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

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Stockholm 2013
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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.


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<td>Cyclic Adenosine Mono-Phosphate</td>
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<tr>
<td>ASB</td>
<td>Ankyrin repeat SOCS-box protein</td>
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<tr>
<td>ATP</td>
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<tr>
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<td>Bel-2-Interacting Mediator of cell death protein</td>
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<td>BMDM</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Dalton</td>
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<td>Ubiquitin activating enzyme</td>
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<td>Elongin C-Cullin-SOCS-box complex</td>
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<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>Hypoxia Inducible Factor</td>
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<td>HOMA-IR</td>
<td>Homeostatic Model of Insulin Resistance</td>
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<td>Description</td>
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<td>IAP</td>
<td>Inhibitor of Apoptosis Protein</td>
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<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<td>Interferon</td>
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<td>Inhibitor of NFκB.</td>
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<td>Interleukin</td>
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<td>Inositol trisphosphate</td>
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<td>ipITT</td>
<td>Intraperitoneal Insulin Tolerance Test</td>
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<td>Interferon Regulatory Factor</td>
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<td>c-Jun amino terminal Kinase</td>
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<td>KIR</td>
<td>Kinase Inhibitory Region</td>
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<td>Lipopolysaccharide</td>
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<td>Nuclear Factor κB</td>
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<td>NK</td>
<td>Natural Killer cell</td>
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<tr>
<td>PI</td>
<td>Phosphatidylinositide</td>
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<td>Phosphoinositide 3-Kinase</td>
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<td>PIAS</td>
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<td>S-phase-Kinase-associated Protein</td>
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<td>SOCS</td>
<td>Suppressor Of Cytokine Signaling</td>
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<td>SOS</td>
<td>Sons Of Sevenless</td>
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<td>Description</td>
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<td>SPIA and Ryanodine receptor domain</td>
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<td>Signal Transducer and Activator of Transcription</td>
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<td>Small Ubiquitin-like Modifier</td>
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<td>TGF-β Activated Kinase</td>
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<td>T helper cell</td>
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<td>Toll-Like Receptor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>von Hippel-Lindau protein</td>
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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
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.
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 somatotrophic 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 p90RSK and p70RSK [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 phosphatidylinositol (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 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 (NFkB) and that this might in part be dependent on PI3K activation of Akt [179]. GH initiated NFkB 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 NFkB 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 de neddylates 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 SPIA 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.

![Domain structure of the SOCS family](image)

**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
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 Bel-2-interacting mediator of cell death (Bim) protein has been demonstrated [311,312].
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

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

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 \textit{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
days. Next, non-adherent cells were washed off; 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 were 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 hours 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 \textit{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.](image)

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\textsuperscript{−/−} 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<sup>−/−</sup> mice and GH transgenic mice the SOCS2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice are protected from HFD induced hepatic steatosis. Hepatic expression of genes involved in TAG assembly was upregulated in SOCS2<sup>−/−</sup> 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<sup>−/−</sup> mice displayed exacerbated insulin resistance compared to HFD fed wildtype mice. Fasting plasma insulin levels were increased in SOCS2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 SOCS2’s 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 SOCS2’s 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-phophotyrosine 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 (E2) 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 E2 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 SS2N 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 SOCS2KO 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 SOCS2KO 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 SOCS2KO 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 SOCS2KO 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 SOCS2KO 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


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.

9 ACKNOWLEDGEMENTS

I would like to take the opportunity to express my sincere gratitude to everyone that has supported me during my time as a PhD candidate and contributed to this thesis. I am especially grateful for the supervision I have received:

Amilcar Flores-Morales, thank you for putting you trust in me and allowing me to develop as a researcher. I have been very lucky to have you as my main supervisor and you have taught me to always critically evaluate my work and to consider all the options. Despite the geographical distance you have been a constant presence and I always feel invigorated and energized after our talks. Thank you.

Gunnar Norstedt, thank you for introducing me to the wonderful universe of science and the fascinating world of GH and SOCS. You have been a fantastic support on this journey and your contagious enthusiasm always cheers me up and motivates me to strive for increased understanding of the issues at hand.

Claes-Göran Östensson, thank you for your support and for inviting me to into your group and including me in your meetings. I greatly appreciate the friendly atmosphere and I have benefited immensely from the scientific discussions on insulin and diabetes.

Stefan Knapp, thank you for your help with all things structural and with the interpretation of interaction data. Your suggestions and comments have been very helpful and forced me to think outside the box on more than one occasion.

Juha Juslin, thank you for accepting to be my mentor. I know I am not the most active mentoree but I have greatly appreciated our talks and I am grateful for your assistance and support and, of course, company on the course.

I would like to thank the following past and present members of the Norstedt research group: Petra Tollet-Egnell, for your cheerful spirit, inquisitive nature and scientific dedication. I learnt a lot from listening to you, and from being enrolled as a research subject in your studies. John Flanagan, for your positive attitude and for contributing to many interesting discussions during group meetings. Ola Nilsson, for excellent talks regarding bone and hockey. Diego Iglesias Gato, for all the help in the lab and for being a good friend. I wish you and Saioa all the best for the future. Yin-Choy Chuan, for teaching me the tricks of the trade and for being such a genuinely helpful and friendly guy. Torun Ekblad, for good collaborative efforts and for creating such a positive atmosphere in the lab. I am very grateful for all your screening work. Fahad Al-Zadjali, for everything that you have done for me. You are a true friend and brother and this would not have been possible without you. I am very lucky to have gotten to work alongside you for so long. I wish you and your wonderful family, Nabila, Eyez, Zainab and Myran all the best. We will come down to visit before long. Jin Hu, for all the interesting late night debates in the lab and for teaching me Chinese. Amira Said Al-Kharusi, for constantly offering me sweets and for all your help in the lab. I wish you all the best with your studies! Erik Wahlström, for pleasant chats in the lab and the office and on the golf course. Torbjörn Persson, for your contributions to this
work and for always being in good spirits. Anna Nilsson, for being so passionate about medicine and for all your contributions and efforts. Sandra Dunger, for all the support in the lab, your contagious laughter and for keeping us all in line. Carolina Gustavsson, for being a good travel-buddy and for quarreling over hockey with me. Michael Chau, for being such an agreeable chap and for keeping an open mind about the wonders of beer. A great big thank you goes out to Louisa, Erik H, Åke, Anna-Karin, Lou, Roxana, Stephan, Anenisia, Kalle, Robert, Kamil, Faheem, Cecilia, Mahnaz, Irina, Julian, Yungang, Zicai, Alireza for all their help in the lab.

A big thank you to all my co-authors and to our collaborators in Gran Canaria Leandro, Ruyman and Mercedes for their contributions. Thanks to Alex Bullock for taking the time to answer all my structurally related questions. And thanks to Sergey, Christina, Tine and Charlotte at CPR for collaborative assistance and cheerful times at Tivoli. Special thanks go to Martin and Berit for your kind interest and all the help with our projects and your SOCS-friendly attitude.

I would also like to thank past and present CMMites: Vladana, for your dedication and assistance with resolving the smallest of cellular components. Ming-Lu, and Agneta for pleasant conversations in the cell lab. All the people that made CMM a great place to be: Tomas Ekström, Catharina Larsson, Lars Terenius, Monira, Mohsen, Atosa, Delphi, Daniel, Jonas, Maria, Tiang Ling, Jamileh, Nimrod, Mats, Oscar, Ebba, Kalle, Felix, Pinar, Omid, Zahidul, Stefano, Anastasios, Weng-Onn, Meng Li, Adam, Luqman, Ming Lu, Hong, Andrii, Caterina, Johanna, Ning Xu and of course Anna Maria who always bring a smile to my face.

I want to thank Ann-Britt, Karolina, Kerstin, Christina, Jan-Erik, Lennart, Jeanette and Robert at MMK for all they’ve done. I am also thankful for my new colleagues at M1 and for their friendliness and hospitality: Mohammed Hamza, Julien, Carole, Fara, Shima, Senthil, Tianwei, Harvest, Elizabeth, Saad, Agneta and Neil.

I am also very grateful for the help I have received from Actar/KDev Exploratory and would like to thank the following persons: Michael Sundström, Björn Kull, Natalia Nekhotiava and Peter Brandt. A big thanks goes out to the GE demo lab for assisting with ITC and especially to Natalia Markova and Tove Alm.

Thanks go to Janne Lehtiö for agreeing to help with mass spectrometry and Rafael Krmár for medical advice.

Furthermore, I am very happy for the support from my friends and special thanks go to: Emeli and Thomas for being such wonderful people and hospitable friends. Jonas B, for your endless chattering, the good times and providing valuable relaxation after work. Jonas B, for your steadfast friendship and all the good times spent together. Richard, for taking a genuine interest in what I do. Owe, for your encouragement and support, for all the pleasant evenings spent wining and dining and for all the laughs on deadly serious quiz-nights.
To anyone I have neglected to mention; I am sorry. Please know that I am grateful for all contributions, even the ones that escape me at the moment.


Alexander, tack för att du alltid får din far att le och på ett rent magiskt vis troller bort all hans stress och får honom att leva i net. Eva, jag kan inte nog starkt uttrycka vad ditt stöd betyder. Du är mitt ljus, min klippa, min kajplats i etern. Jag älskar dig.
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