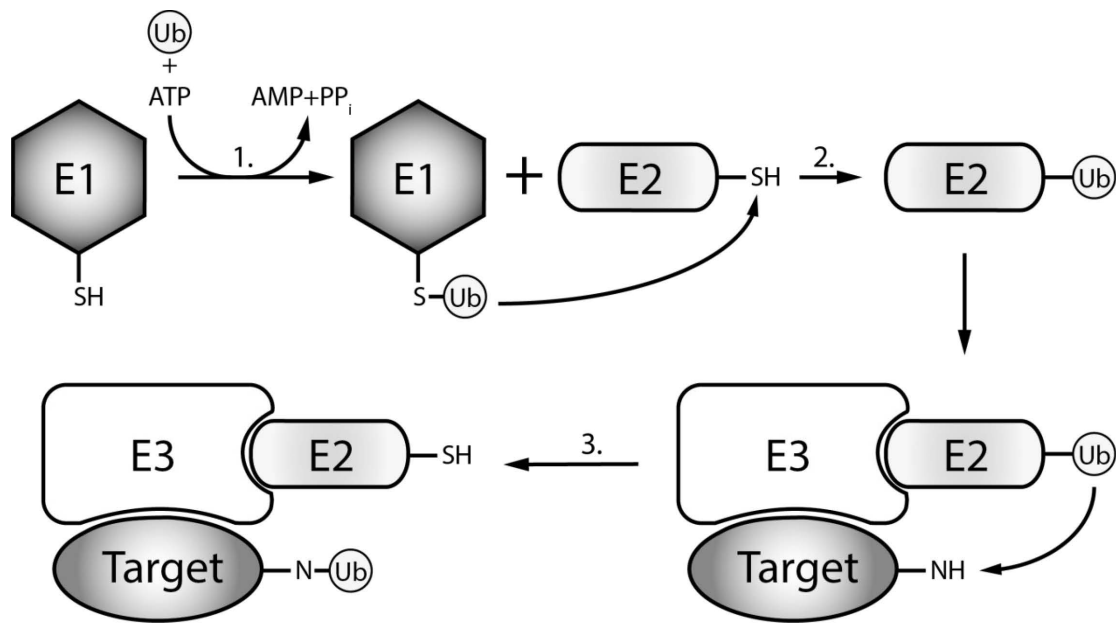
Figure 1.3 The Enzymatic Process of Ubiquitination by RING Ligases. 1. E1 activating enzyme covalently binds ubiquitin on a free serine in an ATP driven reaction. 2. Ubiquitin is transferred from E1 to a serine on the E2 conjugating enzyme. 3. The E2 interacts with the E3 ubiquitin ligase and transfers ubiquitin to a free lysine on the target protein bound to the E3. The process may end after the addition of a single ubiquitin but may also be repeated to generate polyubiquitin chains.

Once a first ubiquitin moiety has been attached to a target protein, several more can be attached to lysines or the N-terminal amine group of the preceding ubiquitin. One ubiquitin molecule can have more than one lysine occupied by other ubiquitin moieties, leading to branched chains. The lysine used for formation of the polyubiquitin chain determines the outcome of the ubiquitination [218]. The two most well characterized chains are Lys48-linked and Lys63-linked chains [219]. The generation of Lys48-linked polyubiquitin chains on a target protein leads to its degradation by the 26S proteasome [220]. The 26S proteasome consists of a central 20S core subunit, which harbors the proteolytic activity of the complex, and two or more 19S subunits that bind to polyubiquitinated targets, deubiquitinates and denatures them and ultimately feeds them into the catalytically active core where they are degraded [221]. Lys63-linked chains primarily play a role in the DNA damage response and in kinase activation. Generation of Lys63-linked chains creates a binding surface for proteins that contain Ubiquitin Binding Domains (UBD) and it can thus promote interactions that are required for signaling propagation [222]. Pathways that involve Lys63-polyubiquitination include IL-1, Toll-like Receptor (TLR) and TNF-α signaling and Lys63 chains have been demonstrated to be involved in NFκB activation [223,224]. The roles of other types of ubiquitin chains are less well elucidated; some are also involved in proteasomal degradation and involvement in mitochondrial function, cell cycle regulation, endocytosis, nuclear translocation and cell signaling cascades have been demonstrated [219].

In humans, 8 different E1 enzymes have been described. Two of them activate ubiquitin and the remaining six activate other ubiquitin-like proteins (UBL), (SUMO and NEDD8 for instance) [225]. The E2 enzymes are more diverse and so far 35 distinct ones have been identified in humans [226], again, some of them conjugate ubiquitin and some of them UBLs. The E3 ligases are responsible for providing the specificity to the system since they interact directly with the target protein, and functional genomic annotation suggests that there may be up to 600 different E3 ligases in humans [227]. While the target recognition domains of E3 ligases vary greatly there are well conserved structural domains that mediate the interaction with E2 enzymes and ubiquitin. Two distinct families have been identified; the Homologous to the E6-AP Carboxyl Terminus (HECT) domain family [228] and the Really Interesting New Gene (RING) finger domain family [229] (including the related PHD finger and U-box containing proteins) [230,231]. The two families differ structurally as well as mechanistically since RING finger containing proteins only associate with the E2 and the E2 directly transfers the ubiquitin to the target protein while the HECT domain is capable of forming a covalent intermediate with ubiquitin [232,233]. E3 ligases based on single peptide chains do exist but many E3 ligases are complexes of several proteins that contribute to different aspects of their activity [234].

1.6.1 Cullin-RING ligases

The GH signal-regulating ubiquitin ligases mentioned above, βTrCP and SOCS proteins, belong to a large sub-family of E3 ligases known as Cullin-RING ligases (CRL) [235,236]. They form multimeric complexes which involve a family of proteins known as Cullins (Cul). The Cullin family contains seven proteins, Cullin1-3, 4A and B, 5 and 7, and they act as a scaffold that links RING-box (Rbx) proteins to the target recognition part of the E3 ligase [237,238].

The RING domain is crucial for the interaction with the E2 and for certain RING domain containing proteins it seems that the affinity for ubiquitin-bound E2s is increased in comparison to free E2s [239]. The detailed mechanism of how RING domains mediate the transfer of ubiquitin to target proteins has remained elusive but recent publications have shown that ubiquitin can fold back and form non-covalent bonds with both the E2 and the RING domain. The current hypothesis is that this serves to stabilize ubiquitin during the transition state before it is bound to a free amine on the target protein [240,241].

The highly conserved C-terminus of the Cullins is responsible for the interaction with Rbx [242]. The N-terminal ends of the Cullin proteins are more variable and they interact with distinct motifs present in the substrate recognizing part of the CRL. Cul1 and Cul7 interact with S-phase-Kinase-associated Protein-1 (Skp1) which is bound to an F-box containing protein that contains a substrate interacting motif of some kind [243,244]. Cul1 containing CRLs are usually referred to as Skp-Cullin-F-box (SCF) complexes, and βTrCP constitutes an example of an F-box protein that participates in SCF complex formation [235,245]. Cul2 and Cul5 interact with SOCS-box containing proteins that interact with Elongin B and C; SOCS proteins exemplify this class of CRLs [236]. Cul3 interacts with proteins that contain Broad complex, Tramtrack, Bric-

a-brac (BTB) domains [246] while Cul4 is believed to interact with the adaptor DNA-Damage-Binding protein-1 (DDB1) [247].

Cullin proteins do not just constitute a passive scaffold for the binding of the other components of the ubiquitination reaction. It has been demonstrated that Cullins can be activated by C-terminal neddylation by the NEDD8 E2, Ubc12 [248,249], and the E3, Defective in Cullin Neddylation (DCN1) [250], in collaboration with the Cullin-bound Rbx protein [251]. Cullin neddylation greatly increases the enzymatic activity of CRLs for a number of substrates [252]. Attachment of NEDD8 induces a conformational change that brings the two ends of the Cullin molecule closer. This allows the Rbx-bound E2 to come in direct contact with the substrate and facilitates the transfer of ubiquitin moieties [253,254]. NEDD8 might also serve as a co-factor for the binding of the E2 to the CRL [252]. Another feature of Cullin activity is dimerization, which is promoted by the N-terminus. Dimerization does not seem to be a complete necessity for CRL activity but it might be important for proper elongation of ubiquitin chains [255].
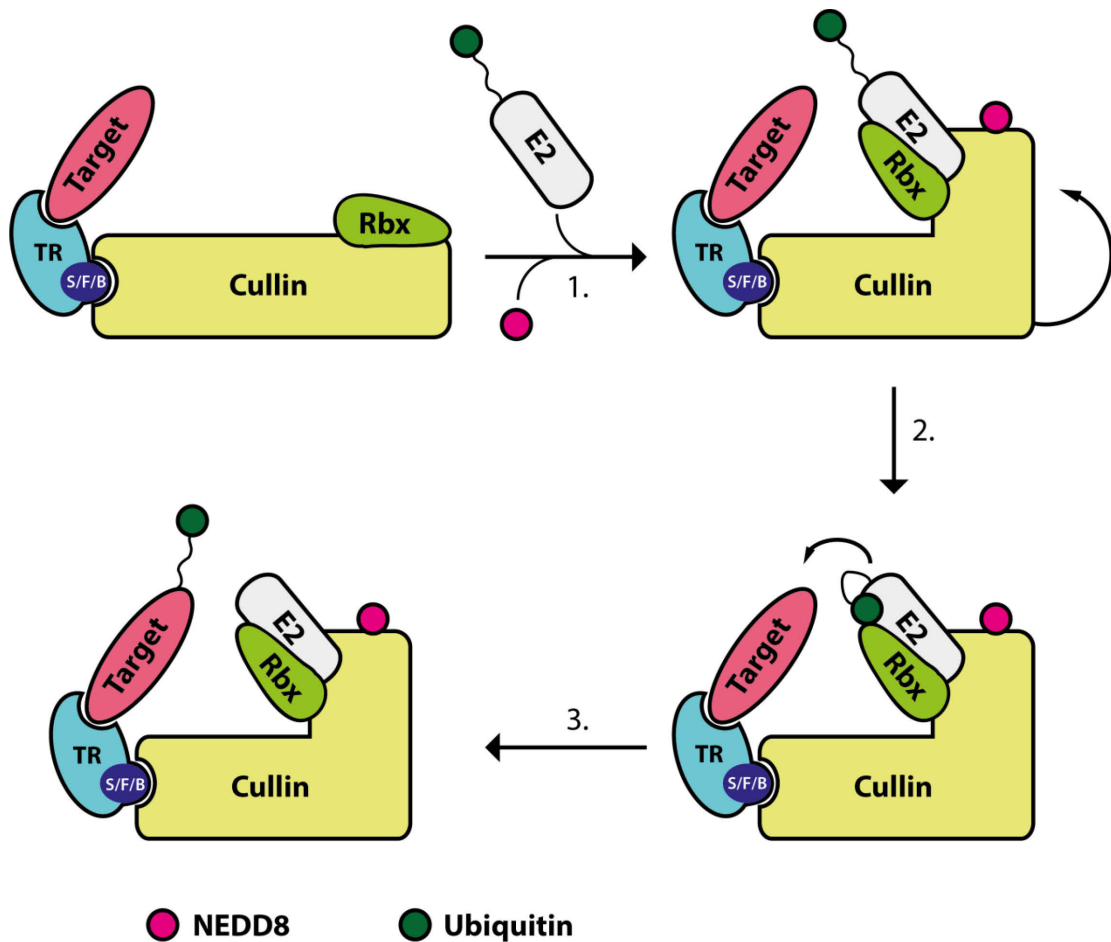

Figure 1.4 Putative mechanism for ubiquitin transfer by Cullin-RING ligases. Cullin interacts with the target recognizing (TR) part of the E3 ligase via the SOCS-box/F-box/BTB domain (S/F/B). Neddylation of Cullin induces a conformational change and promotes the binding of E2-Ub to Rbx (1). Ub is folded in and interacts with the E2, Rbx and Cullin (2). This interaction stabilizes Ub during the transfer to the target (3). The sequence of events might vary.

Two important regulators of Cullin activation are the Cop9 Signalosome and Cullin-Associated and Neddylation-Dissociated 1 (CAND1) protein. The Cop9 Signalosome deneddylates Cullins and can thus be seen as a negative regulator of CRL activity [256]. Similarly, CAND1 is capable of binding to un-neddylated Cul1 and blocking both the Skp1 and the NEDD8 binding sites. It is however not able to bind to neddylated Cul1 due to the conformational change induced by the neddylation and CAND1 has been shown to negatively regulate CRL activity *in vitro* [257]. However, elimination of CAND1 or the Cop9 Signalosome *in vivo* does not increase the activity of CRLs, rather it attenuates the activity [255,258]. This suggests that their roles are more complex than originally expected. It is possible that de-neddylation and re-neddylation of Cullins might be required for proper activity of CRLs and to avoid auto-ubiquitination [255]. Recent reports suggest that CAND1 promotes the assembly of new SCF complexes by triggering the exchange of F-box proteins [259].

1.6.1.1 *SOCS-box proteins*

An interesting group of CRLs are the SOCS-box family of proteins, also referred to as Elongin C-Cullin-SOCS-box (ECS) complexes. The family is believed to encompass up to 80 proteins in humans based on the genomic occurrence of C-terminal SOCS-boxes [260]. ECS complexes consist of a SOCS-box containing protein (with some form of substrate interacting domain) binding to Elongin B and Elongin C with the SOCS-box. The N-terminus of Cul2/5 interacts with both Elongin C and the SOCS-box and it has been shown that sequence variations within the SOCS-box correlate to Cul2 or Cul5 preferential binding [236]. The Elongin B/C-SOCS-box is structurally homologous to the Skp1-F-box structure, indicating that it is a well conserved Cullin interacting conformation [261,262]. The interaction between the SOCS-box and Elongin B/C has been shown to stabilize the ECS complex while disrupting mutations or deletion of the SOCS-box eradicates the E3 ligase activity for several different ECS complexes [194,263-268]. The various substrate interacting domains of ECS complexes include SH2-domains, Ankyrin repeats, Leucine-Rich Repeats (LRR), WD40-domains and SPlA and Ryanodine receptor (SPRY)-domains [260].

Notable examples of SOCS-box proteins are the SOCS family, the von Hippel-Lindau tumor suppressor protein (VHL), and the Ankyrin repeat SOCS-box proteins (ASB). VHL was the first SOCS-box protein to be characterized as an Elongin B/C binding E3 ligase [269,270]. VHL targets the Hypoxia Inducible Factor (Hif) 1α for proteasomal degradation and plays an important role in the regulation of angiogenesis. Mutations in the *vhl* gene that affect the actions of the encoded protein lead to von Hippel-Lindau disease which is characterized by a predisposition for tumor growth [271]. The ASB family consist of 18 proteins, and although less explored than VHL and SOCS proteins, they are important regulators of tumor invasion [272], cell differentiation and protein synthesis [273], arteriogenesis [274] and spermatogenesis [275].

1.6.2 TLR signaling and ubiquitination

The production and release of cytokines is regulated by a myriad of signaling pathways. One interesting family of receptors that control cytokine production in response to invading pathogens is the Toll-like receptor family. TLRs recognize conserved molecular motifs known as pathogen-associated molecular patterns (PAMP). Examples of PAMPs include microbial nucleic acids, parts of bacterial cell walls, lipoproteins and Lipopolysaccharides (LPS) [276]. TLRs are present on either cell surfaces or intracellularly depending on what type of PAMP they are activated by. Here, the signaling of TLR4 will be described.

TLR4 is activated by LPS which is found in the outer membrane of Gram-negative bacteria, but it can also be activated by host-derived molecules, for instance low-density lipoproteins which may contribute to the development of chronic inflammation associated with atherosclerosis and type 2 diabetes [277,278]. Ligand binding to TLR4 recruits the adaptor proteins Myeloid Differentiation factor 88 (MyD88) and MyD88 adaptor-like protein (Mal) to the receptor. They in turn bind IL-1 Receptor Associated Kinases (IRAK) 1 and 4 that become activated upon phosphorylation. Activated IRAKs will interact with TNF Receptor Associated Factor (TRAF) 6 which will associate with and activate TGF-β Activated Kinase (TAK) 1. Here the pathway bifurcates as TAK1 will initiate both MAPK signaling and the activation of NFκB. In addition to the canonical MyD88-pathway, TLR4 can also signal through the TRIF-dependent pathway (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β) which involves signaling through TRAF3 and ultimately leads to interferon production through activation Interferon Regulatory Factor (IRF) 3 [279].

Ubiquitination plays a pivotal role in both the regulation and mediation of signaling in this pathway. TRAF6 is an E3 ligase and will polyubiquitinate target proteins, including itself, with Lys63-linked chains. The TAK1 binding proteins (TAB) 2 and 3 bind to Lys63-polyubiquitin chains and associate with TRAF6, which leads to the activation of TAK1 [279,280]. The TRAF6-generated Lys63-chains can be recognized by the ubiquitin-binding domain of the regulatory subunit of the Inhibitor of NFκB (IκB) kinase (IKK) complex. This allows TAK1 mediated phosphorylation and activation of the complex [281]. The IKK complex goes on to phosphorylate IκB, which allows it to be targeted for ubiquitin-dependent proteasomal degradation by βTrCP. Degradation of IκB exposes the nuclear translocation signal of NFκB and allows it to enter the nucleus and initiate gene transcription [282]. An interesting family of E3 RING-ligases that also affect TLR and NFκB signaling is the Inhibitors of Apoptosis Proteins (IAP). Several proteins in this family contain an ubiquitin binding domain that allows them to interact with already ubiquitinated proteins and one of the more well-studied members, XIAP, has been shown to promote the interaction between TAK1 and IKK through interaction with TAB1 and thus positively affect signaling [283,284]. Two other members of the family, cIAP1 and 2 interact with MyD88 and promotes K48-linked ubiquitination of TRAF3 which has a positive effect on MAPK signaling [276]. Negative ubiquitin related regulation of TLR signaling include deubiquitination of TRAF6 by A20, ubiquitination and proteasomal degradation of TLR4 by Triad3A and K48-linked Mal ubiquitination by SOCS1 [280].

1.7 BIOLOGICAL EFFECTS AND FUNCTIONS OF SOCS PROTEINS

The SOCS proteins were, as the name implies, originally identified as negative regulators of cytokine signaling. They are, as mentioned above, primarily associated with regulating the JAK-STAT pathway which is employed by several of the cytokine family receptors. Since the expression of SOCS proteins is chiefly triggered by STAT signaling they tend to act in a negative feedback loop by downregulating the very pathway that triggers their activation. However, SOCS actions are not limited to the JAK-STAT system and the role of SOCS proteins is not solely that of feedback inhibition. For a summary of factors that induce SOCS expression, pathways regulated by SOCS, and proteins targeted for ubiquitination by SOCS proteins please refer to Table 1.1.
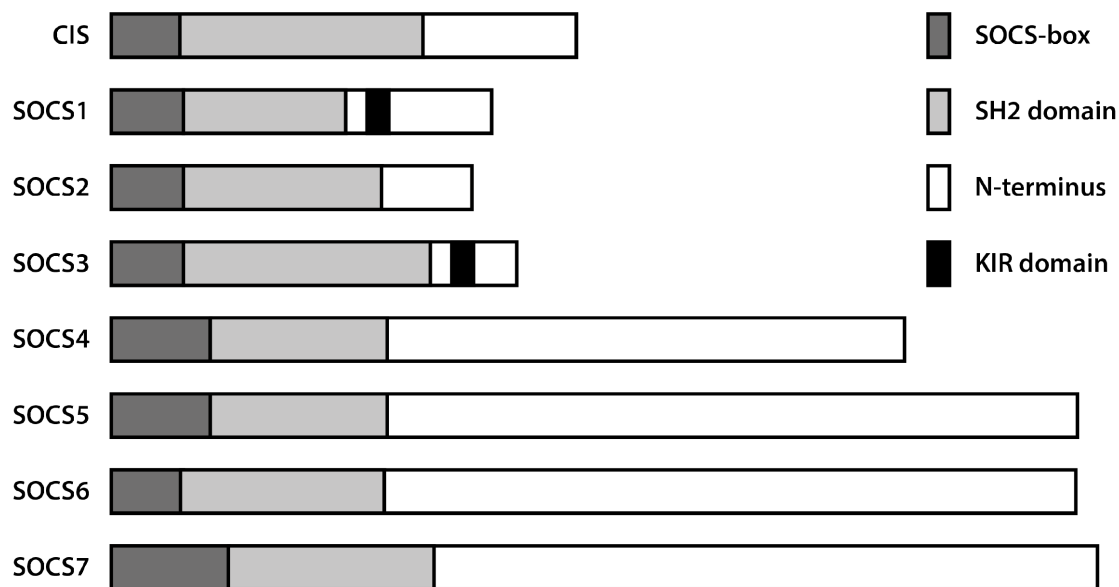

Figure 1.5 Domain structure of the SOCS family. All SOCS proteins contain a C-terminal SOCS-box, a central SH2 domain and an N-terminal domain of varying length. SOCS1 and 3 both have a KIR domain in the N-terminus. Domain sizes are approximate.

1.7.1 SOCS1 and SOCS3

The two most investigated members of the SOCS family are SOCS1 and 3. They have primarily been implicated in the regulation of cytokine signaling in relation to immunological functions but also affect hormone signaling and metabolism. SOCS1$^{-/-}$ mice exhibit stunted growth, liver degeneration and underdeveloped immune responses and they die within 3 weeks of birth [285]. The phenotype can be reverted if the IFN-γ gene is knocked out simultaneously, underscoring the importance of SOCS1 for the regulation of IFN signaling [286]. Deletion of SOCS3 leads to an embryonic lethal phenotype due to functional defects of the placenta [287]. Conditional SOCS3 deletion models have revealed a role for SOCS3 in hematopoiesis and the regulation of Granulocyte-Colony Stimulating Factor (G-CSF) and IL-6 signaling [288,289]. SOCS1 and 3 also negatively regulate insulin signaling by mediating the degradation of IRS-1

20

and 2 [290]. Interestingly, muscle specific deletion of SOCS3 improves HFD induced insulin signaling [291] while liver specific deletion does not and instead promotes lipogenesis, possibly due to altered leptin sensitivity [292].

Ubiquitin ligase activity has been demonstrated for both SOCS1 [293] and 3 [290,294]. However, due to the presence of the KIR domain and their ability to directly inhibit kinases, ubiquitin ligase activity is not crucial for their regulation of cytokine signaling. Deletion of the SOCS-box in SOCS1 and 3 *in vivo* ameliorates the phenotypes observed for the complete knockouts, but does not completely restore the animals [267,295]. Furthermore, mice can be rescued from LPS-induced septic shock by the injection of cell penetrating recombinant SOCS3; an effect likely mediated by the KIR domain [296]. Taken together, this demonstrates the importance of the KIR domain for the *in vivo* actions of SOCS1 and 3.

1.7.2 SOCS 4, 5, 6 and 7

The latest additions to the SOCS family are also its largest members [297]. Specifically, the N-terminal domain of SOCS4-7 is significantly larger than that of the other SOCS family members. However, less is known about the actions of this part of the family. SOCS5$^{-/-}$ mice have no distinctive phenotype [298], while SOCS6$^{-/-}$ mice exhibit slight growth retardation [299]. SOCS7$^{-/-}$ mice are also slightly smaller than wildtype littermates and they suffer from hydrocephalus but have enhanced insulin signaling, likely due to an increase in β-cell mass [300,301]. SOCS4 and 5 have primarily been linked to the regulation of the epidermal growth factor receptor [302]. SOCS6 has a positive effect on insulin signaling, and might have cancer suppressing effects through its regulation of c-Kit receptor signaling [303,304]. SOCS7 on the other hand seems to negatively influence insulin signaling but is a positive marker for breast cancer [300,305].

1.7.3 CIS

The cytokine inducible SH2-containing protein was the first SOCS family member to be described and it is the one most closely related to SOCS2 [306]. It is one of the smaller members of the family and it lacks the KIR domain present in SOCS1 and 3. CIS$^{-/-}$ mice show no obvious phenotype, but transgenic CIS mice exhibit slight growth retardation [307,308]. The actions of CIS overlap with the actions of SOCS2 to a large degree and they likely compete for the same binding sites on the GHR [119]. Besides regulation of GH and PRL signaling [309], CIS also regulates other cytokine signals [306] and is important for the maturation and differentiation of T cells [310] and Natural Killer (NK) cells [307]. CIS can attenuate STAT5 signaling by competing for the same binding sites with its SH2 domain and CIS mediated ubiquitination of the apoptosis triggering Bcl-2-interacting mediator of cell death (Bim) protein has been demonstrated [311,312].

1.7.4 SOCS2

The simultaneous discovery of SOCS1, 2 and 3 was reported by Starr et al in 1997. In line with previous publications on CIS, they were identified as negative feedback regulators of cytokine signaling [313]. The four (at the time described) members were soon identified to be GH regulated and SOCS2 and CIS were found to be reduced in hypophysectomized animals [208,314]. Once knockout animals were generated the paramount importance of SOCS2 for the regulation of longitudinal growth became apparent. SOCS2$^{-/-}$ mice exhibit excessive growth that commences 3 weeks after birth. The males are approximately 40% heavier and the females 20% heavier than wildtype littermates. The weight increase in these animals is not due to any general increase in adiposity, but to a proportional increase in body size. The animals are lean and have perturbed GH signaling. There is no increase in systemic IGF-1 levels but local expression is increased in several organs [315]. Dual-knockout of SOCS2 and STAT5b, and crossing of SOCS2$^{-/-}$ mice with GHRH signaling deficient mice abolished the original phenotype and confirmed that the increased growth observed in SOCS2$^{-/-}$ mice is due to increased sensitivity to GH [195,316]. In humans, SNPs in the vicinity of the SOCS2 gene are associated with stature [317-319]. The SOCS2 gene contains a GH responsive element and its expression is directly regulated by STAT5 [320]. While this is true for several of the other members of the SOCS family, none of the knockout or transgenic models have such a profound effect on postnatal growth as SOCS2. Interestingly, SOCS2 transgenic mice also exhibit enhanced growth and *in vitro* experiments have shown that the inhibitory effect on GH signaling is seen at low levels of SOCS2, while increased levels seem to have a signaling enhancing effect [195,321]. It has been suggested that this might be caused by SOCS2 mediated inhibition of other SOCS proteins, and it has been demonstrated that SOCS2 can counter the inhibitory effects of SOCS1 and 3 [314,322,323]. Similar to CIS, SOCS2 also lacks the KIR domain and thus utilizes other mechanisms for its inhibitory actions. Both competitive inhibition and ubiquitin ligase activity have been suggested; and SOCS2 has been demonstrated to target both the Proline-rich tyrosine Kinase 2 (Pyk2) and GHR for proteasomal degradation [324] (Paper I, [194]). Several factors can induce the expression of SOCS2, either through STAT5, or through independent mechanisms, and thus attenuate GH signaling [313,325].

Besides its actions on GH signaling SOCS2 has been implemented in the regulation of other cytokines (Table 1.1) and immune cell regulation. SOCS2$^{-/-}$ mice are more susceptible to infection and exhibit increased levels of proinflammatory cytokines in response to certain pathogens. Also, expression of SOCS2 is induced by lipoxins and has been demonstrated to mediate some of their anti-inflammatory actions [326]. The role of SOCS2 in the differentiation and maturation of immune cells has come into focus as of late. The proinflammatory, M1 macrophage population is enriched in SOCS2$^{-/-}$ mice. LPS treatment leads to even further enrichment and SOCS2$^{-/-}$ mice are highly susceptible to LPS induced septic shock. Interestingly, macrophage-specific deletion of SOCS3 in mice leads to a diametrically different phenotype and skews the macrophage population towards an anti-inflammatory, M2 polarization which protects the animals from the deleterious effects of LPS [327]. This is in stark contrast to the finding that recombinant SOCS3 can be used to rescue mice from LPS treatment, but might be contributed to increased levels of SOCS1 in SOCS3-ablated macrophages.

Table 1.1 Factors that regulate SOCS expression and factors that are regulated by SOCS

SOCS	Induced by	Negatively regulates signaling by	Demonstrated ubiquitin ligase activity towards
CIS	IL-1, IL-2, IL-3, IL-6, IL-9, IL-10, IL-12, IL-13, LIF, G-CSF, GM-CSF, EGF, CNTF, leptin, IFN-γ, IFN-α, TPO, TNF-α,EPO, TSLP, GH, PRL, LPS, CDCA (FXR)	L-2, IL-3, EPO, GH, PRL, leptin	BimEL
SOCS1	IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-13, IFN-α/β, IFN-γ, LIF, TNF-α, EPO, TPO, TSLP, SCF, CT1, G-CSF, GM-CSF, M-CSF, GH, PRL, insulin, CNTF, TSH, LPS, CpG DNA	IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-15, IFN-α/β, IFN-γ, LIF, M-CSF, TNF-α, EPO, TPO, TSLP, GH, IGF-1, OSM, Flk ligand, SCF, CT1, PRL, insulin, leptin, LPS, CpG DNA	IRS1/2, JAK2, Tel-JAK2, Vav, G-CSFR, p65/RelA
SOCS2	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, G-CSF, GM-CSF, EPO, IFN-α, IFN-γ, LIF, GH, PRL, insulin, CNTF, CT1	IL-6, GH, IGF-1, LIF, PRL	GHR, Pyk2
SOCS3	IL-1, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-22, IFN-α, IFN-γ, LIF, EPO, M-CSF, G-CSF, GM-CSF, GH, PRL, insulin, leptin, TSH, EGF, PDGF, OSM, TPO, TNF-α, CNTF, LPS, CpG DNA	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-11, IFN-α/β, IFN-γ, LIF, OSM, CT1, CNTF, EPO, GH, IGF-1, PRL, insulin, leptin	IRS1/2, G-CSFR, JAK1
SOCS4	EGF	EGF,	
SOCS5	IL-6	EGF, IL-4, IL-6, LIF	
SOCS6	Insulin		IRS4/InsR, c-KIT, Lck, Flt3
SOCS7		IL-6, GH, PRL, Leptin	IRS1/InsR

Abbreviations: CDCA, Chenodeoxycholic acid. CNTF, Ciliary Neurotropic Factor. CT1, Cardiotrophin-1. EGF, Epidermal Growth Factor. EPO, Erythropoeitin. FLT3, FMS-Like Tyrosine kinase 3. G-CSF, Granulocyte-Colony Stimulating Factor. GM-CSF, Granulocyte-Macrophage CSF. M-CSF, Macrophage CSF. GH, Growth Hormone. IFN, Interferon. IL, Interleukin. InsR, Insulin Receptor. IRS, InsR substrate. JAK, Janus Kinase. LIF, Leukemia Inhibitory Factor. LPS, Lipopolysaccharide. OSM, Oncostatin M. PDGF, Platelet Derived Growth Factor PRL, Prolactin. Pyk, Proline Rich Kinase. SCF, Stem Cell Factor. TNF, Tumor Necrosis Factor. TPO, Thrombopoietin . TSH, thyrotropin . TSLP, Thymic stromal lymphopoietin.

SOCS1^{-/-} mice are also highly sensitive to LPS and SOCS1 has been shown to inhibit TLR4 signaling in macrophages [328]. Another example of the effect of SOCS proteins on differentiation is their role in Th cell polarization. Deletion of SOCS2 in mice leads to an increased Th2 population [329], while deletion of SOCS1 increase Th1 differentiation and removal of SOCS3 promotes Th1 and Th17 generation [330]. SOCS2 is also implicated in TLR-ligand induced Dendritic Cell (DC) maturation and activation; however, the effects observed by different studies are contradictory [331,332]. Similarly, SOCS2 has been described as both a negative and a positive regulator of TLR signaling and the exact mechanisms remain to be elucidated [331,333].

While the SOCS2^{-/-} mice do not display any obvious metabolic phenotype, SOCS2 has been linked to metabolism and diabetes. Overexpression of SOCS2 in the β cells of the pancreas leads to diminished insulin secretion and hyperglycemia [334]. In humans, SNPs in the SOCS2 gene have been linked to an increased risk of developing diabetes [335]. Our own studies have also revealed that deletion of SOCS2 protects against hepatic steatosis but worsens insulin sensitivity in mice challenged with a HFD (Paper III, [336]). In addition, previous work has shown that SOCS2 deficiency induces changes in hepatic gene expression that only partially overlap with known GH induced effects, suggesting that not all of the effects attributed to SOCS2 are GH dependent [337]. Further processes affected by SOCS2 include mammary gland development [338], neural differentiation [339], bone mineral density [340] and allergic responses [329].

For detailed references for the factors listed in Table 1.1 please refer to the following publications: [210,260,302,303,311,341-346].

2 AIMS

The general goal of the work presented in this thesis was to determine the mechanism of action for SOCS2 and to investigate its physiological role in the regulation of metabolism. The following aims were therefore set:

1. To determine the molecular mechanism whereby SOCS2 regulates GH signaling. (Paper I).

2. To evaluate the importance of the individual domains in relation to SOCS2 function (Paper I and II).

3. To determine what role SOCS2 plays in hepatic metabolism and the development of insulin resistance and type 2 diabetes (Paper III).

4. To investigate the possibility of interfering with SOCS2 functions with interfering compounds (Supplemental data).

3 METHODS

To achieve the goal of understanding SOCS2 mechanism and actions several different methods were employed. Below follows a brief summary of methods of note, for a more detailed description please refer to the individual papers.

3.1 ANIMAL AND CELLULAR MODELS

For this study several model systems were used to study cell signaling, protein function and metabolic effects. The animal and cellular models used are described below.

3.1.1 Mice

Our animal studies were performed with SOCS2$^{-/-}$ mice (Paper I and III) [315]. They derive from the C57BL/6J strain and wildtype C57BL/6J mice were used as controls. Animal experiments were carried out both at the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet, Sweden, and at the University of Las Palmas de Gran Canaria, Spain. All animal experiments were carried out in accordance with law and local regulations and in compliance with the respective Animal Ethics Committees. Ethical permits were obtained as required. To induce obesity and insulin resistance the mice were fed a HFD *ad libitum* for 2 or 4 months; controls were fed a normal chow diet (Paper III).

3.1.2 Cells

3.1.2.1 Cell lines

All cells were obtained from the American Type Culture Collection. Before stimulation with GH cells were washed and incubated for at least 4 hours in media lacking Fetal Bovine Serum (FBS).

HEK293T cells were used to study the effects of wildtype and mutant version of SOCS on the GHR (Paper I, II and Supplemental data). HEK293T cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 5% CO_2. SuperFect (Qiagen) was employed according to the manufacturer's protocol to carry out transfections.

LNCaP, MCF7 and BRL4 were used to assay the cellular effects of screening hits on GH-signaling (Supplemental data). LNCaP was maintained in RPMI supplemented as above while MCF7 and BRL were cultured in DMEM as above.

3.1.2.2 Bone Marrow Derived Macrophages

To generate Bone Marrow Derived Macrophages (BMDM) from SOCS2$^{-/-}$ and wildtype mice (Paper III) the mice were euthanized and the femur and tibia of the hind legs were dissected and bone marrow cavities flushed out. Bone marrow cells were resuspended in DMEM supplemented as above with the addition of 10 mM HEPES and 20-30% L929 conditioned media (as a source of macrophage colony-stimulating factor). Cells were passed through a cell strainer and cultured at 37°C, 5% CO_2, for 6

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days. Next, non-adherent cells were washed of; the cells were trypsinized, counted and re-plated for experimental purposes.

3.2 WESTERN BLOT

Western blotting is a robust and commonly used technique for the quantification and detection of proteins from various types of samples. It involves electrophoretic gel separation of the proteins in the sample, followed by transfer to a membrane, blocking and probing with antibodies directed at proteins of interest [347,348].

Here, we have used Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) to separate denatured protein containing samples and transferred them to Polyvinylidene Fluoride (PVDF) membranes where they have been probed by antibodies to determine the presence, amount and post-translational modification status of proteins of interest (Paper I – III and Supplemental Data).

3.3 *IN VITRO* UBIQUITINATION ASSAY

The protocol for the *in vitro* ubiquitination assay was adapted from previously published protocols [270,349]. In order to evaluate the general ubiquitin ligase activity of SOCS2 HEK293T cells were transiently transfected with FLAG-tagged SOCS2 (either wildtype or a mutant version), Elongin B and Elongin C (Paper I). Agarose beads coupled to an anti-FLAG antibody were used to immunoprecipitate FLAG-SOCS2 and proteins binding to it. After washing the beads were incubated with the other components necessary for the ubiquitination reaction to occur, namely: E1 activating enzyme, E2 conjugating enzyme (UbcH5b), ubiquitin and HA-tagged ubiquitin and an ATP-regenerating system containing ATP, creatinine phosphate and creatinine kinase. Incubation was carried out for 30 minutes at 30° C and the reaction was stopped by boiling the samples in protein electrophoresis loading buffer. Samples were analyzed by western blot and high-molecular weight conjugates of HA-ubiquitin and target proteins were visualized by probing the PVDF membranes with an anti-HA antibody.

In order to evaluate SOCS2's ability to ubiquitinate GHR HEK293T cells were transfected as above in concert with *Myc*-GHR. The reaction was carried out as described but stopped by the addition of EDTA. Proteins were eluted from the beads by incubation with a FLAG-peptide and GHR was immunoprecipitated from the eluate by an anti-*Myc* antibody. Following washing, samples were boiled in protein electrophoresis loading buffer and analyzed by western blot. GHR was detected by an anti-GHR antibody and ubiquitinated GHR was detected by the same anti-HA antibody as above. The principle for the method is depicted in Figure 3.1.
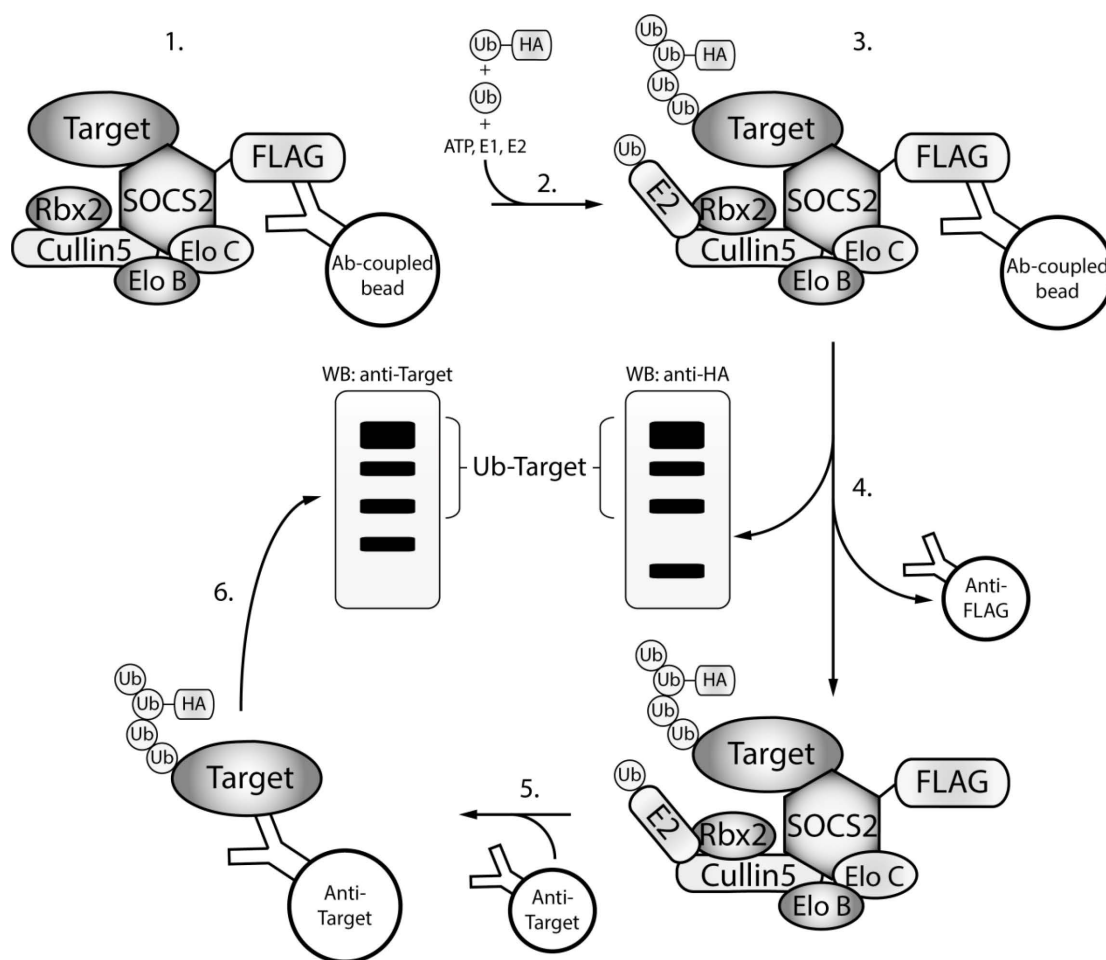
Figure 3.1 *In vitro* ubiquitination assay for SOCS2. (1) FLAG-SOCS2 and bound proteins are immunoprecipitated from cell lysates of cells overexpressing FLAG-SOCS2 and Elongin B/C. (2) Ub, HA-tagged Ub, ATP, E1 and E2 enzymes are added. (3) Incubation for 30 minutes leads to the ubiquitination of SOCS2-bound target proteins. HA-Ub is incorporated in Poly-Ub chains. (4)The reaction is stopped and samples are either eluted or the total ubiquitination activity is visualized by western blot for HA. (5) Eluted samples can be re-precipitated with an antibody that recognizes the target. (6) Western blot detection with anti-HA or anti-Target antibodies can be used to identify the ubiquitinated forms of the target protein

3.4   *IN VITRO* MUTAGENESIS OF PLASMID CONSTRUCTS

Plasmid constructs used have been described earlier [195]. In order to investigate the importance of the various domains of SOCS2 several new constructs with point mutations in the SOCS-box and SH2 domain were generated (Paper I and II). The selection of which residues to mutate was based on previously reported point mutations in related proteins and SOCS2 [350-352].

Mutagenesis was carried out with the Quikchange II kit from Stratagene. In summary, PCR is carried out with a primer pair that contains the desired mutation in a central location and the plasmid that is to be mutated. The reaction generates linear, full length plasmids from the primers that contain the desired mutation. Next, the template, i.e. the original plasmid, is degraded by incubating the samples with *DpnI*, a restriction endonuclease that recognizes methylated sequences. The mutated plasmids are left intact since they are un-methylated. Finally, competent *E. coli* are transformed with the mixture and the mutated plasmids are purified from the bacteria and sequenced to ensure that the mutation is present as desired.

3.5 LUCIFERASE ASSAY

Luciferase reporter assays are frequently used to study gene expression, cellular signaling events and cell physiology. Applications include receptor activity, transcriptional activation and enzymatic activity [353]. In this study the pGL2 Luciferase reporter vector was used; this vector carries the coding region of the wildtype firefly luciferase and it is designed for quantitative analysis of gene expression. We used the luciferase reporter assay to study the effect of SOCS2 levels and SOCS2 mutations on GH signaling (Paper II) and the vector we used had six copies of the GH response elements fused to the minimal TK promoter inserted upstream of the luciferase gene [354]. The vector was transfected into HEK293T cells in concert with different SOCS2 constructs and GHR. Once transfected, the cells were stimulated with GH overnight, lysed and the light intensities in the lysates was measured with a luminometer. A vector expressing the *Renilla* luciferase gene was cotransfected during all experiments to account for variations in transfection efficiency and both luciferases were measured simultaneously using a dual luciferase reporter assay system (Promega).

3.6 METABOLIC PROFILING

To assess the metabolic phenotype of wild type and SOCS2$^{-/-}$ mice, and the effect of the HFD challenge in both strains (Paper III) we utilized several well established methods described below.

3.6.1 Whole body insulin sensitivity

Whole body insulin sensitivity was assessed by intraperitoneal injections of glucose (Glucose Tolerance Test; ipGTT) and insulin (Insulin Tolerance Test; ipITT) followed by measurement of blood glucose levels over time using a glucometer (Paper III). This enables an assessment of: the rate whereby glucose is taken up (ipGTT) and the responsiveness to insulin (ipITT). A slow glucose uptake and a slow or small decrease in blood glucose in response to insulin are indicative of a diabetic state. Measurements of fasting glucose and fasting insulin were also taken and Homeostatic Model of Insulin Resistance (HOMA-IR) values were calculated as follows: fasting insulin (ng/ml) x fasting glucose (mM).

3.6.2 Hepatic triacylglyceride secretion

To determine the Triacylglyceride (TAG) secretion rate mice were injected with Triton WR-1339 to block the hydrolysis of TAG (Paper III). Mice were fasted for 6 hours and injected via the tail vein (500 µg/g of body weight). Blood samples were taken at 0, 30, 60 and 90 minutes and TAG concentration measured by an enzymatic colorimetric assay. The final values were corrected for body weight and the hepatic rate of TAG secretion was calculated from the slope of the curve and expressed as µmol TAG/min.

3.7 SCREENING METHODS

To screen for SOCS2 interacting compounds the EPIC platform was utilized as an initial screening method and hits were confirmed by iso-thermal titration calorimetry (supplemental data).

3.7.1 The EPIC Screening Platform

The initial screen was carried out on the EPIC screening platform (Corning) using 384 well plates. The EPIC plates contain a waveguide at the bottom of the wells and the detection is based on changes in the refractory index of the liquid above the waveguide [355]. Briefly, the wavelength reflected from a well containing an immobilized protein is measured, next an analyte of interest is added and the plate re-measured. If binding to the immobilized protein occurs the refractory index will change and the reflected wavelength will increase. Each well is measured in two areas, one area which contains the immobilized protein and one reference area which lacks the amide coupling chemistry and thus no protein. The signal is calculated from the difference before and after addition of analyte corrected for the internal reference and measured in picometers.

In our experiments recombinant human SOCS2-Elongin C-Elongin B complexes were covalently attached by amine-coupling to the bottom of the wells (20 mM Citrate pH 6.0, 1 hour incubation), followed by washing in 100 mM Tris pH 7.5 to block further binding to the plate. Next, plates were incubated for 4 hour in assay buffer (50 mM HEPES, 100 mM NaCl, 1% DMSO) under temperature stable conditions, measured once before the addition of low molecular weight compounds and 25 minutes after the addition of compound. Experiments were carried out in quadruplicates and positive hits were retested against non-related protein (BSA) and in concentration gradients.

3.7.2 Iso-Thermal Titration Calorimetry

To characterize and confirm the interactions discovered by the EPIC screening platform Iso-thermal calorimetry was employed. The method measures changes in temperature induced by the binding of a ligand to another molecule (protein for instance), or rather the energy required to keep temperatures constant. An iso-thermal calorimeter consists of two separate cells, one for the sample and a reference cell. A molecule of interest is added to the sample cell at a known concentration and with a predetermined volume. The analyte or ligand to be assayed is titrated into the sample cell during stirring and the power required for keeping the temperature difference between the reference and sample cell constant over time is measured [356].

Our experiments were carried out on a MicroCal ITC 200 system (GE). 20 μM of recombinant SOCS2-Elongin C-Elongin B was loaded in the instrument and a 200 μM solution of the compound was injected in 11 increments of 3.5 μl each with 180 seconds delay between injections, stirring speed 1000 rpm, temperature 25°C. All reagents were in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP and 2% DMSO. SOCS2 protein was dialyzed before the experiment and the final buffer used for dialysis was used for ITC; compound was originally dissolved in 100% DMSO and diluted in the same buffer for ITC.

4 RESULTS

4.1 SOCS2 REGULATES GHR TURNOVER - PAPER I

At the start of this project SOCS2 was known to be a negative feedback regulator of GH signaling [315,316] and several mechanisms for its effects had been proposed [196]. The structure of SOCS2 had revealed that it was structurally homologous to other known ubiquitin ligases [264] and our hypothesis was that SOCS2 targeted the GHR or one of the other components of the JAK-STAT pathway for ubiquitination.

The crystal structure of SOCS2 was solved in complex with Elongin B and C [264] and we therefore began by investigating if SOCS2 bound the other proteins present in related ubiquitin ligase complexes, Cullin5 and Rbx2. We found that SOCS2 indeed interacts with these two proteins and that the interaction with the two Elongins serves to stabilize SOCS2. Furthermore, we could show that SOCS2 exhibits ubiquitin ligase activity *in vitro* (Fig 4.1A) and that this activity was dependent on a functional SOCS-box. Next, we investigated what effect SOCS2 has on the other components of the GH-JAK-STAT pathway. Previous reports had determined that SOCS2 binds the GHR [119] and we investigated if this interaction was related to the ubiquitin ligase activity we noted earlier. We found that GHR levels are decreased in the presence of SOCS2 and that this decrease can be halted by the addition of a proteasome inhibitor (Fig 4.1B).
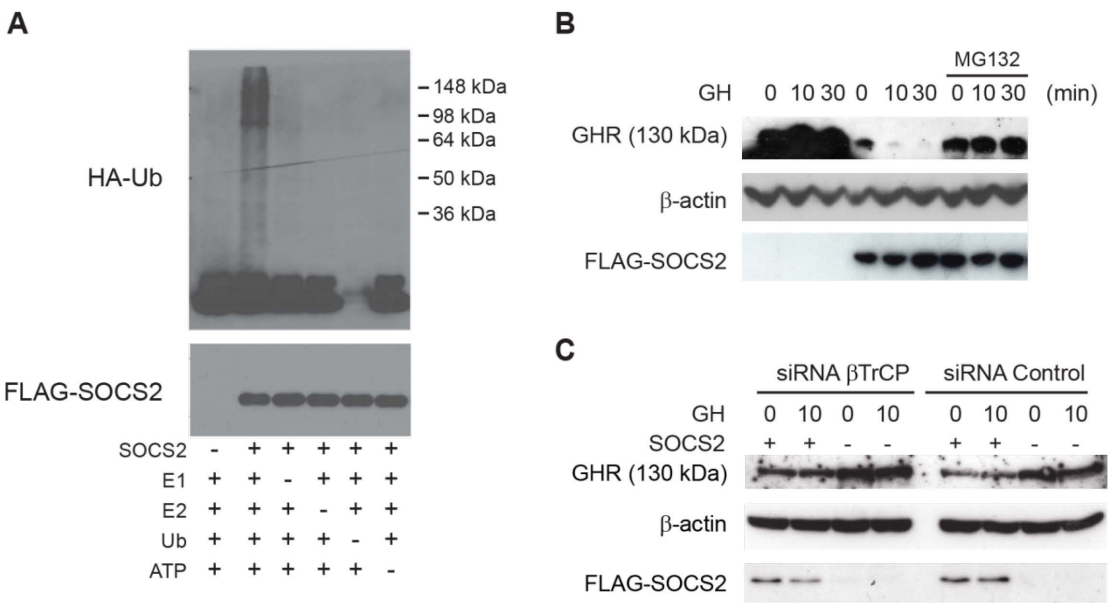
Figure 4.1 SOCS2 regulates GHR levels by ubiquitination. (A) Western blot visualization of high molecular weight conjugates, containing HA-ubiquitin, that are formed when immunoprecipitated SOCS2 is incubated with E1, E2, Ubiquitin and ATP. (B) Western blot of GHR levels in the absence of SOCS2 (lane 1-3) and when SOCS2 is overexpressed (lanes 4-9). Lanes 7-9 are lysates from cells treated with proteasome inhibitor MG132. (C) Western blot showing SOCS2s effect on GHR levels when βTrCP is silenced.

Ger J. Strous and his group had already shown that GHR turnover was dependent on the ubiquitin proteasome system and had identified the ubiquitin ligase βTrCP as involved in GHR ubiquitination [205]. Our experiments showed that silencing of βTrCP indeed increase GHR levels, but that the effect of SOCS2 is independent on βTrCP and we concluded that the two ubiquitin ligases independently regulate the turnover of GHR (Fig 4.1C). We also co-precipitated SOCS2 and GHR and performed an *in vitro* ligase assay where we could observe SOCS2 dependent ubiquitination of the GHR.
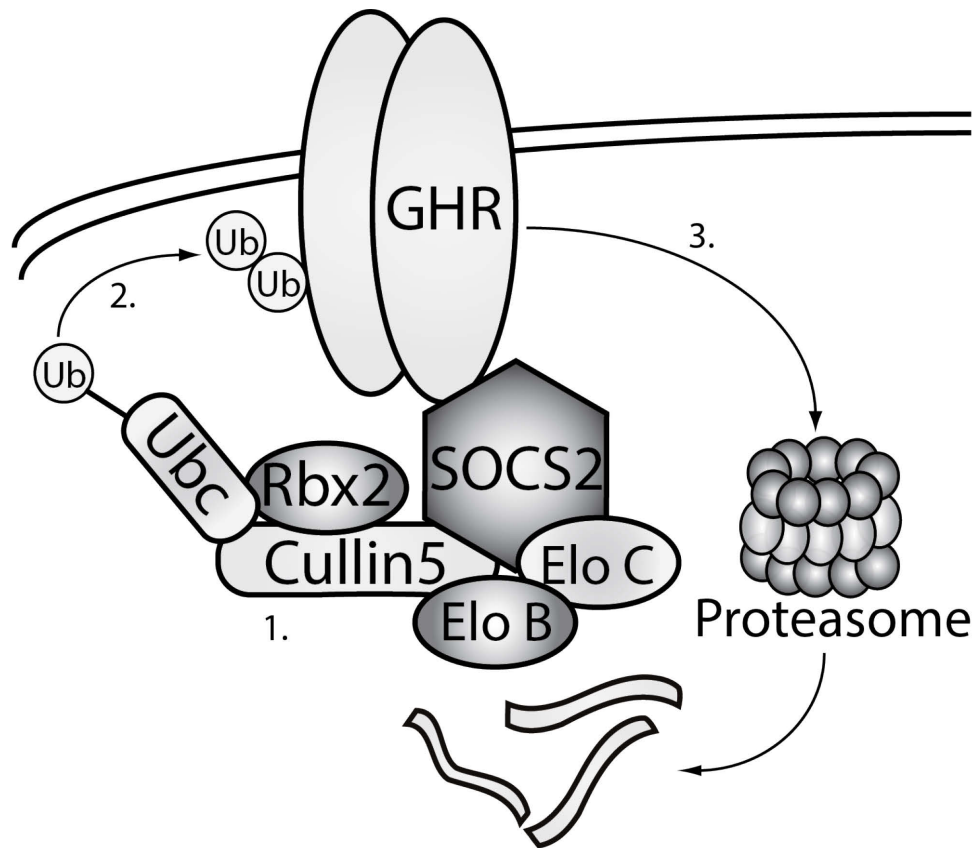
Figure 4.2 SOCS2 binds to the GHR and mediates its ubiquitination. (1) SOCS2 assembles an E3 ubiquitin ligase complex together with Elongin B and C, Cullin5 and Rbx2. Next, SOCS2 binds to phosphorylated tyrosines on the GHR through its SH2 domain. (2) The GHR is poly-ubiquitinated by an E2 conjugating enzyme. (3) The ubiquitinated GHR is degraded by the proteasome.

We also noted that silencing of SOCS2 by siRNA in cells serves to increase GHR levels and that the SOCS2$^{-/-}$ mice have increased levels of GHR in the liver. Finally, we used GHR constructs with mutated tyrosine residues to determine which tyrosines that are crucial for SOCS2s regulation of GHR turnover and found that when Tyr487 is mutated GHR levels are less affected by SOCS2 and that when both Tyr487 and Tyr595 are mutated the GHR level is unaffected by overexpression of SOCS2.

From the experiments conducted in this project we created a model for GHR regulation by SOCS2 which is summarized in Figure 4.2. We conclude that SOCS2 is an ubiquitin ligase that targets the GHR for proteasomal degradation and that this provides a mechanistic explanation for the physiological effects of SOCS2.

4.2 EFFECT OF THE SOCS2 S52N MUTANT ON GH SIGNALLING - PAPER II

In the second paper we investigated the claim that a mutation of SOCS2, S52N, could explain a case of idiopathic acromegaly. A patient in Japan with distinctive signs of acromegaly were recently described [350]. Curiously, no evidence of elevated GH levels or hypothalamic tumors was detected. The patient exhibited a slight elevation in IGF-1 levels and genetic analysis revealed a heterozygous missense mutation in the SOCS2 gene where Ser52 had been replaced with Asn.

To determine if the S52N substitution could be responsible for the reported phenotype we generated FLAG-SOCS2 S52N plasmid vectors and investigated the effect on GHR levels and signaling. In Paper I we noted that the stability of SOCS2 was affected when residues in the SH2 domain were mutated, however, the S52N mutation did not affect protein levels or the association to Elongin B and Cullin5. Next, we investigated if the previously demonstrated ability of SOCS2 to decrease GHR levels was altered for the S52N mutant but again we noted no discernible difference between the WT and the mutant construct. We also employed a luciferase based reporter assay system to quantify the effect on GH signaling but could not detect any significant difference between wildtype SOCS2 and the S52N mutant. Finally, we compared the binding surface of SOCS2 to those of related proteins and noted that a corresponding residue in SOCS3 is involved in a secondary interaction with JAK2 [357]. Since the residue was determined to not be of critical importance and SOCS2 is not known to inhibit JAK2 directly we concluded that it is unlikely to interfere with GHR signaling.

In summary, we were not able to see any effects of the SOCS2 S52N mutation on E3 ligase complex formation, the regulation of GHR levels, GH signaling or association to the GHR. Based on our experiments and a structural assessment of SOCS2 we conclude that the mutation is silent and unlikely to explain the traits of the described patient.

4.3 SOCS2 IN HEPATIC STEATOSIS AND INSULIN RESISTANCE - PAPER III

Despite the similarities in regards to growth between the SOCS2$^{-/-}$ mice and GH transgenic mice the SOCS2$^{-/-}$ mice display no clear metabolic phenotype while GH transgenic mice develop insulin resistance [73,358]. Previous studies from our group have indicated that the hepatic gene expression in SOCS2 mice is altered and that the changes only partially overlap with known GH induced changes [337]. This prompted us to more closely investigate the metabolic phenotype of SOCS2$^{-/-}$ mice. To gain better insight into the roles of SOCS2 and increased GH sensitivity in metabolic processes we provoked the mice with a HFD.

Liver histology revealed that SOCS2$^{-/-}$ mice are protected from HFD induced hepatic steatosis. Hepatic expression of genes involved in TAG assembly was upregulated in SOCS2$^{-/-}$ mice on a HFD, and so was the hepatic *in vivo* TAG secretion rate. As hepatic steatosis is known to be associated with decreased insulin sensitivity we next performed insulin and glucose tolerance tests to investigate this. Surprisingly, we noted that the HFD fed SOCS2$^{-/-}$ mice displayed exacerbated insulin resistance compared to HFD fed wildtype mice. Fasting plasma insulin levels were increased in SOCS2$^{-/-}$ mice on HFD but we did not observe any differences in pancreatic levels of insulin or islet morphology, suggesting that the impaired insulin sensitivity is not caused by altered β cell function. Further analysis of the animals revealed that the HFD induced a marked upregulation of the hepatic expression of proinflammatory cytokines in the SOCS2$^{-/-}$ mice. Plasma levels of proinflammatory cytokines were also increased but we could not detect any enhanced macrophage infiltration of the liver, indicating that the normal population of liver resident macrophages was responsible for the increased secretion.

In vitro studies of macrophages from SOCS2$^{-/-}$ mice demonstrated that deletion of SOCS2 leads to an increased responsiveness to TLR ligands. We assayed the response to LPS which triggers signaling through TLR4 and noted increased secretion of TNF-α, IL-1β and IFN-γ in SOCS2$^{-/-}$ macrophages in response to LPS. The phagocytic activity of the macrophages was also increased and the activation of NFκB, downstream of TLR4, augmented. To confirm our finding we assayed the levels of NFκB phosphorylation *in vivo* and found that they were also increased in SOCS2$^{-/-}$ compared to wildtype mice.

From our experiments we concluded that SOCS2 has a protective role in the development of insulin resistance, likely by suppressing the release of proinflammatory cytokines that are known to contribute to the development of the condition. We describe an interesting model for future studies of the interplay between GH, inflammation and dietary factors in the regulation of glucose and lipid metabolism.

4.3.1 Regulation of TLR signaling by SOCS2

In a follow up investigation to Paper III we have attempted to discern how SOCS2 regulates macrophage activity and specifically how SOCS2 affects LPS-TLR4 signaling. We again compared the LPS sensitivity of SOCS2$^{-/-}$ and wildtype BMDMs (Fig 4.3 A) and found that deletion of SOCS2 increases LPS induced TNF-α secretion. Analysis of the intracellular TLR4 signaling events revealed that the MyD88-dependent NFκB and p38 activation was increased in response to LPS (Fig 4.3 B), but that the MyD88-independent TLR4 activation of IRF3 was unchanged (Fig 4.3 C). To assess if the signaling difference persisted further upstream TAK1 phosphorylation levels were examined in the BMDMs but no SOCS2-dependent effects were discerned (Fig 4.3 D).
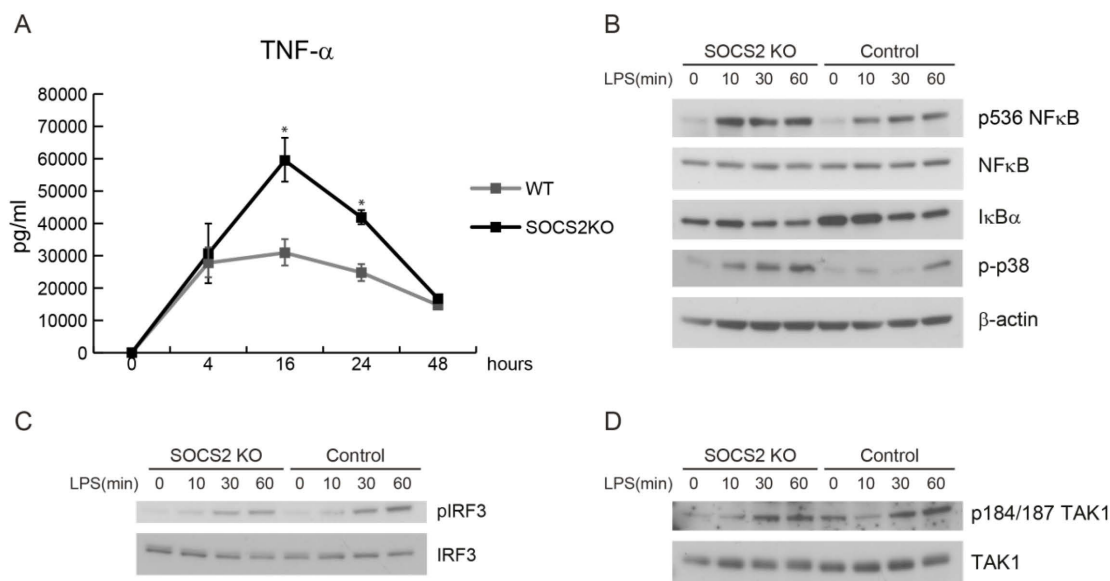
Figure 4.3 SOCS2s effect on TLR4 signaling in Bone Marrow Derived Macrophages. (A) BMDMs from SOCS2KO and WT animals were stimulated with 10 ng/ml of LPS. Media was collected at the indicated timepoints and analyzed with a Milliplex kit to determine TNF-α levels in the media. The experiment was carried out in triplicates. (B), (C) and (D) BMDMs from SOCS2$^{-/-}$ and WT animals were stimulated with 10 ng/ml of LPS and lysed at the indicated timepoints. Whole cell lysates were analyzed by Western blot with the antibodies denoted in the figure.

A lingering question from the published study was to what extent the observed phenotype was related to increased GH signaling and if any of the effects of SOCS2 deletion were GH-independent. To evaluate the role of GH SOCS2$^{-/-}$ and wildtype BMDMs were treated with bovine GH 4 hours before they were stimulated with LPS. Media was collected after 16 hours and analyzed for TNF-α levels. We again saw increased secretion of TNF-α from SOCS2$^{-/-}$ macrophages in response to LPS but no GH effect (Fig 4.4 A).
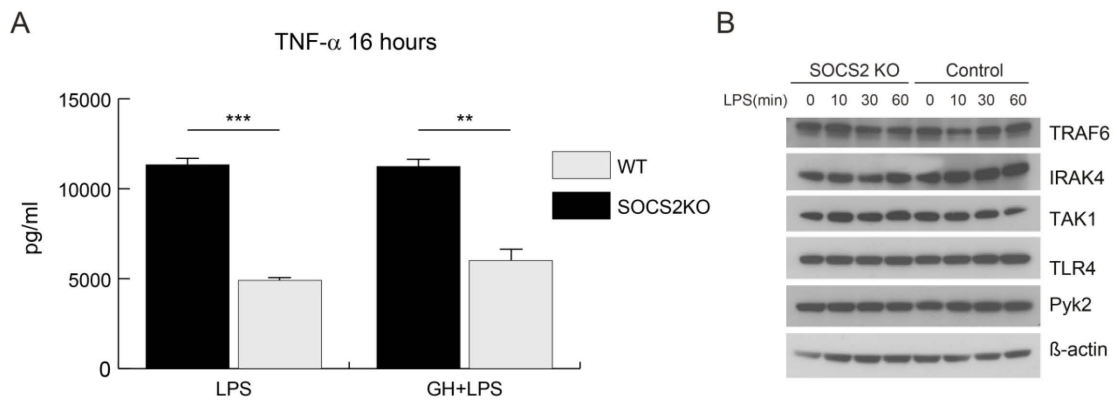
Figure 4.4 Growth Hormone does not affect TNF-α secretion from BMDMs. (A) BMDMs from SOCS2KO and WT animals were either pre-treated with 2 µg/ml of GH and 4 hours later 10 ng/ml of LPS (GH+LPS) or only treated with 10 ng/ml of LPS (LPS). Media was collected 16 hours after LPS stimulation and analyzed with an Elisa kit to determine TNF-α levels in the media. The experiment was carried out in quadruplicates. (B) BMDMs from SOCS2KO and WT animals were stimulated with 10 ng/ml of LPS and lysed at the indicated timepoints. Whole cell lysates were analyzed by Western blot with the antibodies denoted in the figure.

It has been reported that SOCS2 would trigger Lys48-ubiquitination of TRAF6 and thereby regulate the downstream expression of cytokines. To assess if this might explain the increased TLR4 signaling we observe in SOCS2$^{-/-}$ macrophages we investigated the protein levels of TRAF6 and its interacting proteins IRAK4 and TAK1 as well as levels of TLR4 (Fig 4.6 B). We did not observe any SOCS2 dependent changes in the total levels of these four proteins as one would expect if SOCS2 indeed causes Lys48-ubiquitination of them.

As of yet we cannot explain how SOCS2 influences LPS signaling and the activation of macrophages. Given that GH does not seem to affect the response to LPS in our system it is unlikely that the effect is secondary to GH-signaling. Our hypothesis is that SOCS2 has a direct target in the pathway and we are currently evaluating which proteins SOCS2 interacts with in cells stimulated with LPS by proteomic techniques.

4.4 SCREENING FOR SOCS2 MODULATING COMPOUNDS

As part of our studies on SOCS2 actions we have attempted to screen for small, low molecular weight, drug-like compounds that can be used to modulate SOCS2 functions. The aim was to identify compounds that interact with SOCS2 and to test confirmed binders for their ability to affect SOCS2 activity. The screening was carried out in collaboration with Actar on their EPIC instrumentation (Corning) [359]. The screen was carried out against the compound library at Actar which contains low-molecular weight compounds that fulfill the Lipinski rules [360]. A little over 3000 compounds were screened against the recombinant SOCS2-Elongin B-Elongin C complex. Hits were tested against non-related protein (BSA) to exclude constitutive binders.

From the initial screen 11 compounds were further evaluated for their ability to bind SOCS2 by Iso-Thermal Calorimetry (ITC) with the aid of the GE demo-lab. Of the 11 compounds, 1 was confirmed to be a SOCS2 binder (Figure 4.5). The binding of this compound to SOCS2 is endothermic and the affinity was estimated to be in the range $K_D = 0.44 - 2.5 \ \mu M$.
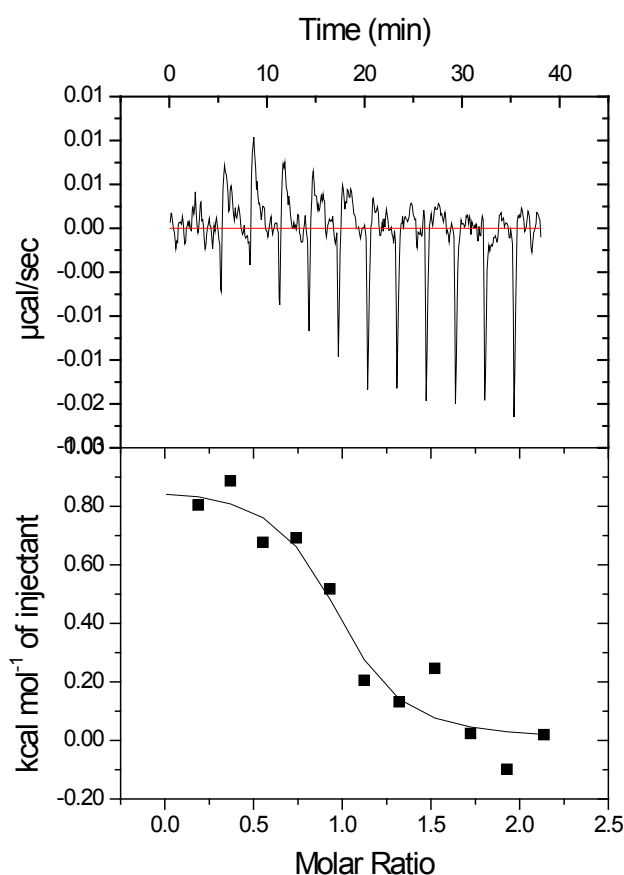
Figure 4.5 Isothermal Calorimetry of recombinant SOCS2-Elongin B-Elongin C and hit 10136. Calorimetry was carried out on a MicroCal ITC 200 system (GE). 20 μM of recombinant SOCS2 was loaded in the instrument and a 200 μM solution of the compound was injected in 11 increments of 3.5 μl each with 180 seconds delay between injections, stirring speed 1000 rpm. All reagents were in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP and 2% DMSO. SOCS2 protein was dialyzed before the experiment and the final buffer used for dialysis was used for ITC; compound was originally dissolved in 100% DMSO and diluted in the same buffer for ITC. Injection of compound in buffer without SOCS2 was also investigated and yielded no signal.

To evaluate the effect on SOCS2 activity we began by investigating the compounds ability to infer with SOCS2s ubiquitin ligase activity *in vitro*. We added the compound to the ubiquitin ligase reaction [194] and evaluated it compared to control (Figure 4.6). We noted a significant inhibition of SOCS2 activity at a concentration of 20 μM of the compound.
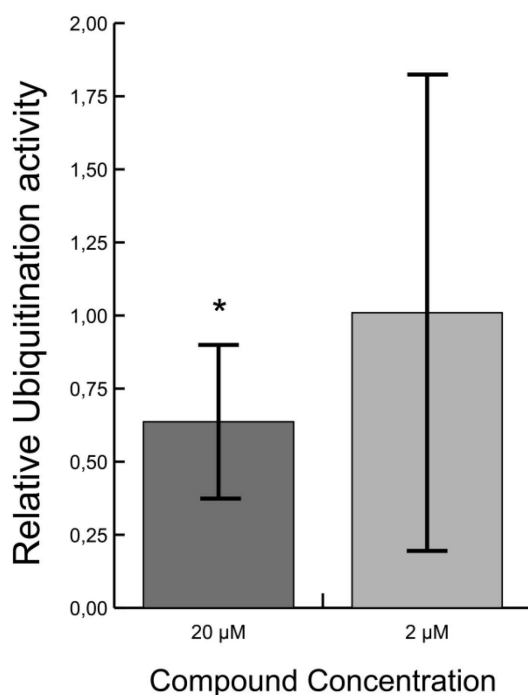

Figure 4.6 Hit 10136 decreases SOCS2s In vitro ubiquitin ligase activity. Quantitation of western blot visualization of high molecular weight (>100 kDa) HA-ubiquitin bands from in vitro ubiquitin ligase activity assay for SOCS2 carried out in the presence of 20 or 2 μM of hit 10136. Data displayed is relative to control (no compound) which is set to 1. N = 5 and error bars denote the 95% confidence interval of the mean.

Next, we moved on to evaluate the compound in different cell systems that respond to GH by STAT5 phosphorylation. However, we were unable to see any effect of the compound even at high (100 μM) concentrations on GH signaling.

Due to the lack of cellular effects we decided to halt our investigation. From the results achieved in the project we do however conclude that the activity of SOCS2 can be targeted and affected by low molecular weight compounds and that the hit we identified constitutes a possible scaffold for the design of such a compound.

5 DISCUSSION

The important role of SOCS2 in the regulation of postnatal growth became obvious with the generation of the first SOCS2$^{-/-}$ mice [315]. No other SOCS protein or phosphatase produces such an obvious growth phenotype when deleted. While the effect has been known for some time, the underlying mechanism for this regulation has been a matter of debate. In Paper I we described how SOCS2 regulates GHR levels and thus terminates downstream signaling events. We demonstrated that SOCS2 ubiquitinates the GHR which ultimately leads to its proteasomal degradation. The finding that SOCS2 is part of an ubiquitin ligase complex and that this activity is important for its effects on GH signaling was not controversial and confirmed previous suspicions [196,264].

We also determined that the SOCS-box is required for the assembly of the E3 ligase complex and that interaction with Elongin C and B are important for the stability of the complex. Similarly, the SOCS-box of SOCS3 has been shown to be unstructured in the absence of Elongin B/C binding and to adopt a stable conformation once engaged by these two proteins [263]. The same is likely the case for SOCS2; we noted that point mutations in the SOCS-box domain led to unstable proteins present at low concentrations while deletion of the entire domain generated a protein with superior stability. The SH2 domain of SOCS2 is involved in the interaction with the target proteins but mutations in this domain also impact stability. Curiously, we also noted that both deletion of the N-terminal domain and point mutations in the SH2 domain abolishes the binding to Elongin C, but not the other components of the ECS complex. The finding could be an artifact, but similar results were obtained for SOCS6 in our lab [304]. An alternative explanation is that Cullin5/Elongin B complexes retain their ability to interact with the C-terminal domain of SOCS proteins. Indeed, independent motifs within the SOCS-box domain seem to mediate Cullin5 and Elongin C binding [236].

In both Paper I and II we investigated the effects of SOCS2 SH2 domain mutants on GHR regulation. The mutant investigated in Paper I contains a mutation of Arg73. Corresponding residues in other SH2 domain containing proteins are involved in the interaction with the phosphotyrosine group and it is well conserved across species and for most human SH2 domains [265,304,357]. We noted that this mutation lead to decreased binding to the GHR, impaired regulation of GHR levels and diminished E3 ligase activity, likely due to decreased affinity for phosphorylated tyrosine residues 487 and 595 in the intracellular domain of GHR. On the other hand, the SOCS2R73E mutant retains some of its activity, suggesting that the SH2-phopshotyrosine interaction might not be the only interaction involved in SOCS2 target recognition. The observation that the SOCS2 dependent decrease in GHR levels was independent of ligand stimulation also suggests that SOCS2 might interact with inactive GHR and mediate its destruction. In Paper II we investigated the importance of Ser52, and specifically serine to asparagine substitution at this position. We could not observe any effect of the mutation suggesting that the position is of little relevance for SOCS2 activity.

The domain that confers the most variation between the SOCS family members is the N-terminal domain. Little is known about this part of the proteins apart from that it contains the KIR domain for SOCS1 and 3 and a nuclear localization motif for SOCS6 [361]. It has not been possible to determine the structure of any of the N-terminal domains in their entirety, likely since they adopt an unstructured conformation in the absence of a binding partner [362]. Further examination of this domain could increase our understanding of SOCS target interactions and possibly yield novel insight into the pleiotropic actions of the SOCS family.

SOCS2 interaction with GHR has been demonstrated previously and in Paper I we showed that of the two phosphotyrosines that are known to interact with SOCS2, Tyr487 is of principal concern for its downregulation of GHR levels. Earlier studies from our group have noted corresponding effects on STAT5 signaling [195]. While our experiments in Paper I demonstrate that ubiquitin mediated proteasomal degradation of the GHR constitute one mechanism for SOCS2s regulation of GH signaling it does not preclude the contribution of other means of regulation. STAT5 interaction with the GHR has been shown to at least partially overlap with SOCS2 binding and even if they do not bind to the same tyrosine, steric hindrance by SOCS2 could still play a role. In particular, the unknown role of the N-terminus might confer such effects.

Another means of SOCS2 regulation of the JAK-STAT pathway that has been suggested is that of direct JAK2 inhibition. Induction of SOCS expression by 17β-estradiol (E_2) leads to decreased GH induced JAK2 phosphorylation [325]. The effect was demonstrated to be dependent on SOCS2 and not SOCS1 or 3. This suggests that SOCS2 might directly inhibit JAK2. However, as we noted in Paper I, SOCS2 mediated turnover of GHR is ligand independent and another possible explanation is that E_2 induces SOCS2 expression, SOCS2 mediates GHR degradation and in the absence of GHR, JAK2 does not become phosphorylated in response to GH. Alas, a recent publication did demonstrate SOCS2 mediated inhibition of JAK2 kinase activity *in vitro* using recombinant proteins [363]. However, these studies did not include a measurement of direct interaction between SOCS2 and JAK2 and the biological relevance of these *in vitro* assays remains unknown. Previous publications have noted that SOCS2 overexpression does not affect JAK2 phosphorylation [314,322] and further experiments are needed to determine how SOCS2 affects JAK2 phosphorylation.

SOCS3 is a known inhibitor of JAK2 with a well characterized inhibitory mechanism. Another recent publication solved the structure of SOCS3 bound both to the gp130 subunit of the IL-6 receptor and to JAK2 [357]. The structure revealed that several residues in the N-terminal end of the SH2 domain, among them Ala50, in SOCS3 is involved in the secondary interaction with JAK2. The corresponding residue in SOCS2 is Ser52 and if SOCS2 indeed has a capacity for direct inhibition it could potentially be involved. This would in that case give credibility to the assumption that the SOCS2 S52N mutation could promote an acromegalic phenotype [350]. However, SOCS3 interaction with the GHR takes place in the membrane proximal part of the receptor, adjacent to the JAK2 binding site on the receptor while SOCS2 interacts in with membrane distal parts of the receptor [309]. It is of course possible that some part of SOCS2 could still interact with JAK2 but the notion seems unlikely. Furthermore, we

did not observe any increase in STAT5 signaling or MAPK activation by the S52N mutant which would be expected if it did indeed lead to diminished JAK2 activation.

As detailed above, several different proteins regulate intracellular GH signaling. While all of these processes do not necessarily influence the actions of GH in all types of cells and under all conditions, it is clear that several mechanisms collaborate to attenuate GH signaling. The regulation of GHR turnover by the ubiquitin ligase βTrCP has been extensively studied and it is clear that βTrCP plays an important role in the regulation of GHR levels. Expression of βTrCP has not been demonstrated to be affected by GH and GHR endocytosis and degradation by βTrCP are independent of ligand stimulation [203]. This suggests that the role of βTrCP is to regulate receptor concentrations under basal conditions and thus the relative responsiveness of the individual cell to GH. SOCS proteins on the other hand, are upregulated in response to GH and through their SH2 domains they preferentially interact with activated receptors. Their role as negative feedback inhibitors is well documented and they contribute to the transient nature of GH induced STAT5 activation. SOCS1 and 3 are the first line of SOCS to be activated and they inhibit JAK2 which leads to a cessation of signaling. SOCS2 (and likely CIS) are upregulated more slowly, but once present they target the GHR. GHR depletion leads to a temporary GH resistant state and is likely important for the propagation of male specific GH pulses. CIS has been shown to be more constitutively upregulated in female livers, and could play a key role in the regulation of sex-specific, GH induced gene expression patterns [364]. The phenotype of the SOCS2$^{-/-}$ mice and our findings in Paper I clearly demonstrate the inhibitory effect of SOCS2 on GH signaling.

In Paper III we describe the phenotype of SOCS2$^{-/-}$ mice under dietary stress. The exacerbated insulin resistance exhibited under these conditions matches the phenotype of GH transgenic mice fed a normal diet. The fact that a provocation in the form of HFD was required for this trait to appear underscores the difference between models of increased hormone secretion and increased hormone sensitivity. We observed that SOCS2$^{-/-}$ mice under normal circumstances have decreased plasma levels of GH compared to wildtype mice but retain normal levels of circulating IGF-1. GH transgenic mice on the other hand have elevated levels of both GH and IGF-1 [365]. Despite increased GH sensitivity the SOCS2$^{-/-}$ mice manage to decrease GH secretion, likely through negative feedback on the pituitary by IGF-1, which allows them to avoid the deleterious diabetogenic effects of elevated GH. The GH transgenic mice do not have the same ability to regulate their GH levels as GH is constitutively produced in these animals. The metabolic phenotypes of several other mouse models of altered GH sensitivity have also been investigated. Mice with a liver specific deletion of GHR, JAK2 or STAT5 spontaneously develop hepatic steatosis [75,366,367]. This is in line with what we observe in Paper III for the SOCS2$^{-/-}$ mice and strongly suggests that the lack of steatosis is due to increased GH sensitivity. Muscle specific deletion of GHR on the other hand protects against diet induced insulin resistance and reveals the tissue specific actions of GH [77]. Again, this correlates to the worsened insulin sensitivity observed in our model and provides a simple explanation for the phenotype: Complete deletion of SOCS2 leads to increased GH sensitivity throughout the body. In the liver the increased GH signaling counters steatosis while the diabetogenic effects of GH on the muscle leads to the observed decrease in insulin sensitivity. However, this model is

overly simplistic and overlooks the contribution of increased GH induced lipolysis and inflammation.

An interesting and recent observation was that the mice with liver specific JAK2 deletion are protected against HFD induced insulin resistance [368]. This differs from the phenotype of mice with liver specific GHR deletion that in addition to hepatic steatosis also develop insulin resistance. An explanation for the discrepancy is that JAK2 is utilized by several other cytokines and in its absence proinflammatory signaling is likely to be decreased. Again, this correlates to the heightened levels of proinflammatory cytokines we observed in the HFD fed SOCS2^{-/-} mice and suggests that this phenotypic feature might be related to GH independent actions of SOCS2. In our follow-up study we investigated the effect of GH to try to elucidate if the increased secretion of TNF-α from SOCS2^{-/-} macrophages is an indirect effect of SOCS2 and related to increased GH sensitivity. We could not observe any GH effect in our *in vitro* cultures and our current hypothesis is that SOCS2 directly regulates TLR4 signaling in macrophages. SOCS2 mediated Lys48 ubiquitination of TRAF6 was reported to attenuate TLR signaling but we have not observed any change in TRAF6 levels in macrophages from SOCS2^{-/-} mice nor any effect on TAK1 phosphorylation [333]. Another putative target for SOCS2 regulation is Pyk2 which has been reported to be degraded by SOCS2 in NK cells and that has also been shown to regulate MyD88-dependent TLR4 signaling [324,369]. Again, we have not observed any SOCS2 dependent change in Pyk2 levels in our cell model and we are currently awaiting mass spectrometrical identification of potential LPS activated, SOCS2 interacting proteins for an unbiased overview of possible targets.

While we have not noted any GH contribution to the augmented LPS signaling in SOCS2^{-/-} macrophages, GH plays a part in the regulation of inflammatory signals. Cell based investigations have reported both pro and anti-inflammatory effects of GH [97,370]. However, it is worth noting that some are based on constitutive overexpression of GH while others use a single bolus dose for treatment which complicates comparative efforts due to the different gene expression patterns triggered by constant and transient GH exposure [371]. *In vivo* experiments have more consistently pointed towards an inflammation promoting role for GH. Transgenic GH mice suffer from chronic inflammation and short term GH treatment of healthy volunteers leads to increased plasma levels of proinflammatory cytokines [372,373]. As pointed out earlier, GH treatment is detrimental for critically ill patients and also suggests a proinflammatory role for GH. Interestingly, a recent publication showed that macrophage-specific deletion of the GHR in mice leads to exacerbated insulin resistance after high fat diet feeding [374]. The described phenotype resembles what we described for the SOCS2^{-/-} mice and suggest that GH has an anti-inflammatory effect during dietary stress. A possible way to reconcile the two models is that in macrophages devoid of GHR there is likely to be low levels of SOCS2 which, as our experiments indicate, could lead to increased secretion of proinflammatory cytokines. Speculatively, SOCS2 might be considered an anti-inflammatory agent that counters the proinflammatory effects of GH, both by direct inhibition of GH signaling but also by decreasing proinflammatory cytokine production. This remains to be shown, but it is interesting to note that the macrophages from both macrophage-specific GHR deletion

and SOCS2$^{-/-}$ mice are both skewed towards M1 polarization suggesting a common link [327,374].

In the supplementary data we give an account of our endeavors to screen for SOCS2-modulating low molecular weight compounds. We have managed to identify a compound which affects SOCS2s ubiquitin ligase activity *in vitro* and work is on-going to improve its actions in cells. The concept of pharmaceutical amplification of hormone sensitivity holds promise of novel therapies that may be better suited for the treatment of hormone resistant states. A key example is diabetes where improving insulin sensitivity is central. GH replacement therapy is also associated with side effects and relying on endogenous hormone levels and improved sensitivity could decrease the occurrence of side effects.

6 CONCLUSIONS AND FUTURE PERSPECTIVE

This thesis explored the molecular basis for the actions of SOCS and its involvement in growth and metabolism. Conclusions are summarized below.

I.   SOCS2 forms an ubiquitin ligase complex with Elongin B and C, Cullin5 and Rbx2. The complex binds to and ubiquitinates the GHR which leads to its proteasomal degradation and a cessation of intracellular GH signaling.

II.   Mutation of the serine residue at position 52 to asparagine in SOCS does not impair SOCS2 mediated regulation of GH signaling. The finding does not support that the mutation contributes to the development of acromegaly.

III.   Deletion of SOCS2 protects against high fat diet induced hepatic steatosis but worsens insulin resistance. Increased levels of proinflammatory cytokines likely contribute to the phenotype.

IV.   SOCS2 is involved in the TLR4 mediated release of proinflammatory cytokines from macrophages *in vitro*. The effect is likely independent of GH.

V.   Screening for and identifying SOCS inhibitory molecules is possible and we have identified a substance that interferes with the actions of SOCS2 *in vitro*. Such inhibitors would be a useful research tool and could potentially constitute a novel class of therapeutic agents.

A suggestion for future investigations based on the findings reported here is to focus on identifying novel targets of SOCS2 that may explain its effects. Several hormone and cytokine signaling pathways are known to be affected by SOCS2 but mechanisms remain unclear. A proper characterization of tissue specific effects of SOCS2 coupled with mass spectrometry to identify targets could prove fruitful in this endeavor. Of key importance is understanding the relationship of SOCS2 and GH in this regulation. Under what conditions is SOCS2 merely a negative regulator of GH actions and under what conditions is it a mediator of GH actions and in what way does SOCS2 act as an independent agent? Several interesting questions remain un-answered related to GH and altered GH sensitivity. Discoveries on the subject of genetically caused GH hypersensitivity due to SOCS2 mutations is likely to be presented in the future and it is also likely that altered GH sensitivity will be more firmly linked to disorders related to specific organ systems. Acquired disturbances of SOCS2 would require further investigations of the hormonal and environmental factors in control of its expression, studies of situations of genomic instability (e.g. tumors) where alteration of SOCS2 would change growth and metabolism. Finally, the concept that SOCS proteins regulate inflammatory signals need to be further investigated. Ultimately one can foresee that the SOCS system will be adequately placed in the context of human physiology and pathology.

7 POPULAR SCIENCE SUMMARY

Several bodily functions are controlled by hormones. Hormones are signaling molecules that are released from specialized glands and travel through the bloodstream to their intended targets. Once they reach their intended organ they interact with specialized receptors on the surface or inside of a target cell. Peptide hormones and cytokines are small proteins that are unable to enter the cell and therefore bind to surface receptors. The binding initiates a signaling cascade inside the cell which leads to the expression of hormone responsive genes. A well-studied example of peptide hormones is Growth Hormone (GH).

It is important that the signaling inside the cell is not to low, but also not exaggerated, and several mechanisms for regulating the signaling therefore exist. An important protein family that regulates hormone and cytokine signals inside the cell is the Suppressors of Cytokine Signaling (SOCS) family. The genes for several SOCS proteins are activated by hormonal signals. This leads to production of SOCS proteins which will start to block the hormonal signaling. This thesis is concerned with the actions of SOCS2, and primarily its regulation of GH signaling. SOCS2 is known to inhibit GH signaling and if the SOCS2 gene is deleted in mice they increase in size by approximately 40%.

In the first paper the detailed molecular mechanism for how SOCS2 blocks GH signaling is described. SOCS2 binds to several other proteins and together they form a large complex. This complex binds to the GH receptor and transfers a small protein called ubiquitin to the receptor. A chain of ubiquitin proteins eventually forms on the receptor, and this chain acts as a flag that marks the receptor for degradation. Flagged receptors are recognized by the proteasome which will begin to break them down. Once the receptors are degraded, the signaling ends. In the second paper a mutant version of SOCS2 is investigated. In this mutant the amino acid serine in position 52 is changed to an asparagine. This mutant has been reported in a patient with acromegaly, a syndrome normally associated with too much GH. However, the reported mutation does not affect SOCS2 activity or GH signaling and is therefore not likely to cause acromegaly.

In the third paper the role of SOCS2 in metabolism is investigated. Mice where the SOCS2 gene is deleted (SOCS2 knock-out mice) were fed a high fat diet in order to induce diabetes. We found that compared to normal mice fed the same diet the SOCS2 knock-out mice had reduced fat in their livers, which is a good sign, but they developed worsened insulin resistance. The reason for this is increased chronic inflammation and points to an important role for GH and SOCS2 in the regulation of inflammatory signals.

In summary, we have elucidated the mechanism of action for SOCS2s regulation of GH signaling. We also report that the SOCS2 Ser52Asn mutant is fully functional and likely does not cause acromegaly. And finally, that SOCS2 knock-out mice are protected from diet induced fatty liver but instead become increasingly diabetic. The concept that SOCS2 regulates sensitivity to hormones can be of relevance to disorders of metabolism, growth and inflammation.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Många av kroppens funktioner styrs av hormoner. Hormoner är signalmolekyler som frisätts från specialiserade körtlar och färdas genom blodbanan till olika organ. När de når sitt avsedda organ så binder de till receptorer på ytan eller inuti sina målceller. Peptidhormoner och cytokiner är små proteiner som inte själva kan ta sig in i cellen och de binder därför till ytreceptorer. När de binder till sin receptor så påbörjas en signalkaskad inuti cellen vilket leder till att hormonaktiverade gener uttrycks. Ett välstuderat exempel på ett peptidhormon är tillväxthormon (GH).

Det är viktigt att hormonsignalen inuti cellen inte är för svag eller överdriven utan lagom och flera mekanismer som reglerar signalen finns därför. En viktig familj av protein som reglerar hormon- och cytokinsignaler är familjen Suppressorer av Cytokinsignalering (SOCS). Generna för flera SOCS proteiner aktiveras av hormonsignaler. Detta leder till produktion av SOCS proteiner som börjar blockera signalen. Denna avhandling rör SOCS2 och framförallt dess reglering av GH signaler. Det är sedan tidigare känt att SOCS2 reglerar GH signalering och om genen för SOCS2 tas bort i möss så ökar deras storlek med ungefär 40%.

I den första artikeln så beskrivs den detaljerade molekylära mekanismen för hur SOCS2 blockerar GH signallering. SOCS2 binder till ett flertal andra proteiner och tillsammans bildar de ett stort komplex. Detta komplex binder till GH receptorn och överför ett litet protein benämnt ubiquitin till receptorn. En kedja av ubiquitin bildas så småningom på receptorn och denna kedja fungerar som en flagga som markerar att receptorn ska brytas ned. Markerade receptorer känns igen av proteasomen som börjar bryta ned dem. När GH receptorerna har brutits ned så avstannar signalen. I den andra artikeln så undersöks en SOCS2 mutant. I denna mutant har aminosyran serin på position 52 bytts ut mot en asparagin. Det har rapporterats att den här mutationen är närvarande i en patient med akromegali, ett syndrom som normalt karakteriseras av för mycket GH. Våra resultat visar dock att mutationen inte påverkar SOCS2s aktivitet eller GH signaleringen och det är därför inte troligt att den orskar akromegali.

I den tredje artikeln undersöks SOCS2s effekt på ämnesomsättningen. Möss där SOCS2-genen tagits bort (så kallade SOCS2-knock-out möss) gavs en diet med högt fettinnehåll för att framkalla diabetes. Jämfört med normala möss som fick samma diet så har SOCS2-knock-out mössen minskad leverförfettning, vilket brukar betraktas som ett gott tecken, men de utvecklar förvärrad insulinresistens. Vi fann att skälet för den förvärrade insulinresistensen är ökad kronisk inflammation i knock-out mössen vilket pekar på att GH och SOCS2 har en viktig roll i regleringen av inflammatoriska signaler.

Sammanfattningsvis så har vi utrett mekanismen för hur SOCS2 reglerar GH signallering. Vi rapporterar även att mutationen Ser52Asn i SOCS2 inte påverkar SOCS2s aktivitet och troligtvis inte orsakar akromegali. Samt att SOCS2-knock-out möss är skyddade från dietorsakad leverförfettning men istället utvecklar förvärrad diabetes. Kunskap kring förändrad känslighet för GH kan vara av betydelse för metabola sjukdomar liksom för cancer och inflammatoriska tillstånd.

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10 REFERENCES

1. Evans H, Long J. The effect of the anterior lobe administered intraperitoneally upon growth, maturity, and oestrous cycles of the rat. *Anat Rec.* 1921;21(1):62-63.
2. Li CH, Evans HM. The Isolation of Pituitary Growth Hormone. *Science.* 1944;99(2566):183-184.
3. Li CH, Papkoff H. Preparation and properties of growth hormone from human and monkey pituitary glands. *Science.* 1956;124(3235):1293-1294.
4. Raben MS. Treatment of a pituitary dwarf with human growth hormone. *The Journal of clinical endocrinology and metabolism.* 1958;18(8):901-903.
5. Sawin CT. Maurice S. Raben and the Treatment of Growth Hormone Deficiency. *Endocrinologist.* 2002;12(2):73-76.
6. Daughaday WH, Herington AC, Phillips LS. The regulation of growth by endocrines. *Annual review of physiology.* 1975;37:211-244.
7. Abdel-Meguid SS, Shieh HS, Smith WW, Dayringer HE, Violand BN, Bentle LA. Three-dimensional structure of a genetically engineered variant of porcine growth hormone. *Proceedings of the National Academy of Sciences of the United States of America.* 1987;84(18):6434-6437.
8. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science.* 1989;244(4908):1081-1085.
9. Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, Wells JA. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science.* 1991;254(5033):821-825.
10. Chen WY, Wight DC, Mehta BV, Wagner TE, Kopchick JJ. Glycine 119 of bovine growth hormone is critical for growth-promoting activity. *Molecular endocrinology.* 1991;5(12):1845-1852.
11. Chen WY, Chen N, Yun J, Wagner TE, Kopchick JJ. In vitro and in vivo studies of the antagonistic effects of human growth hormone analogs. *The Journal of biological chemistry.* 1994;269(32):20806.
12. van der Lely AJ, Kopchick JJ. Growth hormone receptor antagonists. *Neuroendocrinology.* 2006;83(3-4):264-268.
13. Paladini AC, Pena C, Poskus E. Molecular biology of growth hormone. *CRC critical reviews in biochemistry.* 1983;15(1):25-56.
14. Kopchick JJ, Andry JM. Growth hormone (GH), GH receptor, and signal transduction. *Molecular genetics and metabolism.* 2000;71(1-2):293-314.
15. Alsat E, Guibourdenche J, Luton D, Frankenne F, Evain-Brion D. Human placental growth hormone. *American journal of obstetrics and gynecology.* 1997;177(6):1526-1534.
16. Cohen LE, Wondisford FE, Radovick S. Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. *Endocrinology and metabolism clinics of North America.* 1996;25(3):523-540.
17. McCormick A, Brady H, Theill LE, Karin M. Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. *Nature.* 1990;345(6278):829-832.
18. Tannenbaum GS. Neuroendocrine control of growth hormone secretion. *Acta paediatrica Scandinavica. Supplement.* 1991;372:5-16.
19. Guillemin R. Hypothalamic hormones a.k.a. hypothalamic releasing factors. *The Journal of endocrinology.* 2005;184(1):11-28.
20. Takahashi Y, Kipnis DM, Daughaday WH. Growth hormone secretion during sleep. *The Journal of clinical investigation.* 1968;47(9):2079-2090.
21. Jaffe CA, Ocampo-Lim B, Guo W, et al. Regulatory mechanisms of growth hormone secretion are sexually dimorphic. *The Journal of clinical investigation.* 1998;102(1):153-164.
22. Isgaard J, Carlsson L, Isaksson OG, Jansson JO. Pulsatile intravenous growth hormone (GH) infusion to hypophysectomized rats increases insulin-like growth factor I messenger ribonucleic acid in skeletal tissues more effectively than continuous GH infusion. *Endocrinology.* 1988;123(6):2605-2610.

23. Wauthier V, Waxman DJ. Sex-specific early growth hormone response genes in rat liver. *Molecular endocrinology.* 2008;22(8):1962-1974.

24. Jaffe CA, Turgeon DK, Lown K, Demott-Friberg R, Watkins PB. Growth hormone secretion pattern is an independent regulator of growth hormone actions in humans. *American journal of physiology. Endocrinology and metabolism.* 2002;283(5):E1008-1015.

25. Rosenbloom AL. Physiology and disorders of the growth hormone receptor (GHR) and GH-GHR signal transduction. *Endocrine.* 2000;12(2):107-119.

26. Salmon WD, Jr., Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *The Journal of laboratory and clinical medicine.* 1957;49(6):825-836.

27. Casanueva FF. Physiology of growth hormone secretion and action. *Endocrinology and metabolism clinics of North America.* 1992;21(3):483-517.

28. Namba H, Morita S, Melmed S. Insulin-like growth factor-I action on growth hormone secretion and messenger ribonucleic acid levels: interaction with somatostatin. *Endocrinology.* 1989;124(4):1794-1799.

29. Nass R, Gilrain J, Anderson S, et al. High plasma growth hormone (GH) levels inhibit expression of GH secretagogue receptor messenger ribonucleic acid levels in the rat pituitary. *Endocrinology.* 2000;141(6):2084-2089.

30. Gillies G. Somatostatin: the neuroendocrine story. *Trends in pharmacological sciences.* 1997;18(3):87-95.

31. Wren AM, Small CJ, Ward HL, et al. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology.* 2000;141(11):4325-4328.

32. Shuto Y, Shibasaki T, Otagiri A, et al. Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *The Journal of clinical investigation.* 2002;109(11):1429-1436.

33. Meinhardt UJ, Ho KK. Modulation of growth hormone action by sex steroids. *Clinical endocrinology.* 2006;65(4):413-422.

34. Kanaley JA, Weltman JY, Veldhuis JD, Rogol AD, Hartman ML, Weltman A. Human growth hormone response to repeated bouts of aerobic exercise. *Journal of applied physiology (Bethesda, Md. : 1985).* 1997;83(5):1756-1761.

35. Allen DB. Growth suppression by glucocorticoid therapy. *Endocrinology and metabolism clinics of North America.* 1996;25(3):699-717.

36. Tamura H, Kamegai J, Sugihara H, Kineman RD, Frohman LA, Wakabayashi I. Glucocorticoids regulate pituitary growth hormone secretagogue receptor gene expression. *Journal of neuroendocrinology.* 2000;12(6):481-485.

37. Cabello G, Wrutniak C. Thyroid hormone and growth: relationships with growth hormone effects and regulation. *Reproduction, nutrition, development.* 1989;29(4):387-402.

38. Sotiropoulos A, Goujon L, Simonin G, Kelly PA, Postel-Vinay MC, Finidori J. Evidence for generation of the growth hormone-binding protein through proteolysis of the growth hormone membrane receptor. *Endocrinology.* 1993;132(4):1863-1865.

39. Baumbach WR, Horner DL, Logan JS. The growth hormone-binding protein in rat serum is an alternatively spliced form of the rat growth hormone receptor. *Genes & development.* 1989;3(8):1199-1205.

40. Baumann G. Growth hormone binding protein 2001. *Journal of pediatric endocrinology & metabolism : JPEM.* 2001;14(4):355-375.

41. Isaksson OG, Jansson JO, Gause IA. Growth hormone stimulates longitudinal bone growth directly. *Science.* 1982;216(4551):1237-1239.

42. Jorgensen JO, Rubeck KZ, Nielsen TS, et al. Effects of GH in human muscle and fat. *Pediatric nephrology.* 2010;25(4):705-709.

43. Vijayakumar A, Novosyadlyy R, Wu Y, Yakar S, LeRoith D. Biological effects of growth hormone on carbohydrate and lipid metabolism. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2010;20(1):1-7.

44. Nam SY, Lobie PE. The mechanism of effect of growth hormone on preadipocyte and adipocyte function. *Obesity reviews : an official journal of the International Association for the Study of Obesity.* 2000;1(2):73-86.

45. Madsen K, Friberg U, Roos P, Eden S, Isaksson O. Growth hormone stimulates the proliferation of cultured chondrocytes from rabbit ear and rat rib growth cartilage. *Nature.* 1983;304(5926):545-547.

46. Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. *Endocrine reviews.* 1996;17(5):481-517.

47. Smaniotto S, Martins-Neto AA, Dardenne M, Savino W. Growth hormone is a modulator of lymphocyte migration. *Neuroimmunomodulation.* 2011;18(5):309-313.

48. Kelley KW, Arkins S, Minshall C, Liu Q, Dantzer R. Growth hormone, growth factors and hematopoiesis. *Hormone research.* 1996;45(1-2):38-45.

49. Yoshizato H, Fujikawa T, Soya H, Tanaka M, Nakashima K. The growth hormone (GH) gene is expressed in the lateral hypothalamus: enhancement by GH-releasing hormone and repression by restraint stress. *Endocrinology.* 1998;139(5):2545-2551.

50. Creyghton WM, van Dam PS, Koppeschaar HP. The role of the somatotropic system in cognition and other cerebral functions. *Seminars in vascular medicine.* 2004;4(2):167-172.

51. Parkinson C, Renehan AG, Ryder WD, O'Dwyer ST, Shalet SM, Trainer PJ. Gender and age influence the relationship between serum GH and IGF-I in patients with acromegaly. *Clinical endocrinology.* 2002;57(1):59-64.

52. Schalch DS, Reichlin S. Plasma growth hormone concentration in the rat determined by radioimmunoassay: influence of sex, pregnancy, lactation, anesthesia, hypophysectomy and extrasellar pituitary transplants. *Endocrinology.* 1966;79(2):275-280.

53. Pilecka I, Whatmore A, Hooft van Huijsduijnen R, Destenaves B, Clayton P. Growth hormone signalling: sprouting links between pathways, human genetics and therapeutic options. *Trends in endocrinology and metabolism: TEM.* 2007;18(1):12-18.

54. Villemure I, Stokes IA. Growth plate mechanics and mechanobiology. A survey of present understanding. *Journal of biomechanics.* 2009;42(12):1793-1803.

55. Karimian E, Savendahl L. Estrogen signaling in growth plate cartilage. *Endocrine development.* 2011;21:42-51.

56. Zhou Y, Xu BC, Maheshwari HG, et al. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(24):13215-13220.

57. Alba M, Fintini D, Salvatori R. Effects of recombinant mouse growth hormone treatment on growth and body composition in GHRH knock out mice. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2005;15(4):275-282.

58. Alba M, Salvatori R. A mouse with targeted ablation of the growth hormone-releasing hormone gene: a new model of isolated growth hormone deficiency. *Endocrinology.* 2004;145(9):4134-4143.

59. Walker DG, Simpson ME, Asling CW, Evans HM. Growth and differentiation in the rat following hypophysectomy at 6 days of age. *Anat Rec.* 1950;106(4):536-554.

60. Zapf J. Growth promotion by insulin-like growth factor I in hypophysectomized and diabetic rats. *Molecular and cellular endocrinology.* 1998;140(1-2):143-149.

61. Daughaday WH, Hall K, Raben MS, Salmon WD, Jr., van den Brande JL, van Wyk JJ. Somatomedin: proposed designation for sulphation factor. *Nature.* 1972;235(5333):107.

62. Klapper DG, Svoboda ME, Van Wyk JJ. Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinology.* 1983;112(6):2215-2217.

63. Biller BM, Samuels MH, Zagar A, et al. Sensitivity and specificity of six tests for the diagnosis of adult GH deficiency. *The Journal of clinical endocrinology and metabolism.* 2002;87(5):2067-2079.

64. van Buul-Offers SC, Kooijman R. The role of growth hormone and insulin-like growth factors in the immune system. *Cellular and molecular life sciences : CMLS.* 1998;54(10):1083-1094.

65. Powell-Braxton L, Hollingshead P, Warburton C, et al. IGF-I is required for normal embryonic growth in mice. *Genes & development.* 1993;7(12B):2609-2617.

66. Kaplan SA, Cohen P. The somatomedin hypothesis 2007: 50 years later. *The Journal of clinical endocrinology and metabolism.* 2007;92(12):4529-4535.

67. Yakar S, Liu JL, Stannard B, et al. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proceedings of the National Academy of Sciences of the United States of America.* 1999;96(13):7324-7329.

68. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. *Endocrine reviews.* 2001;22(1):53-74.

69. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A. Roles of Growth Hormone and Insulin-like Growth Factor 1 in Mouse Postnatal Growth. *Developmental Biology.* 2001;229(1):141-162.

70. Isaksson OG, Eden S, Jansson JO. Mode of action of pituitary growth hormone on target cells. *Annual review of physiology.* 1985;47:483-499.

71. Richelsen B. Action of growth hormone in adipose tissue. *Hormone research.* 1997;48 Suppl 5:105-110.

72. Kostyo JL. Rapid effects of growth hormone on amino acid transport and protein synthesis. *Annals of the New York Academy of Sciences.* 1968;148(2):389-407.

73. Bartke A, Chandrashekar V, Bailey B, Zaczek D, Turyn D. Consequences of growth hormone (GH) overexpression and GH resistance. *Neuropeptides.* 2002;36(2-3):201-208.

74. LeRoith D, Yakar S. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. *Nature clinical practice. Endocrinology & metabolism.* 2007;3(3):302-310.

75. Fan Y, Menon RK, Cohen P, et al. Liver-specific deletion of the growth hormone receptor reveals essential role of growth hormone signaling in hepatic lipid metabolism. *The Journal of biological chemistry.* 2009;284(30):19937-19944.

76. Kostyo JL, Hotchkiss J, Knobil E. Stimulation of amino acid transport in isolated diaphragm by growth hormone added in vitro. *Science.* 1959;130(3389):1653-1654.

77. Vijayakumar A, Wu Y, Sun H, et al. Targeted loss of GHR signaling in mouse skeletal muscle protects against high-fat diet-induced metabolic deterioration. *Diabetes.* 2012;61(1):94-103.

78. List EO, Berryman DE, Funk K, et al. The role of GH in adipose tissue: lessons from adipose-specific GH receptor gene-disrupted mice. *Molecular endocrinology.* 2013;27(3):524-535.

79. Wu Y, Liu C, Sun H, et al. Growth hormone receptor regulates beta cell hyperplasia and glucose-stimulated insulin secretion in obese mice. *The Journal of clinical investigation.* 2011;121(6):2422-2426.

80. Jacob R, Barrett E, Plewe G, Fagin KD, Sherwin RS. Acute effects of insulin-like growth factor I on glucose and amino acid metabolism in the awake fasted rat. Comparison with insulin. *The Journal of clinical investigation.* 1989;83(5):1717-1723.

81. Ranke MB, Savage MO, Chatelain PG, Preece MA, Rosenfeld RG, Wilton P. Long-term treatment of growth hormone insensitivity syndrome with IGF-I. Results of the European Multicentre Study. The Working Group on Growth Hormone Insensitivity Syndromes. *Hormone research.* 1999;51(3):128-134.

82. Schoenle EJ, Zenobi PD, Torresani T, Werder EA, Zachmann M, Froesch ER. Recombinant human insulin-like growth factor I (rhIGF I) reduces hyperglycaemia in patients with extreme insulin resistance. *Diabetologia.* 1991;34(9):675-679.

83. Fryburg DA. Insulin-like growth factor I exerts growth hormone- and insulin-like actions on human muscle protein metabolism. *The American journal of physiology.* 1994;267(2 Pt 1):E331-336.

84. Brown-Borg HM, Borg KE, Meliska CJ, Bartke A. Dwarf mice and the ageing process. *Nature.* 1996;384(6604):33.

85. Brown-Borg HM, Bartke A. GH and IGF1: roles in energy metabolism of long-living GH mutant mice. *The journals of gerontology. Series A, Biological sciences and medical sciences.* 2012;67(6):652-660.

86. Hattori N. Expression, regulation and biological actions of growth hormone (GH) and ghrelin in the immune system. *Growth Hormone & IGF Research.* 2009;19(3):187-197.

87. Wiedermann CJ, Reinisch N, Braunsteiner H. Stimulation of monocyte chemotaxis by human growth hormone and its deactivation by somatostatin. *Blood.* 1993;82(3):954-960.

88. Mercola KE, Cline MJ, Golde DW. Growth hormone stimulation of normal and leukemic human T-lymphocyte proliferation in vitro. *Blood.* 1981;58(2):337-340.

54

89. Sumita K, Hattori N, Inagaki C. Effects of growth hormone on the differentiation of mouse B-lymphoid precursors. *Journal of pharmacological sciences.* 2005;97(3):408-416.

90. Gonzalo JA, Mazuchelli R, Mellado M, et al. Enterotoxin septic shock protection and deficient T helper 2 cytokine production in growth hormone transgenic mice. *Journal of immunology.* 1996;157(8):3298-3304.

91. Masternak MM, Bartke A. Growth hormone, inflammation and aging. *Pathobiology of aging & age related diseases.* 2012;2.

92. Uronen-Hansson H, Allen ML, Lichtarowicz-Krynska E, et al. Growth hormone enhances proinflammatory cytokine production by monocytes in whole blood. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2003;13(5):282-286.

93. Bozzola M, De Benedetti F, De Amici M, et al. Stimulating effect of growth hormone on cytokine release in children. *European journal of endocrinology / European Federation of Endocrine Societies.* 2003;149(5):397-401.

94. Takala J, Ruokonen E, Webster NR, et al. Increased mortality associated with growth hormone treatment in critically ill adults. *The New England journal of medicine.* 1999;341(11):785-792.

95. de Groof F, Joosten KF, Janssen JA, et al. Acute stress response in children with meningococcal sepsis: important differences in the growth hormone/insulin-like growth factor I axis between nonsurvivors and survivors. *The Journal of clinical endocrinology and metabolism.* 2002;87(7):3118-3124.

96. Onenli-Mungan N, Yildizdas D, Yapicioglu H, Topaloglu AK, Yuksel B, Ozer G. Growth hormone and insulin-like growth factor 1 levels and their relation to survival in children with bacterial sepsis and septic shock. *Journal of paediatrics and child health.* 2004;40(4):221-226.

97. Haeffner A, Thieblemont N, Deas O, et al. Inhibitory effect of growth hormone on TNF-alpha secretion and nuclear factor-kappaB translocation in lipopolysaccharide-stimulated human monocytes. *Journal of immunology.* 1997;158(3):1310-1314.

98. Serri O, St-Jacques P, Sartippour M, Renier G. Alterations of monocyte function in patients with growth hormone (GH) deficiency: effect of substitutive GH therapy. *The Journal of clinical endocrinology and metabolism.* 1999;84(1):58-63.

99. Kirk J. Indications for growth hormone therapy in children. *Archives of disease in childhood.* 2012;97(1):63-68.

100. Laron Z, Pertzelan A, Mannheimer S. Genetic pituitary dwarfism with high serum concentation of growth hormone--a new inborn error of metabolism? *Israel journal of medical sciences.* 1966;2(2):152-155.

101. Amselem S, Sobrier M-L, Dastot F, Duquesnoy P, Duriez B, Goossens M. Molecular basis of inherited growth hormone resistance in childhood. *Baillière's Clinical Endocrinology and Metabolism.* 1996;10(3):353-369.

102. Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, et al. Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. *Science translational medicine.* 2011;3(70):70ra13.

103. Backeljauw PF, Chernausek SD. Treatment of insulin-like growth factor deficiency with IGF-I: studies in humans. *Hormone research.* 2006;65 Suppl 1:21-27.

104. Ayuk J, Sheppard MC. Growth hormone and its disorders. *Postgraduate medical journal.* 2006;82(963):24-30.

105. Pearce JM. A note on Pierre Marie (1853-1940). *Journal of neurology, neurosurgery, and psychiatry.* 2004;75(11):1583.

106. Marie P. Sur deux cas d'acromégalie: hypertrophie singulière non congénitale des extrémités supérieures, inférieures et céphalique. *Rev Med Paris* 1886;vi:297–333.

107. Chanson P, Salenave S. Acromegaly. *Orphanet journal of rare diseases.* 2008;3:17.

108. Ribeiro-Oliveira A, Jr., Barkan A. The changing face of acromegaly--advances in diagnosis and treatment. *Nature reviews. Endocrinology.* 2012;8(10):605-611.

109. Gent J, van Kerkhof P, Roza M, Bu G, Strous GJ. Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent endocytosis. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99(15):9858-9863.

110. Brown RJ, Adams JJ, Pelekanos RA, et al. Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nature structural & molecular biology.* 2005;12(9):814-821.
111. Silva CM, Day RN, Weber MJ, Thorner MO. Human growth hormone (GH) receptor is characterized as the 134-kilodalton tyrosine-phosphorylated protein activated by GH treatment in IM-9 cells. *Endocrinology.* 1993;133(5):2307-2312.
112. van den Eijnden MJ, Lahaye LL, Strous GJ. Disulfide bonds determine growth hormone receptor folding, dimerisation and ligand binding. *Journal of cell science.* 2006;119(Pt 15):3078-3086.
113. Harding PA, Wang XZ, Kelder B, Souza S, Okada S, Kopchick JJ. In vitro mutagenesis of growth hormone receptor Asn-linked glycosylation sites. *Molecular and cellular endocrinology.* 1994;106(1-2):171-180.
114. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proceedings of the National Academy of Sciences of the United States of America.* 1990;87(18):6934-6938.
115. Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. *Science.* 2002;296(5573):1653-1655.
116. Argetsinger LS, Campbell GS, Yang X, et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell.* 1993;74(2):237-244.
117. Matsuda T, Feng J, Witthuhn BA, Sekine Y, Ihle JN. Determination of the transphosphorylation sites of Jak2 kinase. *Biochemical and biophysical research communications.* 2004;325(2):586-594.
118. Xu BC, Wang X, Darus CJ, Kopchick JJ. Growth hormone promotes the association of transcription factor STAT5 with the growth hormone receptor. *The Journal of biological chemistry.* 1996;271(33):19768-19773.
119. Uyttendaele I, Lemmens I, Verhee A, et al. Mammalian protein-protein interaction trap (MAPPIT) analysis of STAT5, CIS, and SOCS2 interactions with the growth hormone receptor. *Molecular endocrinology.* 2007;21(11):2821-2831.
120. Flores-Morales A, Pircher TJ, Silvennoinen O, et al. In vitro interaction between STAT 5 and JAK 2; dependence upon phosphorylation status of STAT 5 and JAK 2. *Molecular and cellular endocrinology.* 1998;138(1-2):1-10.
121. Wood TJ, Sliva D, Lobie PE, et al. Mediation of growth hormone-dependent transcriptional activation by mammary gland factor/Stat 5. *The Journal of biological chemistry.* 1995;270(16):9448-9453.
122. Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, Darnell JE, Jr. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell.* 1994;76(5):821-828.
123. Meyer DJ, Campbell GS, Cochran BH, et al. Growth hormone induces a DNA binding factor related to the interferon-stimulated 91-kDa transcription factor. *The Journal of biological chemistry.* 1994;269(7):4701-4704.
124. Finbloom DS, Petricoin EF, 3rd, Hackett RH, et al. Growth hormone and erythropoietin differentially activate DNA-binding proteins by tyrosine phosphorylation. *Molecular and cellular biology.* 1994;14(3):2113-2118.
125. Smit LS, Meyer DJ, Billestrup N, Norstedt G, Schwartz J, Carter-Su C. The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. *Molecular endocrinology.* 1996;10(5):519-533.
126. Mui AL, Wakao H, O'Farrell AM, Harada N, Miyajima A. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *The EMBO journal.* 1995;14(6):1166-1175.
127. Moriggl R, Gouilleux-Gruart V, Jahne R, et al. Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. *Molecular and cellular biology.* 1996;16(10):5691-5700.
128. Boucheron C, Dumon S, Santos SC, et al. A single amino acid in the DNA binding regions of STAT5A and STAT5B confers distinct DNA binding specificities. *The Journal of biological chemistry.* 1998;273(51):33936-33941.
129. Grimley PM, Dong F, Rui H. Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine & growth factor reviews.* 1999;10(2):131-157.
130. Gebert CA, Park SH, Waxman DJ. Regulation of signal transducer and activator of transcription (STAT) 5b activation by the temporal pattern of growth hormone stimulation. *Molecular endocrinology.* 1997;11(4):400-414.

56

131. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(19):8831-8835.

132. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell.* 1996;84(3):443-450.

133. Meraz MA, White JM, Sheehan KC, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell.* 1996;84(3):431-442.

134. Park C, Li S, Cha E, Schindler C. Immune response in Stat2 knockout mice. *Immunity.* 2000;13(6):795-804.

135. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(8):3801-3804.

136. Akira S. Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene.* 2000;19(21):2607-2611.

137. Grusby MJ. Stat4- and Stat6-deficient mice as models for manipulating T helper cell responses. *Biochemical Society transactions.* 1997;25(2):359-360.

138. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes & development.* 1997;11(2):179-186.

139. Udy GB, Towers RP, Snell RG, et al. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(14):7239-7244.

140. Davey HW, Park SH, Grattan DR, McLachlan MJ, Waxman DJ. STAT5b-deficient mice are growth hormone pulse-resistant. Role of STAT5b in sex-specific liver p450 expression. *The Journal of biological chemistry.* 1999;274(50):35331-35336.

141. Kofoed EM, Hwa V, Little B, et al. Growth hormone insensitivity associated with a STAT5b mutation. *The New England journal of medicine.* 2003;349(12):1139-1147.

142. Scaglia PA, Martinez AS, Feigerlova E, et al. A novel missense mutation in the SH2 domain of the STAT5B gene results in a transcriptionally inactive STAT5b associated with severe IGF-I deficiency, immune dysfunction, and lack of pulmonary disease. *The Journal of clinical endocrinology and metabolism.* 2012;97(5):E830-839.

143. Flores-Morales A, Stahlberg N, Tollet-Egnell P, et al. Microarray analysis of the in vivo effects of hypophysectomy and growth hormone treatment on gene expression in the rat. *Endocrinology.* 2001;142(7):3163-3176.

144. Woelfle J, Rotwein P. In vivo regulation of growth hormone-stimulated gene transcription by STAT5b. *American journal of physiology. Endocrinology and metabolism.* 2004;286(3):E393-401.

145. Davey HW, McLachlan MJ, Wilkins RJ, Hilton DJ, Adams TE. STAT5b mediates the GH-induced expression of SOCS-2 and SOCS-3 mRNA in the liver. *Molecular and cellular endocrinology.* 1999;158(1-2):111-116.

146. Lahuna O, Rastegar M, Maiter D, Thissen JP, Lemaigre FP, Rousseau GG. Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. *Molecular endocrinology.* 2000;14(2):285-294.

147. Engblom D, Kornfeld JW, Schwake L, et al. Direct glucocorticoid receptor-Stat5 interaction in hepatocytes controls body size and maturation-related gene expression. *Genes & development.* 2007;21(10):1157-1162.

148. Winston LA, Hunter T. JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. *The Journal of biological chemistry.* 1995;270(52):30837-30840.

149. VanderKuur J, Allevato G, Billestrup N, Norstedt G, Carter-Su C. Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2. *The Journal of biological chemistry.* 1995;270(13):7587-7593.

150. Vanderkuur JA, Butch ER, Waters SB, Pessin JE, Guan KL, Carter-Su C. Signaling molecules involved in coupling growth hormone receptor to mitogen-activated protein kinase activation. *Endocrinology.* 1997;138(10):4301-4307.

151. Winston LA, Bertics PJ. Growth hormone stimulates the tyrosine phosphorylation of 42- and 45-kDa ERK-related proteins. *The Journal of biological chemistry.* 1992;267(7):4747-4751.

152. Harding PA, Wang XZ, Kopchick JJ. Growth hormone (GH)-induced tyrosine-phosphorylated proteins in cells that express GH receptors. *Receptor.* 1995;5(2):81-92.

153. Barclay JL, Kerr LM, Arthur L, et al. In vivo targeting of the growth hormone receptor (GHR) Box1 sequence demonstrates that the GHR does not signal exclusively through JAK2. *Molecular endocrinology.* 2010;24(1):204-217.

154. Rowlinson SW, Yoshizato H, Barclay JL, et al. An agonist-induced conformational change in the growth hormone receptor determines the choice of signalling pathway. *Nature cell biology.* 2008;10(6):740-747.

155. Pircher TJ, Petersen H, Gustafsson JA, Haldosen LA. Extracellular signal-regulated kinase (ERK) interacts with signal transducer and activator of transcription (STAT) 5a. *Molecular endocrinology.* 1999;13(4):555-565.

156. Anderson NG. Growth hormone activates mitogen-activated protein kinase and S6 kinase and promotes intracellular tyrosine phosphorylation in 3T3-F442A preadipocytes. *The Biochemical journal.* 1992;284 (Pt 3):649-652.

157. Anderson NG. Simultaneous activation of p90rsk and p70s6k S6 kinases by growth hormone in 3T3-F442A preadipocytes. *Biochemical and biophysical research communications.* 1993;193(1):284-290.

158. Tollet P, Hamberg M, Gustafsson JA, Mode A. Growth hormone signaling leading to CYP2C12 gene expression in rat hepatocytes involves phospholipase A2. *The Journal of biological chemistry.* 1995;270(21):12569-12577.

159. Zhu T, Lobie PE. Janus kinase 2-dependent activation of p38 mitogen-activated protein kinase by growth hormone. Resultant transcriptional activation of ATF-2 and CHOP, cytoskeletal re-organization and mitogenesis. *The Journal of biological chemistry.* 2000;275(3):2103-2114.

160. Zhu T, Goh EL, LeRoith D, Lobie PE. Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and CrkII. Resultant activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). *The Journal of biological chemistry.* 1998;273(50):33864-33875.

161. Souza SC, Frick GP, Yip R, Lobo RB, Tai LR, Goodman HM. Growth hormone stimulates tyrosine phosphorylation of insulin receptor substrate-1. *The Journal of biological chemistry.* 1994;269(48):30085-30088.

162. Argetsinger LS, Norstedt G, Billestrup N, White MF, Carter-Su C. Growth hormone, interferon-gamma, and leukemia inhibitory factor utilize insulin receptor substrate-2 in intracellular signaling. *The Journal of biological chemistry.* 1996;271(46):29415-29421.

163. Yamauchi T, Kaburagi Y, Ueki K, et al. Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. *The Journal of biological chemistry.* 1998;273(25):15719-15726.

164. Liang L, Jiang J, Frank SJ. Insulin receptor substrate-1-mediated enhancement of growth hormone-induced mitogen-activated protein kinase activation. *Endocrinology.* 2000;141(9):3328-3336.

165. Zhu T, Goh EL, Graichen R, Ling L, Lobie PE. Signal transduction via the growth hormone receptor. *Cellular signalling.* 2001;13(9):599-616.

166. Okada S, Kopchick JJ. Biological effects of growth hormone and its antagonist. *Trends in molecular medicine.* 2001;7(3):126-132.

167. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Molecular and cellular biology.* 1994;14(7):4902-4911.

168. Costoya JA, Finidori J, Moutoussamy S, Searis R, Devesa J, Arce VM. Activation of growth hormone receptor delivers an antiapoptotic signal: evidence for a role of Akt in this pathway. *Endocrinology.* 1999;140(12):5937-5943.

169. Ammit AJ, Panettieri RA, Jr. Invited review: the circle of life: cell cycle regulation in airway smooth muscle. *Journal of applied physiology (Bethesda, Md. : 1985).* 2001;91(3):1431-1437.

170. Yokota I, Hayashi H, Matsuda J, et al. Effect of growth hormone on the translocation of GLUT4 and its relation to insulin-like and anti-insulin action. *Biochimica et biophysica acta.* 1998;1404(3):451-456.

171. Dominici FP, Argentino DP, Munoz MC, Miquet JG, Sotelo AI, Turyn D. Influence of the crosstalk between growth hormone and insulin signalling on the modulation of insulin sensitivity. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2005;15(5):324-336.

172. MacKenzie S, Fleming I, Houslay MD, Anderson NG, Kilgour E. Growth hormone and phorbol esters require specific protein kinase C isoforms to activate mitogen-activated protein kinases in 3T3-F442A cells. *The Biochemical journal.* 1997;324 (Pt 1):159-165.

173. Kahler CM, Pischel AB, Haller T, et al. Signal transduction pathways in directed migration of human monocytes induced by human growth hormone in vitro. *International immunopharmacology.* 2001;1(7):1351-1361.

174. Wakai K, Tsushima T, Murakami H, et al. Protein kinase C (PKC)-mediated growth hormone (GH) actions. *Endocrine journal.* 1998;45 Suppl:S97-99.

175. Gaur S, Yamaguchi H, Goodman HM. Growth hormone increases calcium uptake in rat fat cells by a mechanism dependent on protein kinase C. *The American journal of physiology.* 1996;270(5 Pt 1):C1485-1492.

176. Guan JL, Chen HC. Signal transduction in cell-matrix interactions. *International review of cytology.* 1996;168:81-121.

177. Zhu T, Goh EL, Lobie PE. Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. *The Journal of biological chemistry.* 1998;273(17):10682-10689.

178. Ilic D, Damsky CH, Yamamoto T. Focal adhesion kinase: at the crossroads of signal transduction. *Journal of cell science.* 1997;110 (Pt 4):401-407.

179. Jeay S, Sonenshein GE, Kelly PA, Postel-Vinay MC, Baixeras E. Growth hormone exerts antiapoptotic and proliferative effects through two different pathways involving nuclear factor-kappaB and phosphatidylinositol 3-kinase. *Endocrinology.* 2001;142(1):147-156.

180. Wu S, Morrison A, Sun H, De Luca F. Nuclear factor-kappaB (NF-kappaB) p65 interacts with Stat5b in growth plate chondrocytes and mediates the effects of growth hormone on chondrogenesis and on the expression of insulin-like growth factor-1 and bone morphogenetic protein-2. *The Journal of biological chemistry.* 2011;286(28):24726-24734.

181. Sodhi A, Tripathi A. Prolactin and growth hormone induce differential cytokine and chemokine profile in murine peritoneal macrophages in vitro: involvement of p-38 MAP kinase, STAT3 and NF-kappaB. *Cytokine.* 2008;41(2):162-173.

182. Rui L, Carter-Su C. Identification of SH2-bbeta as a potent cytoplasmic activator of the tyrosine kinase Janus kinase 2. *Proceedings of the National Academy of Sciences of the United States of America.* 1999;96(13):7172-7177.

183. Rytinki MM, Kaikkonen S, Pehkonen P, Jaaskelainen T, Palvimo JJ. PIAS proteins: pleiotropic interactors associated with SUMO. *Cellular and molecular life sciences : CMLS.* 2009;66(18):3029-3041.

184. Okubo S, Hara F, Tsuchida Y, et al. NMR structure of the N-terminal domain of SUMO ligase PIAS1 and its interaction with tumor suppressor p53 and A/T-rich DNA oligomers. *The Journal of biological chemistry.* 2004;279(30):31455-31461.

185. Liu B, Yang R, Wong KA, et al. Negative regulation of NF-kappaB signaling by PIAS1. *Molecular and cellular biology.* 2005;25(3):1113-1123.

186. Rakesh K, Agrawal DK. Controlling cytokine signaling by constitutive inhibitors. *Biochemical pharmacology.* 2005;70(5):649-657.

187. Pasquali C, Curchod ML, Walchli S, et al. Identification of protein tyrosine phosphatases with specificity for the ligand-activated growth hormone receptor. *Molecular endocrinology.* 2003;17(11):2228-2239.

188. Gu F, Dube N, Kim JW, et al. Protein tyrosine phosphatase 1B attenuates growth hormone-mediated JAK2-STAT signaling. *Molecular and cellular biology.* 2003;23(11):3753-3762.

189. Choi JH, Kim HS, Kim SH, et al. Phospholipase Cgamma1 negatively regulates growth hormone signalling by forming a ternary complex with Jak2 and protein tyrosine phosphatase-1B. *Nature cell biology*. 2006;8(12):1389-1397.

190. Hackett RH, Wang YD, Sweitzer S, Feldman G, Wood WI, Larner AC. Mapping of a cytoplasmic domain of the human growth hormone receptor that regulates rates of inactivation of Jak2 and Stat proteins. *The Journal of biological chemistry*. 1997;272(17):11128-11132.

191. Ram PA, Waxman DJ. Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. *The Journal of biological chemistry*. 1997;272(28):17694-17702.

192. Kim SO, Jiang J, Yi W, Feng GS, Frank SJ. Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signaling. *The Journal of biological chemistry*. 1998;273(4):2344-2354.

193. Stofega MR, Herrington J, Billestrup N, Carter-Su C. Mutation of the SHP-2 binding site in growth hormone (GH) receptor prolongs GH-promoted tyrosyl phosphorylation of GH receptor, JAK2, and STAT5B. *Molecular endocrinology*. 2000;14(9):1338-1350.

194. Vesterlund M, Zadjali F, Persson T, et al. The SOCS2 ubiquitin ligase complex regulates growth hormone receptor levels. *PloS one*. 2011;6(9):e25358.

195. Greenhalgh CJ, Rico-Bautista E, Lorentzon M, et al. SOCS2 negatively regulates growth hormone action in vitro and in vivo. *The Journal of clinical investigation*. 2005;115(2):397-406.

196. Flores-Morales A, Greenhalgh CJ, Norstedt G, Rico-Bautista E. Negative regulation of growth hormone receptor signaling. *Molecular endocrinology*. 2006;20(2):241-253.

197. Frank SJ. Growth hormone signalling and its regulation: Preventing too much of a good thing. *Growth Hormone & IGF Research*. 2001;11(4):201-212.

198. Vleurick L, Pezet A, Kuhn ER, Decuypere E, Edery M. A beta-turn endocytic code is required for optimal internalization of the growth hormone receptor but not for alpha-adaptin association. *Molecular endocrinology*. 1999;13(11):1823-1831.

199. Lobie PE, Sadir R, Graichen R, Mertani HC, Morel G. Caveolar internalization of growth hormone. *Experimental cell research*. 1999;246(1):47-55.

200. Sachse M, van Kerkhof P, Strous GJ, Klumperman J. The ubiquitin-dependent endocytosis motif is required for efficient incorporation of growth hormone receptor in clathrin-coated pits, but not clathrin-coated lattices. *Journal of cell science*. 2001;114(Pt 21):3943-3952.

201. Gorin E, Goodman HM. Turnover of growth hormone receptors in rat adipocytes. *Endocrinology*. 1985;116(5):1796-1805.

202. Lesniak MA, Roth J. Regulation of receptor concentration by homologous hormone. Effect of human growth hormone on its receptor in IM-9 lymphocytes. *The Journal of biological chemistry*. 1976;251(12):3720-3729.

203. da Silva Almeida AC, Strous GJ, van Rossum AG. betaTrCP controls GH receptor degradation via two different motifs. *Molecular endocrinology*. 2012;26(1):165-177.

204. Allevato G, Billestrup N, Goujon L, et al. Identification of phenylalanine 346 in the rat growth hormone receptor as being critical for ligand-mediated internalization and down-regulation. *The Journal of biological chemistry*. 1995;270(29):17210-17214.

205. van Kerkhof P, Putters J, Strous GJ. The ubiquitin ligase SCF(betaTrCP) regulates the degradation of the growth hormone receptor. *The Journal of biological chemistry*. 2007;282(28):20475-20483.

206. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews. Immunology*. 2007;7(6):454-465.

207. Krebs DL, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. *Journal of cell science*. 2000;113 (Pt 16):2813-2819.

208. Tollet-Egnell P, Flores-Morales A, Stavreus-Evers A, Sahlin L, Norstedt G. Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. *Endocrinology*. 1999;140(8):3693-3704.

209. Adams TE, Hansen JA, Starr R, Nicola NA, Hilton DJ, Billestrup N. Growth hormone preferentially induces the rapid, transient expression of SOCS-3, a novel inhibitor of cytokine receptor signaling. *The Journal of biological chemistry*. 1998;273(3):1285-1287.

60

210. Fujimoto M, Naka T. Regulation of cytokine signaling by SOCS family molecules. *Trends in immunology.* 2003;24(12):659-666.

211. Hershko A, Ciechanover A, Heller H, Haas AL, Rose IA. Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences of the United States of America.* 1980;77(4):1783-1786.

212. Matunis MJ, Coutavas E, Blobel G. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *The Journal of cell biology.* 1996;135(6 Pt 1):1457-1470.

213. Kumar S, Yoshida Y, Noda M. Cloning of a cDNA which encodes a novel ubiquitin-like protein. *Biochemical and biophysical research communications.* 1993;195(1):393-399.

214. Dolloff NG, Talamo G. Targeted therapy of multiple myeloma. *Advances in experimental medicine and biology.* 2013;779:197-221.

215. Schlesinger DH, Goldstein G, Niall HD. The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry.* 1975;14(10):2214-2218.

216. Wilkinson KD, Audhya TK. Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly. *The Journal of biological chemistry.* 1981;256(17):9235-9241.

217. Dye BT, Schulman BA. Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. *Annual review of biophysics and biomolecular structure.* 2007;36:131-150.

218. Kravtsova-Ivantsiv Y, Ciechanover A. Non-canonical ubiquitin-based signals for proteasomal degradation. *Journal of cell science.* 2012;125(Pt 3):539-548.

219. Kulathu Y, Komander D. Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nature reviews. Molecular cell biology.* 2012;13(8):508-523.

220. Chau V, Tobias JW, Bachmair A, et al. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science.* 1989;243(4898):1576-1583.

221. Tanaka K, Mizushima T, Saeki Y. The proteasome: molecular machinery and pathophysiological roles. *Biological chemistry.* 2012;393(4):217-234.

222. Chen ZJ, Sun LJ. Nonproteolytic functions of ubiquitin in cell signaling. *Molecular cell.* 2009;33(3):275-286.

223. Wu CJ, Ashwell JD. NEMO recognition of ubiquitinated Bcl10 is required for T cell receptor-mediated NF-kappaB activation. *Proceedings of the National Academy of Sciences of the United States of America.* 2008;105(8):3023-3028.

224. Newton K, Matsumoto ML, Wertz IE, et al. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell.* 2008;134(4):668-678.

225. Schulman BA, Harper JW. Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nature reviews. Molecular cell biology.* 2009;10(5):319-331.

226. van Wijk SJ, Timmers HT. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2010;24(4):981-993.

227. Li W, Bengtson MH, Ulbrich A, et al. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PloS one.* 2008;3(1):e1487.

228. Schwarz SE, Rosa JL, Scheffner M. Characterization of human hect domain family members and their interaction with UbcH5 and UbcH7. *The Journal of biological chemistry.* 1998;273(20):12148-12154.

229. Freemont PS. RING for destruction? *Current biology : CB.* 2000;10(2):R84-87.

230. Hatakeyama S, Nakayama KI. U-box proteins as a new family of ubiquitin ligases. *Biochemical and biophysical research communications.* 2003;302(4):635-645.

231. Coscoy L, Ganem D. PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends in cell biology.* 2003;13(1):7-12.

232. Scheffner M, Nuber U, Huibregtse JM. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature.* 1995;373(6509):81-83.

233. Zheng N, Wang P, Jeffrey PD, Pavletich NP. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell.* 2000;102(4):533-539.

234. Sun Y. Targeting E3 ubiquitin ligases for cancer therapy. *Cancer biology & therapy.* 2003;2(6):623-629.

235. Latres E, Chiaur DS, Pagano M. The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. *Oncogene.* 1999;18(4):849-854.

236. Kamura T, Maenaka K, Kotoshiba S, et al. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. *Genes & development.* 2004;18(24):3055-3065.

237. Sarikas A, Hartmann T, Pan ZQ. The cullin protein family. *Genome biology.* 2011;12(4):220.

238. Petroski MD, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. *Nature reviews. Molecular cell biology.* 2005;6(1):9-20.

239. Spratt DE, Wu K, Kovacev J, Pan ZQ, Shaw GS. Selective recruitment of an E2~ubiquitin complex by an E3 ubiquitin ligase. *The Journal of biological chemistry.* 2012;287(21):17374-17385.

240. Plechanovova A, Jaffray EG, Tatham MH, Naismith JH, Hay RT. Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature.* 2012;489(7414):115-120.

241. Pruneda JN, Littlefield PJ, Soss SE, et al. Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. *Molecular cell.* 2012;47(6):933-942.

242. Ohta T, Michel JJ, Schottelius AJ, Xiong Y. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Molecular cell.* 1999;3(4):535-541.

243. Lyapina SA, Correll CC, Kipreos ET, Deshaies RJ. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(13):7451-7456.

244. Dias DC, Dolios G, Wang R, Pan ZQ. CUL7: A DOC domain-containing cullin selectively binds Skp1.Fbx29 to form an SCF-like complex. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99(26):16601-16606.

245. Winston JT, Strack P, Beer-Romero P, Chu CY, Elledge SJ, Harper JW. The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes & development.* 1999;13(3):270-283.

246. Geyer R, Wee S, Anderson S, Yates J, Wolf DA. BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Molecular cell.* 2003;12(3):783-790.

247. Shiyanov P, Nag A, Raychaudhuri P. Cullin 4A associates with the UV-damaged DNA-binding protein DDB. *The Journal of biological chemistry.* 1999;274(50):35309-35312.

248. Osaka F, Kawasaki H, Aida N, et al. A new NEDD8-ligating system for cullin-4A. *Genes & development.* 1998;12(15):2263-2268.

249. Liakopoulos D, Doenges G, Matuschewski K, Jentsch S. A novel protein modification pathway related to the ubiquitin system. *The EMBO journal.* 1998;17(8):2208-2214.

250. Kurz T, Ozlu N, Rudolf F, et al. The conserved protein DCN-1/Dcn1p is required for cullin neddylation in C. elegans and S. cerevisiae. *Nature.* 2005;435(7046):1257-1261.

251. Kamura T, Conrad MN, Yan Q, Conaway RC, Conaway JW. The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes & development.* 1999;13(22):2928-2933.

252. Kawakami T, Chiba T, Suzuki T, et al. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *The EMBO journal.* 2001;20(15):4003-4012.

253. Duda DM, Borg LA, Scott DC, Hunt HW, Hammel M, Schulman BA. Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. *Cell.* 2008;134(6):995-1006.

254. Saha A, Deshaies RJ. Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Molecular cell.* 2008;32(1):21-31.

255. Merlet J, Burger J, Gomes JE, Pintard L. Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization. *Cellular and molecular life sciences : CMLS.* 2009;66(11-12):1924-1938.
256. Lyapina S, Cope G, Shevchenko A, et al. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science.* 2001;292(5520):1382-1385.
257. Liu J, Furukawa M, Matsumoto T, Xiong Y. NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Molecular cell.* 2002;10(6):1511-1518.
258. Lo SC, Hannink M. CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1. *Molecular and cellular biology.* 2006;26(4):1235-1244.
259. Pierce NW, Lee JE, Liu X, et al. Cand1 Promotes Assembly of New SCF Complexes through Dynamic Exchange of F Box Proteins. *Cell.* 2013;153(1):206-215.
260. Linossi EM, Nicholson SE. The SOCS box-adapting proteins for ubiquitination and proteasomal degradation. *IUBMB life.* 2012;64(4):316-323.
261. Stebbins CE, Kaelin WG, Jr., Pavletich NP. Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science.* 1999;284(5413):455-461.
262. Schulman BA, Carrano AC, Jeffrey PD, et al. Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature.* 2000;408(6810):381-386.
263. Babon JJ, Sabo JK, Soetopo A, et al. The SOCS box domain of SOCS3: structure and interaction with the elonginBC-cullin5 ubiquitin ligase. *Journal of molecular biology.* 2008;381(4):928-940.
264. Bullock AN, Debreczeni JE, Edwards AM, Sundstrom M, Knapp S. Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103(20):7637-7642.
265. Bullock AN, Rodriguez MC, Debreczeni JE, Songyang Z, Knapp S. Structure of the SOCS4-ElonginB/C complex reveals a distinct SOCS box interface and the molecular basis for SOCS-dependent EGFR degradation. *Structure.* 2007;15(11):1493-1504.
266. Haan S, Ferguson P, Sommer U, et al. Tyrosine phosphorylation disrupts elongin interaction and accelerates SOCS3 degradation. *The Journal of biological chemistry.* 2003;278(34):31972-31979.
267. Boyle K, Egan P, Rakar S, et al. The SOCS box of suppressor of cytokine signaling-3 contributes to the control of G-CSF responsiveness in vivo. *Blood.* 2007;110(5):1466-1474.
268. Boyle K, Zhang JG, Nicholson SE, et al. Deletion of the SOCS box of suppressor of cytokine signaling 3 (SOCS3) in embryonic stem cells reveals SOCS box-dependent regulation of JAK but not STAT phosphorylation. *Cellular signalling.* 2009;21(3):394-404.
269. Kibel A, Iliopoulos O, DeCaprio JA, Kaelin WG, Jr. Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. *Science.* 1995;269(5229):1444-1446.
270. Iwai K, Yamanaka K, Kamura T, et al. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proceedings of the National Academy of Sciences of the United States of America.* 1999;96(22):12436-12441.
271. Maher ER, Neumann HP, Richard S. von Hippel-Lindau disease: a clinical and scientific review. *European journal of human genetics : EJHG.* 2011;19(6):617-623.
272. Tokuoka M, Miyoshi N, Hitora T, et al. Clinical significance of ASB9 in human colorectal cancer. *International journal of oncology.* 2010;37(5):1105-1111.
273. McDaneld TG, Hannon K, Moody DE. Ankyrin repeat and SOCS box protein 15 regulates protein synthesis in skeletal muscle. *American journal of physiology. Regulatory, integrative and comparative physiology.* 2006;290(6):R1672-1682.
274. Boengler K, Pipp F, Fernandez B, Richter A, Schaper W, Deindl E. The ankyrin repeat containing SOCS box protein 5: a novel protein associated with arteriogenesis. *Biochemical and biophysical research communications.* 2003;302(1):17-22.
275. Kim SK, Rhim SY, Lee MR, et al. Stage-specific expression of ankyrin and SOCS box protein-4 (Asb-4) during spermatogenesis. *Molecules and cells.* 2008;25(2):317-321.
276. Damgaard RB, Gyrd-Hansen M. Inhibitor of apoptosis (IAP) proteins in regulation of inflammation and innate immunity. *Discovery medicine.* 2011;11(58):221-231.

277. Miller YI, Choi SH, Wiesner P, Bae YS. The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL. *British journal of pharmacology.* 2012;167(5):990-999.

278. Kim JK. Fat uses a TOLL-road to connect inflammation and diabetes. *Cell metabolism.* 2006;4(6):417-419.

279. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology.* 2010;11(5):373-384.

280. Lowe EL, Doherty TM, Karahashi H, Arditi M. Ubiquitination and de-ubiquitination: role in regulation of signaling by Toll-like receptors. *Journal of endotoxin research.* 2006;12(6):337-345.

281. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature.* 2001;412(6844):346-351.

282. Chen ZJ. Ubiquitin signalling in the NF-kappaB pathway. *Nature cell biology.* 2005;7(8):758-765.

283. Gyrd-Hansen M, Darding M, Miasari M, et al. IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. *Nature cell biology.* 2008;10(11):1309-1317.

284. Lu M, Lin SC, Huang Y, et al. XIAP induces NF-kappaB activation via the BIR1/TAB1 interaction and BIR1 dimerization. *Molecular cell.* 2007;26(5):689-702.

285. Starr R, Metcalf D, Elefanty AG, et al. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(24):14395-14399.

286. Alexander WS, Starr R, Fenner JE, et al. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell.* 1999;98(5):597-608.

287. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, Ihle JN. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *The EMBO journal.* 2003;22(3):372-384.

288. Croker BA, Krebs DL, Zhang JG, et al. SOCS3 negatively regulates IL-6 signaling in vivo. *Nature immunology.* 2003;4(6):540-545.

289. Kimura A, Kinjyo I, Matsumura Y, et al. SOCS3 is a physiological negative regulator for granulopoiesis and granulocyte colony-stimulating factor receptor signaling. *The Journal of biological chemistry.* 2004;279(8):6905-6910.

290. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *The Journal of biological chemistry.* 2002;277(44):42394-42398.

291. Jorgensen SB, O'Neill HM, Sylow L, et al. Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. *Diabetes.* 2013;62(1):56-64.

292. Sachithanandan N, Fam BC, Fynch S, et al. Liver-specific suppressor of cytokine signaling-3 deletion in mice enhances hepatic insulin sensitivity and lipogenesis resulting in fatty liver and obesity. *Hepatology.* 2010;52(5):1632-1642.

293. De Sepulveda P, Ilangumaran S, Rottapel R. Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation. *The Journal of biological chemistry.* 2000;275(19):14005-14008.

294. Orr SJ, Morgan NM, Elliott J, et al. CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover. *Blood.* 2007;109(3):1061-1068.

295. Zhang JG, Metcalf D, Rakar S, et al. The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(23):13261-13265.

296. Jo D, Liu D, Yao S, Collins RD, Hawiger J. Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. *Nature medicine.* 2005;11(8):892-898.

297. Hilton DJ, Richardson RT, Alexander WS, et al. Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(1):114-119.

298. Brender C, Columbus R, Metcalf D, et al. SOCS5 is expressed in primary B and T lymphoid cells but is dispensable for lymphocyte production and function. *Molecular and cellular biology.* 2004;24(13):6094-6103.

299. Krebs DL, Uren RT, Metcalf D, et al. SOCS-6 binds to insulin receptor substrate 4, and mice lacking the SOCS-6 gene exhibit mild growth retardation. *Molecular and cellular biology.* 2002;22(13):4567-4578.

300. Banks AS, Li J, McKeag L, et al. Deletion of SOCS7 leads to enhanced insulin action and enlarged islets of Langerhans. *The Journal of clinical investigation.* 2005;115(9):2462-2471.

301. Krebs DL, Metcalf D, Merson TD, et al. Development of hydrocephalus in mice lacking SOCS7. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(43):15446-15451.

302. Kario E, Marmor MD, Adamsky K, et al. Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. *The Journal of biological chemistry.* 2005;280(8):7038-7048.

303. Li L, Gronning LM, Anderson PO, et al. Insulin induces SOCS-6 expression and its binding to the p85 monomer of phosphoinositide 3-kinase, resulting in improvement in glucose metabolism. *The Journal of biological chemistry.* 2004;279(33):34107-34114.

304. Zadjali F, Pike AC, Vesterlund M, et al. Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase. *The Journal of biological chemistry.* 2011;286(1):480-490.

305. Sasi W, Jiang WG, Sharma A, Mokbel K. Higher expression levels of SOCS 1,3,4,7 are associated with earlier tumour stage and better clinical outcome in human breast cancer. *BMC cancer.* 2010;10:178.

306. Yoshimura A, Ohkubo T, Kiguchi T, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *The EMBO journal.* 1995;14(12):2816-2826.

307. Matsumoto A, Seki Y, Kubo M, et al. Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. *Molecular and cellular biology.* 1999;19(9):6396-6407.

308. Yoshimura A. Negative regulation of cytokine signaling. *Clinical reviews in allergy & immunology.* 2005;28(3):205-220.

309. Ram PA, Waxman DJ. SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *The Journal of biological chemistry.* 1999;274(50):35553-35561.

310. Li S, Chen S, Xu X, et al. Cytokine-induced Src homology 2 protein (CIS) promotes T cell receptor-mediated proliferation and prolongs survival of activated T cells. *The Journal of experimental medicine.* 2000;191(6):985-994.

311. Zhang W, Cheng GZ, Gong J, et al. RACK1 and CIS mediate the degradation of BimEL in cancer cells. *The Journal of biological chemistry.* 2008;283(24):16416-16426.

312. Ram PA, Waxman DJ. Role of the cytokine-inducible SH2 protein CIS in desensitization of STAT5b signaling by continuous growth hormone. *The Journal of biological chemistry.* 2000;275(50):39487-39496.

313. Starr R, Willson TA, Viney EM, et al. A family of cytokine-inducible inhibitors of signalling. *Nature.* 1997;387(6636):917-921.

314. Favre H, Benhamou A, Finidori J, Kelly PA, Edery M. Dual effects of suppressor of cytokine signaling (SOCS-2) on growth hormone signal transduction. *FEBS letters.* 1999;453(1-2):63-66.

315. Metcalf D, Greenhalgh CJ, Viney E, et al. Gigantism in mice lacking suppressor of cytokine signalling-2. *Nature.* 2000;405(6790):1069-1073.

316. Greenhalgh CJ, Bertolino P, Asa SL, et al. Growth enhancement in suppressor of cytokine signaling 2 (SOCS-2)-deficient mice is dependent on signal transducer and activator of transcription 5b (STAT5b). *Molecular endocrinology.* 2002;16(6):1394-1406.

317. Weedon MN, Lango H, Lindgren CM, et al. Genome-wide association analysis identifies 20 loci that influence adult height. *Nature genetics.* 2008;40(5):575-583.

318. Lango Allen H, Estrada K, Lettre G, et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. *Nature.* 2010;467(7317):832-838.

319. Gudbjartsson DF, Walters GB, Thorleifsson G, et al. Many sequence variants affecting diversity of adult human height. *Nature genetics.* 2008;40(5):609-615.

320. Vidal OM, Merino R, Rico-Bautista E, et al. In vivo transcript profiling and phylogenetic analysis identifies suppressor of cytokine signaling 2 as a direct signal

transducer and activator of transcription 5b target in liver. *Molecular endocrinology.* 2007;21(1):293-311.

321. Greenhalgh CJ, Metcalf D, Thaus AL, et al. Biological evidence that SOCS-2 can act either as an enhancer or suppressor of growth hormone signaling. *The Journal of biological chemistry.* 2002;277(43):40181-40184.

322. Pezet A, Favre H, Kelly PA, Edery M. Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. *The Journal of biological chemistry.* 1999;274(35):24497-24502.

323. Tannahill GM, Elliott J, Barry AC, Hibbert L, Cacalano NA, Johnston JA. SOCS2 can enhance interleukin-2 (IL-2) and IL-3 signaling by accelerating SOCS3 degradation. *Molecular and cellular biology.* 2005;25(20):9115-9126.

324. Lee SH, Yun S, Piao ZH, et al. Suppressor of cytokine signaling 2 regulates IL-15-primed human NK cell function via control of phosphorylated Pyk2. *Journal of immunology.* 2010;185(2):917-928.

325. Leong GM, Moverare S, Brce J, et al. Estrogen up-regulates hepatic expression of suppressors of cytokine signaling-2 and -3 in vivo and in vitro. *Endocrinology.* 2004;145(12):5525-5531.

326. Machado FS, Johndrow JE, Esper L, et al. Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nature medicine.* 2006;12(3):330-334.

327. Spence S, Fitzsimons A, Boyd CR, et al. Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization. *Immunity.* 2013;38(1):66-78.

328. Nakagawa R, Naka T, Tsutsui H, et al. SOCS-1 participates in negative regulation of LPS responses. *Immunity.* 2002;17(5):677-687.

329. Knosp CA, Carroll HP, Elliott J, et al. SOCS2 regulates T helper type 2 differentiation and the generation of type 2 allergic responses. *The Journal of experimental medicine.* 2011;208(7):1523-1531.

330. Palmer DC, Restifo NP. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends in immunology.* 2009;30(12):592-602.

331. Hu J, Winqvist O, Flores-Morales A, Wikstrom AC, Norstedt G. SOCS2 influences LPS induced human monocyte-derived dendritic cell maturation. *PloS one.* 2009;4(9):e7178.

332. Posselt G, Schwarz H, Duschl A, Horejs-Hoeck J. Suppressor of cytokine signaling 2 is a feedback inhibitor of TLR-induced activation in human monocyte-derived dendritic cells. *Journal of immunology.* 2011;187(6):2875-2884.

333. McBerry C, Gonzalez RM, Shryock N, Dias A, Aliberti J. SOCS2-induced proteasome-dependent TRAF6 degradation: a common anti-inflammatory pathway for control of innate immune responses. *PloS one.* 2012;7(6):e38384.

334. Lebrun P, Cognard E, Gontard P, et al. The suppressor of cytokine signalling 2 (SOCS2) is a key repressor of insulin secretion. *Diabetologia.* 2010;53(9):1935-1946.

335. Kato H, Nomura K, Osabe D, et al. Association of single-nucleotide polymorphisms in the suppressor of cytokine signaling 2 (SOCS2) gene with type 2 diabetes in the Japanese. *Genomics.* 2006;87(4):446-458.

336. Zadjali F, Santana-Farre R, Vesterlund M, et al. SOCS2 deletion protects against hepatic steatosis but worsens insulin resistance in high-fat-diet-fed mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2012;26(8):3282-3291.

337. Rico-Bautista E, Greenhalgh CJ, Tollet-Egnell P, et al. Suppressor of cytokine signaling-2 deficiency induces molecular and metabolic changes that partially overlap with growth hormone-dependent effects. *Molecular endocrinology.* 2005;19(3):781-793.

338. Sutherland KD, Lindeman GJ, Visvader JE. Knocking off SOCS genes in the mammary gland. *Cell cycle.* 2007;6(7):799-803.

339. Turnley AM. Role of SOCS2 in growth hormone actions. *Trends in endocrinology and metabolism: TEM.* 2005;16(2):53-58.

340. Lorentzon M, Greenhalgh CJ, Mohan S, Alexander WS, Ohlsson C. Reduced bone mineral density in SOCS-2-deficient mice. *Pediatric research.* 2005;57(2):223-226.

341. Kazi JU, Sun J, Phung B, Zadjali F, Flores-Morales A, Ronnstrand L. Suppressor of Cytokine Signaling 6 (SOCS6) Negatively Regulates Flt3 Signal Transduction through Direct Binding to Phosphorylated Tyrosines 591 and 919 of Flt3. *The Journal of biological chemistry.* 2012;287(43):36509-36517.

342. Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. *Nature reviews. Immunology.* 2002;2(6):410-416.

343. Ge D, Gao AC, Zhang Q, Liu S, Xue Y, You Z. LNCaP prostate cancer cells with autocrine interleukin-6 expression are resistant to IL-6-induced neuroendocrine differentiation due to increased expression of suppressors of cytokine signaling. *The Prostate.* 2012;72(12):1306-1316.

344. Martens N, Uzan G, Wery M, Hooghe R, Hooghe-Peters EL, Gertler A. Suppressor of cytokine signaling 7 inhibits prolactin, growth hormone, and leptin signaling by interacting with STAT5 or STAT3 and attenuating their nuclear translocation. *The Journal of biological chemistry.* 2005;280(14):13817-13823.

345. Xu Z, Huang G, Gong W, et al. Activation of farnesoid X receptor increases the expression of cytokine inducible SH2-containing protein in HepG2 cells. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research.* 2012;32(11):517-523.

346. Harris J, Stanford PM, Sutherland K, et al. Socs2 and elf5 mediate prolactin-induced mammary gland development. *Molecular endocrinology.* 2006;20(5):1177-1187.

347. Renart J, Reiser J, Stark GR. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proceedings of the National Academy of Sciences of the United States of America.* 1979;76(7):3116-3120.

348. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America.* 1979;76(9):4350-4354.

349. Ciechanover A, Heller H, Elias S, Haas AL, Hershko A. ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences of the United States of America.* 1980;77(3):1365-1368.

350. Suda K, Iguchi G, Yamamoto M, et al. Suppressor of cytokine signaling 2 missense mutation identified in a patient with gigantism without growth hormone producing pituitary adenoma. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2012;22(Supplement 1):S85.

351. Kamura T, Sato S, Haque D, et al. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes & development.* 1998;12(24):3872-3881.

352. Kamura T, Burian D, Yan Q, et al. Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase. *The Journal of biological chemistry.* 2001;276(32):29748-29753.

353. Fan F, Wood KV. Bioluminescent assays for high-throughput screening. *Assay and drug development technologies.* 2007;5(1):127-136.

354. Wood TJ, Sliva D, Lobie PE, et al. Specificity of transcription enhancement via the STAT responsive element in the serine protease inhibitor 2.1 promoter. *Molecular and cellular endocrinology.* 1997;130(1-2):69-81.

355. Yuen PK, Fontaine NH, Quesada MA, Mazumder P, Bergman R, Mozdy EJ. Self-referencing a single waveguide grating sensor in a micron-sized deep flow chamber for label-free biomolecular binding assays. *Lab on a chip.* 2005;5(9):959-965.

356. Freire E, Mayorga OL, Straume M. Isothermal titration calorimetry. *Analytical Chemistry.* 1990;62(18):950A-959A.

357. Kershaw NJ, Murphy JM, Liau NP, et al. SOCS3 binds specific receptor-JAK complexes to control cytokine signaling by direct kinase inhibition. *Nature structural & molecular biology.* 2013;20(4):469-476.

358. Rico-Bautista E, Flores-Morales A, Fernandez-Perez L. Suppressor of cytokine signaling (SOCS) 2, a protein with multiple functions. *Cytokine & growth factor reviews.* 2006;17(6):431-439.

359. O'Malley SM, Xie X, Frutos AG. Label-free high-throughput functional lytic assays. *Journal of biomolecular screening.* 2007;12(1):117-125.

360. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews.* 2001;46(1-3):3-26.

361. Hwang MN, Min CH, Kim HS, et al. The nuclear localization of SOCS6 requires the N-terminal region and negatively regulates Stat3 protein levels. *Biochemical and biophysical research communications.* 2007;360(2):333-338.

362. Feng ZP, Chandrashekaran IR, Low A, Speed TP, Nicholson SE, Norton RS. The N-terminal domains of SOCS proteins: a conserved region in the disordered N-termini of SOCS4 and 5. *Proteins.* 2012;80(3):946-957.

363. Sen B, Peng S, Woods DM, et al. STAT5A-mediated SOCS2 expression regulates Jak2 and STAT3 activity following c-Src inhibition in head and neck squamous carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2012;18(1):127-139.

364. Thangavel C, Shapiro BH. A molecular basis for the sexually dimorphic response to growth hormone. *Endocrinology.* 2007;148(6):2894-2903.

365. Palmiter RD, Norstedt G, Gelinas RE, Hammer RE, Brinster RL. Metallothionein-human GH fusion genes stimulate growth of mice. *Science.* 1983;222(4625):809-814.

366. Cui Y, Hosui A, Sun R, et al. Loss of signal transducer and activator of transcription 5 leads to hepatosteatosis and impaired liver regeneration. *Hepatology.* 2007;46(2):504-513.

367. Sos BC, Harris C, Nordstrom SM, et al. Abrogation of growth hormone secretion rescues fatty liver in mice with hepatocyte-specific deletion of JAK2. *The Journal of clinical investigation.* 2011;121(4):1412-1423.

368. Shi SY, Martin RG, Duncan RE, et al. Hepatocyte-specific deletion of Janus kinase 2 (JAK2) protects against diet-induced steatohepatitis and glucose intolerance. *The Journal of biological chemistry.* 2012;287(13):10277-10288.

369. Xi CX, Xiong F, Zhou Z, Mei L, Xiong WC. PYK2 interacts with MyD88 and regulates MyD88-mediated NF-kappaB activation in macrophages. *Journal of leukocyte biology.* 2010;87(3):415-423.

370. Tripathi A, Sodhi A. Growth hormone-induced production of cytokines in murine peritoneal macrophages in vitro: role of JAK/STAT, PI3K, PKC and MAP kinases. *Immunobiology.* 2009;214(6):430-440.

371. Mode A, Wiersma-Larsson E, Strom A, Zaphiropoulos PG, Gustafsson JA. A dual role of growth hormone as a feminizing and masculinizing factor in the control of sex-specific cytochrome P-450 isozymes in rat liver. *The Journal of endocrinology.* 1989;120(2):311-317.

372. Andreassen M, Frystyk J, Faber J, Kristensen LO. GH activity and markers of inflammation: a crossover study in healthy volunteers treated with GH and a GH receptor antagonist. *European journal of endocrinology / European Federation of Endocrine Societies.* 2012;166(5):811-819.

373. Friedbichler K, Themanns M, Mueller KM, et al. Growth-hormone-induced signal transducer and activator of transcription 5 signaling causes gigantism, inflammation, and premature death but protects mice from aggressive liver cancer. *Hepatology.* 2012;55(3):941-952.

374. Lu C, Kumar PA, Sun J, et al. Targeted Deletion of Growth Hormone(GH) Receptor in Macrophage Reveals Novel Osteopontin-Mediated Effects of GH on Glucose Homeostasis and Insulin Sensitivity in Diet-Induced Obesity. *The Journal of biological chemistry.* 2013.