Posttranslational modifications in the regulation of GHR and Jak2

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Posttranslational modifications in the regulation of Growth Hormone Receptor and Jak2

Posttranslationale modificaties in de regulatie van groeihormoonreceptor en Jak2

(met een samenvatting in het Nederlands)

Posttranslacyjne modyfikacje w regulacji receptora hormonu wzrostu i kinazy Jak2

(z podsumowaniem po polsku)

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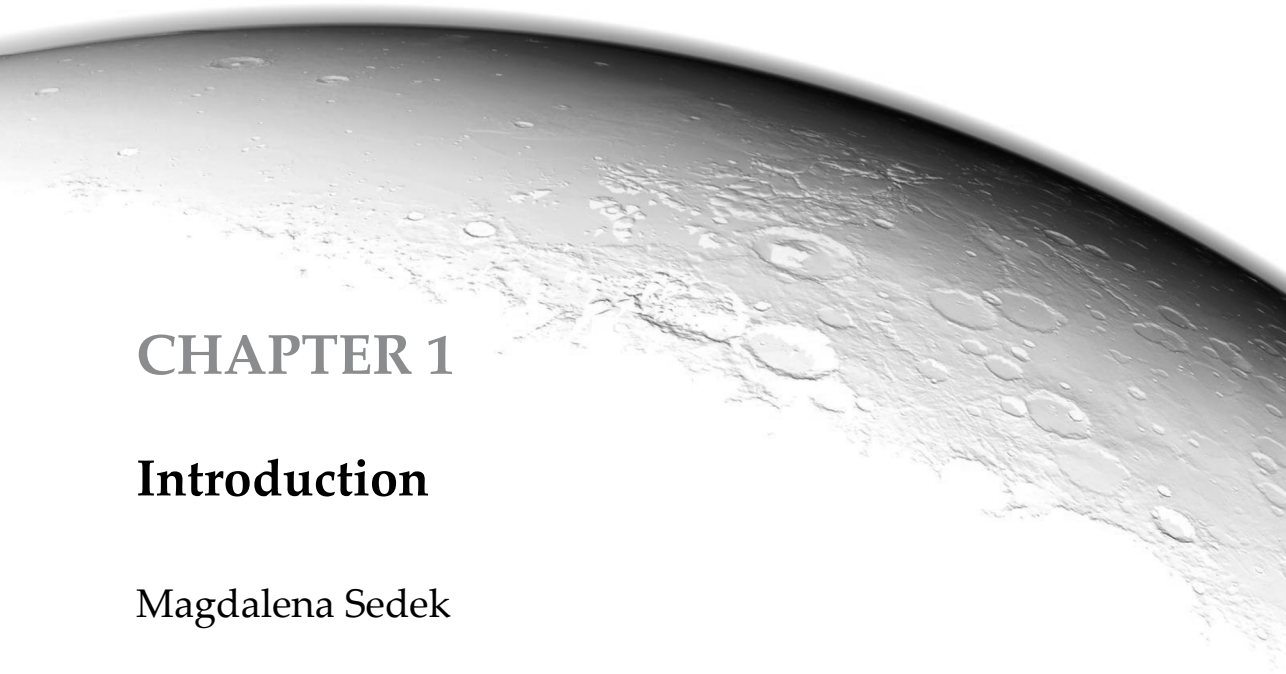
Abbreviations

BN-PAGE	blue native electrophoresis
βTrCP	beta-transducing repeat protein
DUB	deubiquitylating enzyme
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ESCRTs	endosomal sorting complexes required for transport
GH	growth hormone
GHR	growth hormone receptor
HECT	Homologous to E6-AP C-terminus
IGFs	insulin-like growth factors
JAK	Janus kinase
MVB	multivesicular bodies
NEM	N-ethylmaleimide
PDSM	phosphorylation-dependent sumoylation motif
RING	really interesting new gene
RNF4	ring finger protein 4
SCF	SKP1-CUL1-F box protein
SENP	sentrin/SUMO-specific protease
SIM	SUMO-interacting motif
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
TfR	Transferrin receptor
TGN	trans-Golgi-network
Ub	ubiquitin
UbE	ubiquitin dependent endocytosis motif



CHAPTER 1

Introduction

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1. Growth Hormone

Growth hormone (GH) also known as somatotropin is a peptide hormone that is synthetized and secreted by the somatotroph cells of the anterior lobe of the pituitary gland. The most well-known action of GH is regulation of longitudinal growth. Apart from that, it is important for regulating lipid and carbohydrates metabolism, especially by liver and adipose tissue. GH is important for normal reproductive function [1] and additionally, it acts on cardiovascular and immune systems [2, 3].
GH is a member of a gene family that includes prolactin and the placental lactogens, proliferin and proliferin-related protein, and decidual prolactin-like protein [4-8]. The protein consists of 191 amino acids and has a molecular mass of about 22 kDa. Crystallography data show that the three dimensional structure of human GH consists of four α-helices and three shorter connective helices [9]. The helices within the four-helical bundle are arranged in an up-up-down-down topology. Two autonomous receptor binding sites are localized on two opposite surfaces of the GH (Figure 1) [9, 10]. The elucidation of the crystal structure laid the foundation for structural studies of other members of the GH family.

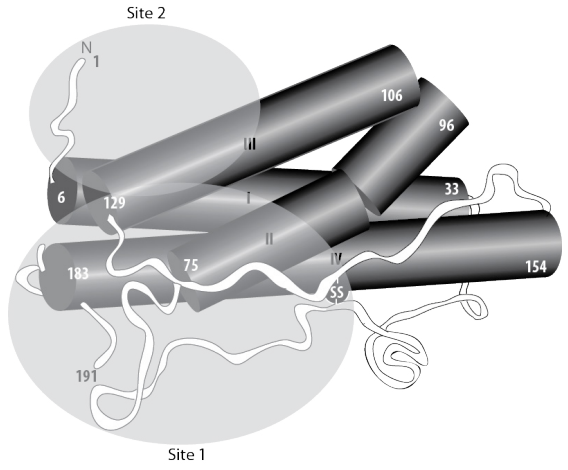

Figure 1. Crystal structure of porcine GH. *Growth hormone consists of four α-helices (I-IV, grey rollers) joined by three connective helices (white ribbons). Disulfide bridges are between cysteines 53-165 and 182-189. Grey highlights show site 1 and 2, corresponding to two independent GH receptor binding sites (Courtesy of da Silva Almeida).*

1.1. Availability and physiological relevance of GH

The pituitary gland is the major source of GH in circulation [11], but some extra-pituitary tissues (a.o. neural, immune, reproductive, alimentary, respiratory systems) have also been found to produce GH [12]. GH is released in a pulsatile manner, and amplitudes of pulses vary with age and between the genders [13]. Sex-dependent GH release has been associated with sexual dimorphism [14-16].
GH release is regulated by opposing actions of GH-releasing hormone and somatostatin, also known as GH-inhibiting hormone. Both hormones are synthesized in the brain and GH-releasing hormone stimulates GH release, while somatostatin inhibits its secretion [17]. Additionally, ghrelin, produced by stomach tissue, is able to suppress GH synthesis [18]. Apart from that, GH stimulates insulin-like growth factor (IGF) release that is able to inhibit GH secretion in a negative feedback loop [19]. GH secretion is also affected by other factors such as physical stress, body composition, metabolic status and others [20-22]. Secretion of GH is maximal at puberty [23] and declines with age both in humans

and animal experimental models [17, 24].
GH induces IGF-1 transcription that has a central role in mediating the growth actions of GH. Administration of IGF-1 has been used as a substitute for GH in the promotion of linear growth and is used, clinically, in the treatment of GH insensitivity caused by loss-of-function mutations in the GH receptor. However, administration of GH is more effective than IGF-1, showing that GH can acts also independently of IGF-1, through many additional signaling processes and mediators [25]. Figure 2 is summarizing the main physiological actions of GH and IGF-1.

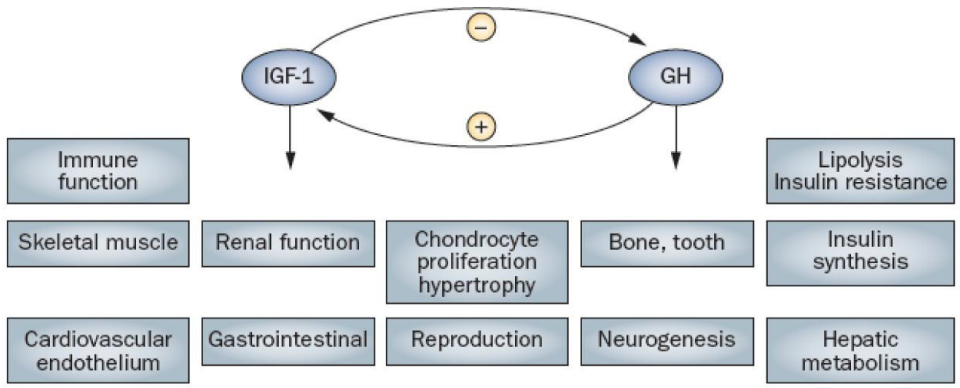

Figure 2. Major physiological actions of growth hormone (GH) and insulin-like growth factor 1 (IGF-1). *Often, it is difficult to distinguish which biological effects are mediated by which hormone. Thus, actions that involve more direct GH activity are placed to the right and the ones involving more IGF-1 are placed to the left. GH and IGF-1 affect each other's secretion. GH stimulates IGF-1 secretion while IGF-1 inhibits GH release. Figure adapted after [26].*

1.2. Role of GH in disease

GH related disorders can be categorized into three major groups: diseases caused either by overproduction (excess) of the hormone, its shortage (deficiency) or hormone insensitivity. Acromegaly is a rare disease, characterized by excess secretion of GH and increased circulating IGF-1 concentrations. The disease is associated with increased morbidity and premature mortality: the therapy includes administration of GH receptor antagonists. GH deficiency (GHD) is a condition where the body produces GH in low amounts. In early childhood GHD is responsible for children with short stature, together with relative adiposity. Both childhood and adult GHD can be treated with the administration of recombinant GH.
The term GH insensitivity syndrome (GHIS) refers to a group of inherited disorders characterized by a reduction in the biological effects of GH. However, GH serum concentrations are normal or elevated. The first described case of GHIS was by Laron in 1966. Laron syndrome is a disorder characterized by insensitivity to GH caused by mutation in gene coding the GH receptor. The syndrome is associated with short stature, and a remarkable resistance to diabetes and cancer [27].
More and more data are accumulating supporting a key role of GH/IGF-1 in mammary cancer. Studies on animals show that GH-deficient rats treated with carcinogens develop malignancies only in the presence of GH [28]. Other studies show that withdrawal of GH

cause tumor regression, clearly demonstrating that advanced rat mammary cancers rely on GH for their survival [29]. Since GH induces IGF-1 production, it has been debated whether IGF-1 is responsible for tumorigenesis. Currently, experimental data support a possibility of direct actions of GH on breast cancers. Autocrine GH expression have been reported in breast cancers [30]. Additionally, Mukhina et al showed that autocrine production of hGH by mammary carcinoma cells is sufficient for generation of an invasive phenotype [31].
Interestingly, in humans, there has not been a single malignancy reported in people suffering from Laron syndrome [32]. Additionally, population studies demonstrated a clear association between height and cancer occurrence. Several studies showed that taller people have higher risk of developing a cancer [33, 34].

2. Growth Hormone Receptor

2.1. Cytokine superfamily

GH acts via GH receptor (GHR), known also as somatogenic or somatotropin receptor that is present on the plasma membrane of virtually every cell of the body. GHR belongs to the class I cytokine receptors that currently comprises 15 members, including erythropoietin, prolactin, granulocyte colony stimulating factor, several of the interleukins, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M and leptin receptors [35]. All members of the family share certain common characteristics. They all lack intrinsic kinase activity. Therefore, they associate with non-receptor tyrosine kinases from the Janus family in order to conduct signaling cascade. They have a single transmembrane domain and the extracellular domain consists of two or more fibronectin III modules. In

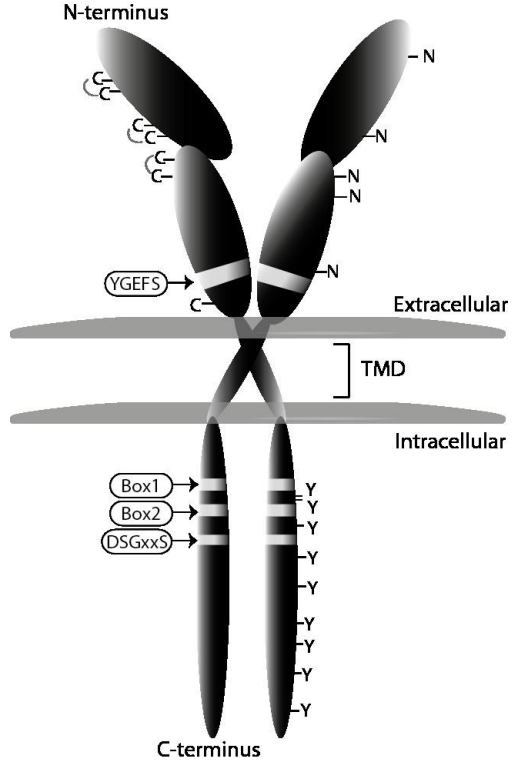

Figure 3. Schematic representation of GHR. *GHR exists on the plasma membrane as a dimer. The receptor consists of extracellular part, a transmembrane domain (TMD) and an intracellular part. In extracellular part there are cysteine residues (C), glycosylation sites (N) and YGEFS sequence. In intracellular part there is Box1, Box2 and DSGxxS motif and several tyrosines subjected to phosphorylation upon GH stimulation (courtesy of da Silva Almeida).*

the extracellular part, they possess two or three pairs of cysteins, a conserved tryptophan and WSXWS or equivalent motif. Additionally, there are two conserved membrane proximal sequences in the cytoplasmic domain, referred to as boxes 1 and 2. Box 1 is proline rich and is central to signaling, because it binds the Jak kinases [25].
The GHR is 620 residues long in its mature form, with 246 residues of the extracellular, 24 residues of transmembrane domain and 350 residues of the cytoplasmic domain. It comprises two fibronectin beta sandwich domains in its extracellular region and possesses a variant of WSXWS sequence, YGEFS, which is involved in stabilizing the lower beta sandwich and GH binding (Figure 3) [36]. The GHR extracellular domain contains 3 disulfide bridges, formed by 6 of its 7 cysteine residues [37]. In case of GHR, Box1 is responsible for Jak2 binding that is essential for signal transduction and Box2 was identified as the ubiquitin-dependent endocytosis (UbE) motif, essential for receptor endocytosis [38]. Additionally, a conserved DSGxxS degradation motif is present, downstream of Box 2 (Thesis da Silva Almeida).

2.2. GHR life cycle

The GHR is synthesized in the ER (Figure 4). Here, disulphide bonds are formed, two receptors dimerize [39] and they become glycosylated with high-mannose oligosaccharides, important for the process of quality control in the ER. When correctly folded, GHR continues its route to the Golgi complex, where the high mannose oligosaccharides are processed into complex oligosaccharides. ER form of receptor is called the precursor and the complex-glycosylated GHR the mature form. Due to the

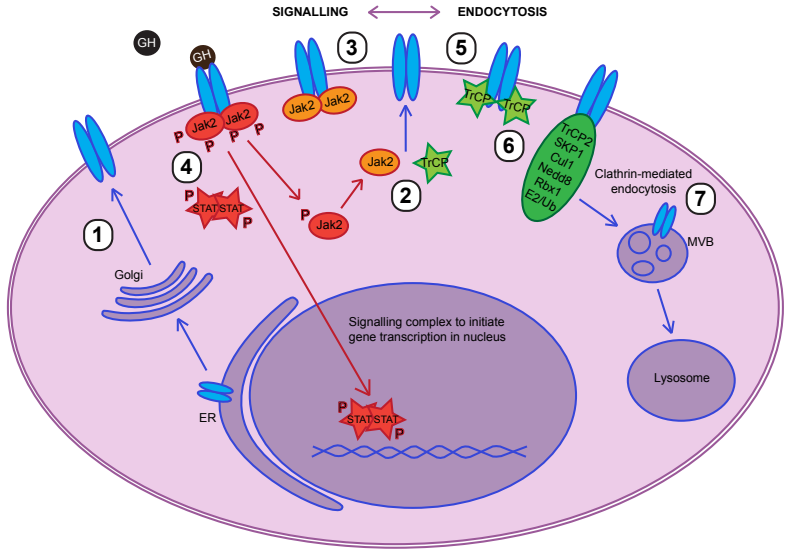

Figure 4. Regulation of Growth Hormone Receptor (GHR) signalling and endocytosis. *GHR immediately after being synthesized, assembles into a dimer in the ER, and, after complex glycosylation in the Golgi, appears on the plasma membrane in a mature form (1). Here, dependent on their cytosolic concentrations, either Jak2 or βTrCP2 will bind to GHR (2). Binding of Jak2 kinase prevents GHR from being endocytosed, Jak2 binding keeps the GHR at the plasma membrane until GH binding triggers the signalling cascade via STAT/MAPK (4). Phosphorylated Jak2 detaches from the phosphorylated GHR. Binding of βTrCP2 causes the GHR complex to become endocytosis-competent (5) and initiates assembly of the full SCF complex (6). GHR is internalised via clathrin-mediated endocytosis (7) and degraded in the lysosome.*

differences in glycosylation, the immature form of the receptor runs as two bands at 110 kDa, while the mature form runs as single disperse band of 130 kDa on SDS-PAGE.
From the Golgi, the receptor travels to the plasma membrane. GHR is constitutively endocytosed, independently of GH with a half-life of 20 min [40, 41]. Binding of GH to GHR triggers the signaling cascade via Jak2 kinase, which is followed by receptor ubiquitylation with SCFβTrCP2 ubiquitin ligase [38, 42]. Subsequently, the receptor is endocytosed via clathrin-coated pits, sorted into multivesicular bodies (MVB) and, eventually, degraded in the lysosomes [43].

2.3. Activation of GHR

GHR lacks kinase activity. Thus, it utilizes Jak2 kinase activity in order to perform signaling cascade. In the absence of GH, Jak2 can bind GHR as became apparent by the finding that Jak2 stabilizes the receptor on the plasma membrane [44, 45].
GH binding starts the GHR activation (Figure 5). One GH molecule binds to the extracellular domains of the GHR dimer [46]. Originally, it was thought that GH is being bound by the monomeric GHR on the cellular surface, and that this event induces the recruitment of the second GHR and its subsequent dimerization. This so called ligand induced dimerization model became a paradigm of activation for class I cytokine receptors. However, studies of Gent and colleagues proved this model to be inadequate [39]. GHR dimerization occurs in the ER. Thus, dimerization of the receptor is not sufficient to initiate signaling cascade. Study of Brown and colleagues compared the crystal structure of the ligand bound and unbound human GHR extracellular domains

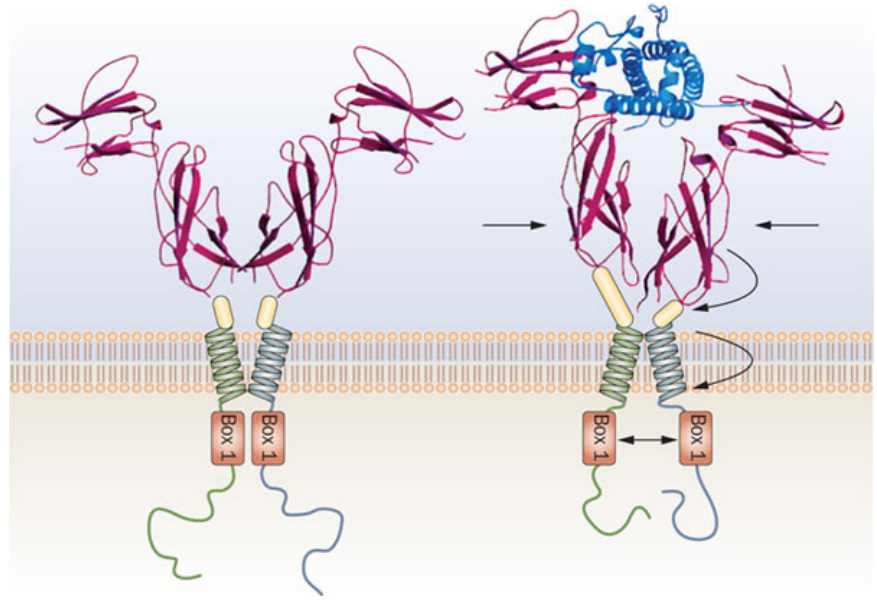

Figure 5. Model of GHR activation by GH. *GH binding to the extracellular domain of the GHR reorients the pre-existing homodimers so that one GHR subunit rotates relative to the other. This structural reorientation is transmitted through the transmembrane domain resulting in a repositioning of tyrosine kinases bound to the Box1 of the intracellular domain of GHR. Data using fluorescence energy transfer suggest that the distance between the Box1 motifs increases between inactive and active state and this movement is fundamental for Jak2 activation. Figure adapted after [26].*

and showed that there is no substantial change upon ligand binding [47]. They proposed the following model of GHR activation. Initially, GH binds via site 1 to one receptor molecule; binding via site 2 to the second receptor lifts and rotates the one receptor molecule relative to the other. This structural reorientation is transmitted through the transmembrane domain, resulting in a repositioning of the Jak2 molecules bound to intracellular part of the receptors. This event brings two (Jak2) kinase molecules in close proximity which leads to their activation. Activated Jak2 molecules transphosphorylate each other, and subsequently, the tyrosines on GHR.

2.4. GHR signaling pathways

The interaction of the ligand (GH) with the dimeric GHR initiates the cascade of events leading to signal transduction. Activation of Jak2 is a key step in starting GH signaling [48]. A number of signaling proteins and pathways are thought to be initiated, at least in part, as a consequence of binding to activated GHR-Jak2 complexes. The best characterized are STAT, MAPK and phosphatidylinositol 3′-kinase (PI3K) pathways, which will be described in more detail below.

2.4.1. The STAT pathway

GH binding to GHR stimulates Jak2 autophosphorylation [49, 50] and subsequently, phosphorylation of tyrosines in the intracellular part of the receptor [51]. The activated GHR-Jak2 complex provides docking sites for SH2 domain-containing signaling proteins, like signal transducers and activators of transcription (STATs) [52]. Binding leads to tyrosine phosphorylation of STAT molecules by Jak2. Next, STAT proteins homo- or heterodimerize and translocate to the nucleus, where they bind to specific DNA sequences in the promoter region of their respective target genes [53-55]. STAT 1, 3, 5a and 5b are the main mediators of GH signaling. Many studies have shown that activation of STAT5a and STAT5b is critical for a variety of GH functions, including changes in metabolism, body growth and sex-dependent liver gene regulation [1, 52]. Additionally, STAT5b directly stimulates IGF-1 expression by binding to its gene promoter elements [56, 57].

2.4.2. The MAPK pathway

The Ras/MAPK or ERK/MAPK pathway has also been shown to be activated by GH. Binding of GH to the receptor stimulates the binding of Src homology 2 domain-containing transforming protein C (Shc) to the activated GHR-Jak2 complex [58]. This event facilitates the phosphorylation of Shc and its subsequent binding to growth factor receptor-bound protein 2 (Grb2). Grb2, in turn, binds the guanine nucleotide exchange factor Son of Sevenless (SOS). Subsequently, Ras, Raf, mitogen-activated protein kinase/ extracellular-regulated protein kinase (MEK), and ultimately, MAPKs are sequentially activated [59]. Phosphorylated MAPKs translocate to the nucleus, where they transactivate transcription factors. This leads to changes in gene expression, which promote growth and differentiation or mitosis.

2.4.3. The PI3K pathway

In addition to activating the Ras/MAP kinase pathway, GH also stimulates the PI-3 kinase pathway via several ways that lead to PI-3 kinase activation. One possible

mechanism is through phosphorylation of the large adaptor proteins, designated insulin receptor substrate (IRS) proteins, because of their role in insulin signaling. IRS-1, IRS-2 and IRS-3 become phosphorylated in response to GH stimulation, which leads to their association with multiple signaling molecules, including the p85 subunit of PI-3 kinase. Other data suggest that GH activates PI-3 kinase through a CrkII-IRS-1 interaction [60]. Apart from that, it was shown that p85a and p85β subunits of PI-3 kinase can directly bind to phosphotyrosine residues of GHR [61]. Additionally, activation of PI3 kinase has been linked to GH stimulation of the glucose transport via glucose transporter-4 (GLUT4) [62]. GH also inhibits apoptosis via stimulation of anti-apoptotic serine kinase Akt, which event depends on PI3K activation [63]. GH-induced activation of p70S6K, a kinase involved in the control of cell proliferation and differentiation, has also been shown to be activated in PI-3 kinase-dependent and PKC-dependent manners [64-66].

3. Janus kinase family

Cytokines exert their function via plasma membrane receptors. Cytokine receptors lack intrinsic kinase activity. Therefore, they associate with members of Janus family of non-receptor tyrosine kinases. There are four mammalian Janus family members of cytoplasmic tyrosine kinases: Jak1, Jak2, Jak3 and Tyk2. They associate with a cytoplasmic region of signal transducing cytokine receptor subunits. Three of them, Jak1, Jak2 and Tyk2 are widely expressed, whereas Jak3 expression is restricted to the hematopoietic system [53, 54].

3.1. Jak2 structure

Based on sequence similarities, several Jak homology (JH) domains have been described [67], which match the domain structure of the Jaks only partially (Figure 6). The

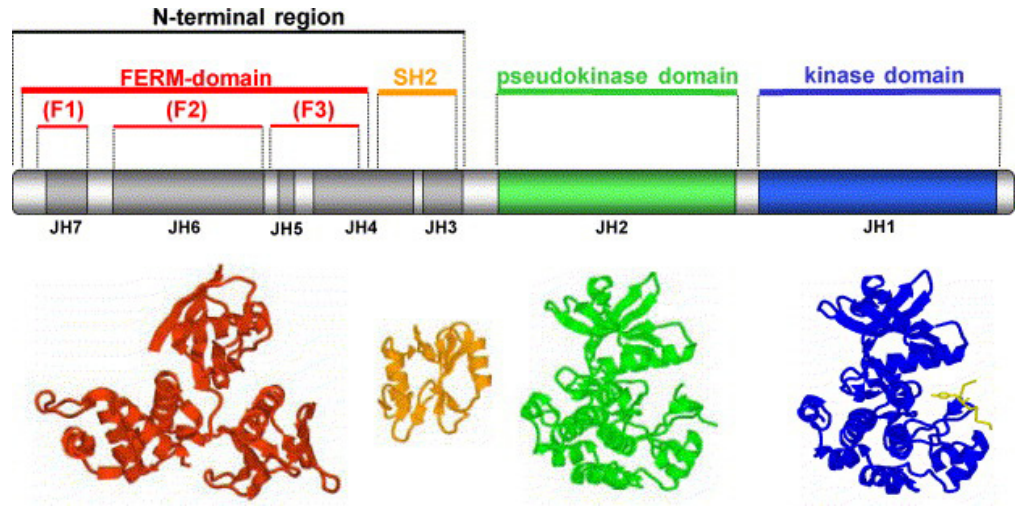

Figure 6. The putative structural organization of Jaks inferred from sequence similarity studies. *The kinase consists of FERM domain responsible for association with cytokine receptors, SH2 domain and pseudokinase domain that is regulatory for the kinase domain. The ribbon of the solved structures of the moesin FERM domain (red), the src-kinase SH2 domain (orange), and the insulin receptor kinase domain (green and blue) give the general idea of the globular organization of Janus kinases. Figure adapted after [78].*

C-terminal JH1 region corresponds to a classical kinase domain. Region JH2 has a kinase fold, but lacks crucial residues for catalytic activity and nucleotide binding. Therefore, it is referred to as pseudokinase domain. It is generally accepted that this domain regulates the function of the kinase domain [68, 69]. The N-terminal part of Jak2, comprising regions JH3-JH7, is involved in binding to cytokine receptors and other signaling effector and modulator proteins [70-74]. The N-terminal region of the Jaks shares significant sequence similarity with the so called four-point-one, ezrin, radixin and moesin (FERM) domains [75] and is responsible for association with corresponding cytokine receptors [70, 72, 73, 76, 77].

3.2. Regulation of Jak2 activity

Activation of Jak2 is a key step in GH signaling [48]. Upon cytokine stimulation Jak2 transphosphorylates itself [49, 50] and, subsequently, tyrosines on the receptor [51]. Some of the autophosphorylation sites like Y966 [79] are thought to serve as docking sites for signaling molecules containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains.

Phosphorylation of some of the tyrosines of Jak2 is thought to cause conformational change in the kinase that alters its activity. Many autophosphorylation sites were reported to regulate kinase function. Mutation of Y221 was shown to increase Jak2 activity [49, 80]. Phosphorylation of tyrosine 1007 is thought to expose the substrate and/or ATP binding sites and is necessary for full kinase activity [81]. Phosphorylation of tyrosine 119 is thought to promote dissociation of Jak2 from its receptor [82]. Autophosphorylation of tyrosine 813 appears to enhance Jak2 activity as a consequence of recruiting the adaptor protein SH2B1 [50]. SH2B1 was hypothesized to stabilize the active conformation of Jak2 [83] or promote the dimerization of Jak2 [84].

Lack of Y972 phosphorylation significantly reduces the levels of both Jak2 total tyrosine phosphorylation and phosphorylation of Y1007/Y1008. Thus, Y972 phosphorylation is important for maximal kinase function. Interestingly, in response to classical cytokine activation, the Jak2 Y972F mutant exhibited a moderately impaired level of activation. However, when Jak2 was activated via a GPCR ligand, the ability of the Y972F mutant to be activated was completely lost, suggesting a different role of Y972 in Jak2 activation. Finally, it was suggested that phosphorylation of Y972 enhances Jak2 kinase function via a mechanism that stabilizes the active conformation of the protein or its dimerization [85]. Similar mechanism was suggested for Y868, Y966, Y972, Y372 and Y373 [86, 87]. Mutation of Y637, within the pseudokinase domain, decreased Jak2 signaling and activity, and suggested a role for Y637 phosphorylation in the release of JH2 domain-mediated suppression of Jak2 kinase activity during cytokine stimulation [88].

On the other hand, phosphorylation on Y317, Y570 and Y913 was shown to inhibit Jak2 activity [49, 80, 88, 89]. Comparison of several regulatory phosphorylation sites on Jak2 revealed a dominant role of Y317 in the attenuation of Jak2 signaling [88]. Additionally, two independent groups identified S523 in Jak2 to be a substrate for phosphorylation. The serine seems to be constitutively phosphorylated in basal conditions. It has also been shown that phosphorylation of S523 is inhibitory for Jak2 activity [90, 91].

Together, these observations suggest a model of phosphorylation-dependent regulation of Jak2 (Figure 7). The baseline phosphorylation of S523 preserves the relative inactivity of Jak2 in the absence of cytokine stimulation. This relatively modest inhibition suffices

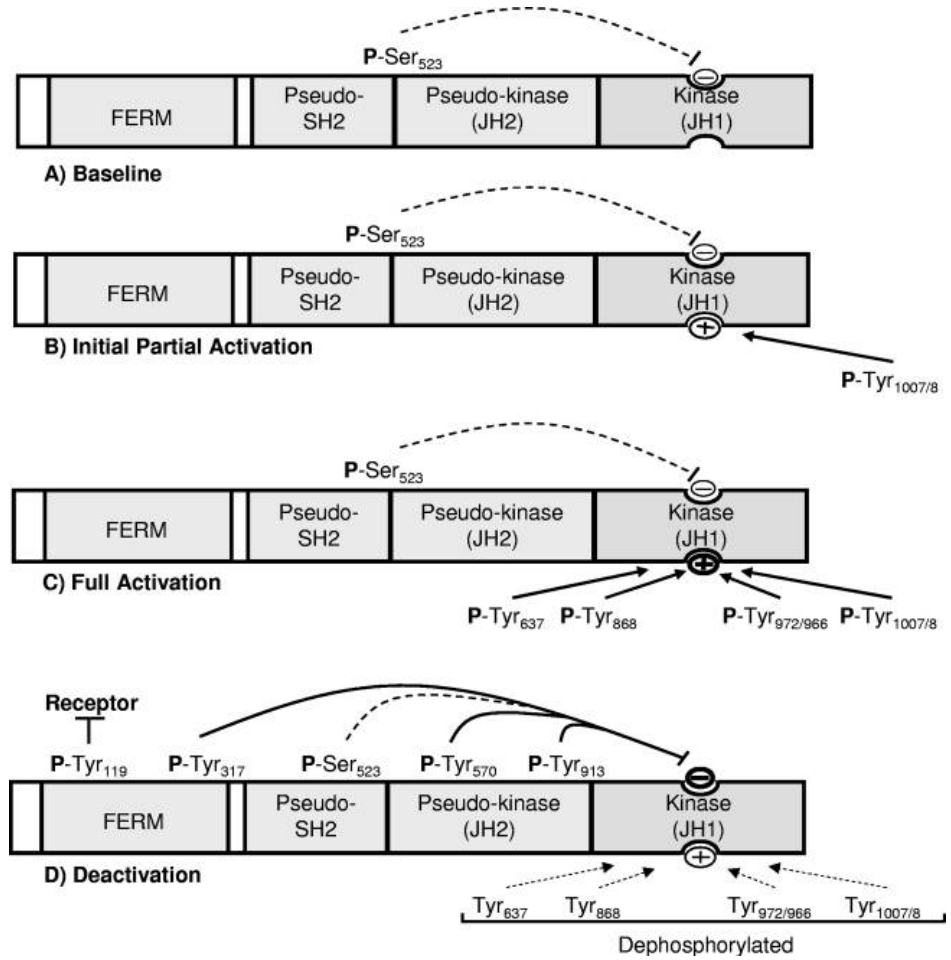

Figure 7. Model of phosphorylation-dependent regulation of Jak2 activity. A. *Without stimulation, Jak2 is essentially unphosphorylated on most sites, except Ser523 that is constitutively phosphorylated, providing a baseline of negative feedback to block kinase activity in the absence of stimulation.* ***B.*** *Upon cytokine stimulation, Tyr1007/8 (the activation loop sites) are rapidly phosphorylated to promote partial activation of the kinase domain.* ***C.*** *The phosphorylation of Tyr1007/1008 permits the phosphorylation of additional activating sites, including Tyr637, as well as other sites in kinase domain, leading to full activation of Jak2.* ***D.*** *After acute activation, strongly inhibitory sites, including Tyr317, Tyr570, Tyr913, and Tyr119, are phosphorylated to limit the extent of Jak2 activity, concomitant with the dephosphorylation of activating sites. Figure adapted after [88].*

to suppress Jak2 in the absence of ligand binding. However, a strong cytokine-mediated stimulus can overcome this weak inhibition. Cytokine stimulation causes rapid phosphorylation of Y1007/8 that alters the conformation of the JH1 domain activation loop and partially activates the kinase. This event allows phosphorylation of Y637 and other sites in the kinase domain like Y868, Y966 and Y972. Kinase becomes fully active. Dephosphorylation of these stimulatory sites coincides with the relatively slower phosphorylation of strong inhibitory sites like Y317 and Y570, providing inhibition to limit the duration of the signal. It was shown that phosphorylation of Y119, mediating Jak2 dissociation from the receptor, and phosphorylation of the inhibitory Y913 in the

kinase domain are slightly delayed and more sustained compared to phosphorylation on Y1007/1008 [82, 89]. Thus, they are likely to function together with Y317 and Y570 to limit the amplitude and duration of Jak2-dependent cytokine signaling.
Funakoshi-Tago and colleagues [92] proposed a model where Jak2 that is not bound to the receptor has a closed conformation. In this situation, the FERM domain interacts with the JH1-JH2 domains, preventing inappropriate activation of the kinase. Binding to the receptor via the FERM domain leads to the relief of inhibitory constraints, exerted by the FERM domain on the JH1/JH2 domains. Upon cytokine binding to the receptor, the JH2 domain releases the JH1 domain, which leads to full activation of the kinase. Therefore, the activation of Jak2 can be seen as gradual relief of multiple intramolecular constrains. Thus, a substantial subset of phosphorylation sites on Jak2 functions to regulate Jak2 kinase activity. After stimulation, the autophosphorylation of many tyrosines stabilise the active conformation of kinase, which is crucial for full activity. One possible explanation is that phosphorylation sites regulate the release of inhibitory constrains within the kinase to ensure the proper conformation both for activation and deactivation of Jak2.

3.3. Jak2 association with myeloproliferative diseases

Myeloproliferative disorders are clonal haematopoietic stem cell malignancies characterized by independency or hypersensitivity of haematopoietic cells to numerous cytokines [93, 94]. Polycythaemia vera is an acquired myeloproliferative disorder, characterized by the presence of polycythaemia, diversely associated with thrombocytosis, leukocytosis and splenomegaly [95]. The molecular basis of most myeloproliferative disorders is unknown.
The most common mutation of Jak2, associated with several myeloproliferative disorders, is substitution of valine 617 to phenylalanine in the pseudokinase domain [96-100]. This mutation occurs in approximately 99% of the patients with polycythemia vera and 50% of the patients with essential thrombocytopenia and idiopathic myelofibrosis [101]. Studies show that the mutation causes constitutive activation of kinase, which is independent of cytokine stimulation. Study of Lu et al suggested that V617F Jak2 needs a scaffold cytokine receptor to mediate cytokine independence transformation of the cells [102]. However, study of Funakoshi-Tago et al did not support these findings [92].

3.4. Role of nuclear pool of Jak2

Work of Dawson et al confirmed that Jak2 has a previously undiscovered function in the nucleus [103]. They found that nuclear Jak2 phosphorylates histone H3 at tyrosine 41, and phospho-H3Y41 releases heterochromatin protein 1α (HP1α) from chromatin. This event results in transcription of LMO2, which is originally repressed by HP1α. Typically, HP1 is an important component of heterochromatin and plays an indispensable part in heterochromatin-mediated gene silencing [104]. Recently, it has been proven to serve as a tumor suppressor protein [105]. In addition, Dawson et al. used two specific Jak2 inhibitors (TG101209 and AT9283) to block Jak2 activity of human leukemic cells and observed that both the expression of LMO2 and the phosphorylation of H3Y41 decreased, while the binding of HP1a at the Y41 site increased [103, 106]. These studies uncover a direct relationship between Jak2 and LMO2, two oncogenic genes, responsible for normal and leukemic hematopoiesis [107]. Meanwhile, Rinaldi et al reported that nuclear Jak2 is only present in Jak2 V617F-positive myeloproliferative neoplasia patients and

Jak2 V617F-positive cells, but not in patients with wild type Jak2, indicating that V617F mutation may affect localization of the kinase [108]. In addition, their work showed that nuclear Jak2 upregulates expression of LMO2 in cells with the V617F mutation, and that the selective Jak2 inhibitor AG490 plays an anti-tumor role by normalizing LMO2 levels and restoring the cytoplasmic localization of Jak2. This suggests that alteration of Jak2 activity interferes with the nuclear localization of Jak2. Additionally, Nilsson et al presented data suggesting that nuclear Jak2 can act as a tumor suppressor by stabilizing NF1-C2 in the mammary gland [109]. In the same study they also showed that active nuclear Jak2 suppresses tumorigenesis by repressing Forkhead box F1. Thus, Jak2 in the nucleus of mammary epithelial cells appears to be required for tumor-suppressing ability, and the activation of nuclear Jak2 could result in decreased tumor growth showing that Jak2 can act as a tumor suppressor.

4. Posttranslational modifications of proteins

Posttranslational modification (PTM) is the chemical modification of a protein that occurs after its translation. There are many different types of PTMs involving addition of functional groups (e.g. phosphorylation, glycosylation, acetylation, palmitoylation and others), addition of other protein or peptides (e.g. ubiquitylation, sumoylation, neddylation and others), involving changing the chemical nature of amino acids (like deamidation) and involving structural changes (like disulfide bridge formation and proteolytic cleavage) within the protein. Below, two PTMs, involving covalent attachment of ubiquitin and SUMO molecule to a substrate, are described in more detail as they are important for the understanding of the thesis.

5. The ubiquitylation pathway

Ubiquitylation pathway was initially discovered as alternative mean of protein degradation [110]. Soon, it became obvious that attachment of ubiquitin molecules to a substrate protein may lead to a variety of different outcomes.
In the ubiquitylation process a protein, called ubiquitin, is covalently attached to lysine residue of a substrate protein accomplished by the consecutive actions of several different enzymes (Figure 8). Ubiquitin is expressed as precursor, either as a fusion of four ubiquitin copies or as a fusion between ubiquitin and ribosomal proteins. A subfamily of deubiquitylating enzymes (DUBs) – the ubiquitin carboxy-terminal hydrolases – are important for processing these immature ubiquitin to their mature form [111]. The free ubiquitin molecule has to be activated in an ATP-dependent manner with the formation of thiol-ester linkage between ubiquitin-activating enzyme (E1) and the carboxyl terminus of ubiquitin. Next, an ubiquitin-conjugating enzyme (E2) accepts ubiquitin from E1 by a transthiolation reaction again involving the carboxyl terminus of ubiquitin. Finally, an ubiquitin ligase (E3) catalyzes the transfer of ubiquitin from the E2 to an ε-amino group of a lysine residue on the substrate protein [112]. There are two main types of E3 ubiquitin ligases containing conserved domains. The presence of a catalytic cysteine residue within the HECT (homologous to E6-AP carboxyl terminus) domain allows transfer of ubiquitin from E2 to E3 via formation of thioester bond. Thus, HECT E3 ligases directly ubiquitylate the substrate [113]. Members of the second class contain RING (really interesting new

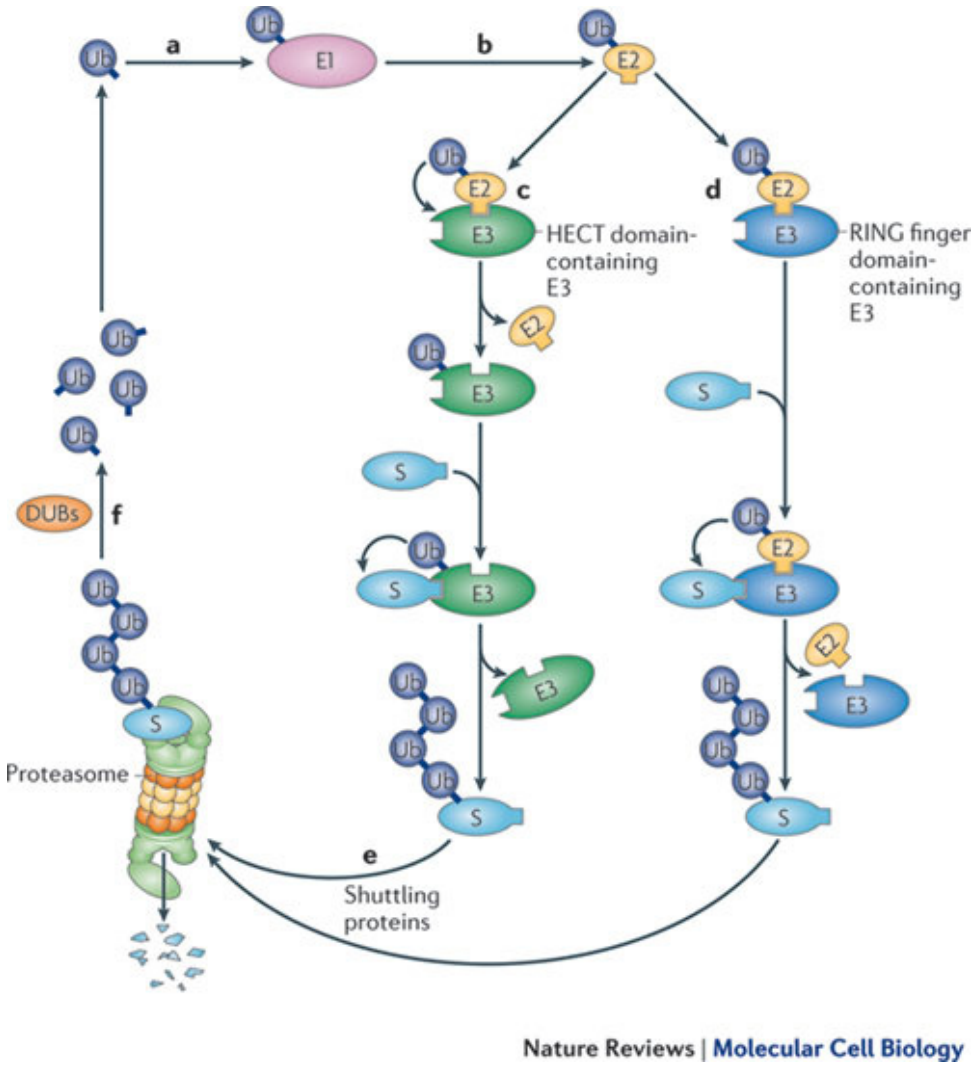

Figure 8. Ubiquitin-proteasome pathway. a. *Ubiquitin (Ub) is activated by the ubiquitin-activating enzyme (E1), which uses ATP to form a high-energy, labile E1– thiol ester intermediate.* ***b.*** *The activated ubiquitin is transferred to an ubiquitin conjugating enzyme (E2) to generate a similar thiol ester intermediate.* ***c.*** *Activated ubiquitin can be transferred from the E2 to a HECT domain-containing ubiquitin-protein ligase (E3) to generate a third labile thiol ester intermediate. It is then transferred to the substrate (S), where, in most cases, a stable isopeptide bond is formed between the activated carboxy-terminal Gly77 of ubiquitin and an εNH2 group of an internal Lys residue in the substrate. Additional ubiquitin moieties are then added to generate a polyubiquitin chain.* ***d.*** *Alternatively, activated ubiquitin can be transferred directly from E2 to an internal Lys residue in a substrate that is bound to a RING finger domain-containing ubiquitin ligase.* ***e.*** *Degradation of the ubiquitin-conjugated substrate to short peptides is performed by the 26S proteasome. The binding of ubiquitylated proteins to the proteasome can be direct, or mediated or enhanced by shuttling proteins.* ***f.*** *Most of the ubiquitin chain is disassembled by deubiquitylating enzymes (DUBs). Figure adapted after [115].*

gene) domain lacking catalytic activity. They mediate the transfer of ubiquitin from E2 to target protein [114]. This process is repeated to generate polyubiquitin chains, which may act as signals that target conjugated proteins for degradation by the 26S proteasome. Ubiquitylation is a dynamic and reversible process. DUBs cleave ubiquitin from proteins and also from residual proteasome-associated peptides and disassemble multi-ubiquitin chains.

The best known function of protein ubiquitylation is proteasome dependent degradation. However, ubiquitylation is involved in a variety of processes, such as cell cycle progression, organelle biogenesis, apoptosis, regulated cell proliferation, cellular differentiation, quality control in the endoplasmic reticulum, protein transport, inflammation, antigen processing, DNA repair and stress responses. There are only few E1 identified, 30-40 E2 enzymes and hundreds of E3s. At each step the number of proteins that can potentially be involved in the process increases. Thus, specificity of ubiquitylation process is mediated mainly by E3 ligases that determine, alone or with their partner E2, the sensitivity and specificity of substrate recognition. The system comprises ~1,000 components (~5% of the genome), and aberrations in their functions underlie the causes of many diseases, including malignancies, inflammatory disorders and neurodegeneration.

5.1. Ubiquitin

Ubiquitin is a 76-amino-acid globular protein that is highly conserved throughout eukaryotes, with only three amino-acid changes from yeast to human. Covalent attachment of ubiquitin to proteins serves as a versatile regulatory signal and controls the stability, localization or activation status of many cellular proteins. Proteins can be modified by ubiquitin monomers (monoubiquitination and multimonoubiquitination) or by ubiquitin polymers (polyubiquitination), in which ubiquitin moieties are most often connected through lysine-mediated isopeptide linkages [116]. The best studied ubiquitin chains are linked via K48 and K63 residues [117, 118].

Lys48-linked chains conjugated to degradation targets are recognized by a series of ubiquitin-binding shuttling proteins, which are believed to ferry the ubiquitylated protein to the proteasome for degradation [119, 120]. In contrast, Lys63-linked chains are primarily thought to impart regulatory information to the target protein, with distinct ubiquitin-binding proteins, controlling downstream signaling in DNA repair and inflammatory pathways, among others [121-123]. We now know, however, that all seven lysine residues in ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63), as well as the N-terminal methionine (Met1) both on substrate protein as well as on ubiquitin (linear polyubiquitin chains), are used as ubiquitin acceptors in vivo [124].

6. Ubiquitin-proteasome system in GHR trafficking

The GHR has been shown to be subjected to K48-mediated ubiquitylation [45, 125]. An active ubiquitin proteasome system is necessary for GHR endocytosis and its subsequent degradation in lysosomes. However, studies with a GHR truncation (after amino acid 399) that had all lysines mutated to arginines showed that the ubiquitylation of the receptor itself is not necessary for receptor endocytosis [126]. This mutant was normally endocytosed in an ubiquitin-system dependent manner. This led to the model where a yet to be identified substrate protein needs to be ubiquitylated and degraded by the

proteasome in order for the receptor to be endocytosed [127].
Recently, we identified two E3 ubiquitin ligases necessary for the GHR endocytosis [38]. βTrCP2 binds to both UbE motif and DSGxxS in the intracellular part of the receptor and is able to directly ubiquitylate the receptor (da Silva Almeida, in preparation). Additionally, our studies with GHR truncated after dileucine motif (GHR349) that endocytose independently of ubiquitin system showed that βTrCP2 is also responsible for GHR selection to multivesicular bodies (MVB) [128]. Recent studies implicated the E3 ubiquitin ligase CHIP as well as E2 conjugating enzyme Ubc13 in GHR endocytosis. Ubc13 is responsible for K63 ubiquitin chain formation, which suggests that K63-linked ubiquitin chains take part in GHR internalization as well.

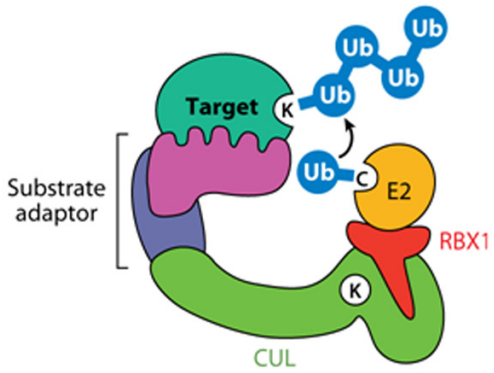

Figure 9. Schematic representation of SCF^{βTrCP2} E3 ubiquitin ligase. *Green represents Cul1, red Rbx1, yellow E2, purple Skp1 and pink TrCP2. Figure adapted after [133].*

βTrCP2 is a F-box protein that is responsible for substrate recognition. It is a part of a SCF (Skp1-Cul1-F-box protein) complex of RING E3 ubiquitin ligases (Figure 9) [129]. Apart from βTrCP2, the SCF complex consists of Skp1, Cul1, Nedd8, Rbx1 and an E2. Cul1 acts as a scaffold protein that interacts with adapter protein Skp1 at its N-terminus and with a RING-finger protein Rbx1 at its C-terminus. Rbx1 binds a specific E2 ubiquitin conjugating enzyme such as Ubc3, Ubc4 or Ubc5 that is responsible for substrate ubiquitylation. Skp1 stabilizes the F-box protein. Specific F-box protein binds its substrate via WD40 domain and brings the substrate in close proximity of the E2 for subsequent ubiquitin attachment. The whole complex needs to be assembled before effective substrate ubiquitylation can occur. Recent studies show that SCF complex can be subjected to dimerization and most likely can generate K48 polyubiquitin chains [130-132].

7. Sumoylation pathway

Another type of posttranslational modification is the attachment of small ubiquitin-related modifier (SUMO). The sumoylation pathway shares features with ubiquitylation pathway described above. SUMO proteins are synthesized as inactive precursors. In a process, called maturation, the C-terminal residues are removed by mammalian SENP (sentrin/SUMO-specific protease) or yeast Ulp enzymes, exposing the di-glycine motif that allows SUMO proteins to be conjugated to target proteins (Figure 10). SUMO becomes activated by the heterodimeric Uba2/Aos1 E1 activating enzyme in an ATP-dependent process. This step involves formation of a thioester bond between the active site cysteine residue of a heterodimer and the C-terminal glycine residue of SUMO. Subsequently, SUMO is transferred to the active site cysteine residue of E2 conjugating enzyme, Ubc9, again via a thoester linkage. Finally, SUMO is attached to a lysine residue of the target protein in a process that may be facilitated by E3 ligase. However, Ubc9 itself can efficiently sumoylate some targets without E3. Sumoylation is a reversible process. In

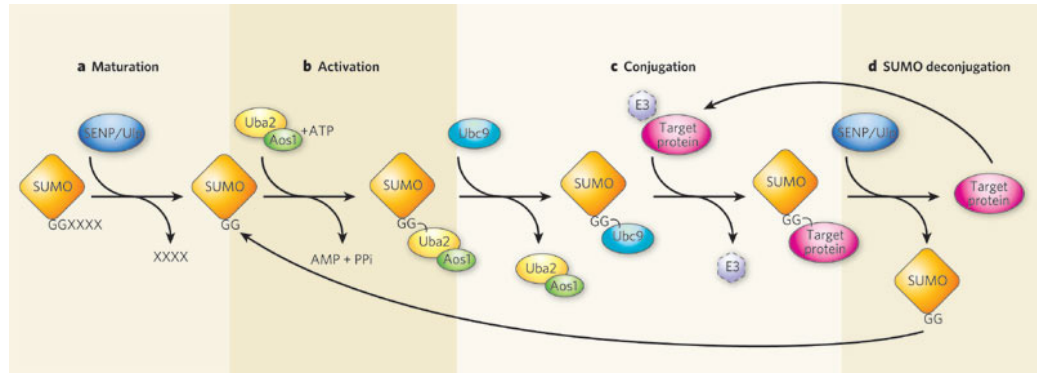

Figure 10. SUMO conjugation and deconjugation pathways. ***a.*** *Newly synthesized SUMO molecule need to have C-terminal residues remove to expose the di-glycine motif. This maturation process is performed by enzymes SENP (mammals) or Ulp (yeast).* ***b.*** *Mature SUMO has to be activated by an E1 activating enzyme Uba2/Aos1 in a process depending on energy transfer from ATP.* ***c.*** *Subsequently, the activated SUMO molecule is transferred to E2 conjugating enzyme, Ubc9. SUMO is conjugated to target protein in a process facilitated by E3 ligases.* ***d.*** *SENP/Ulp enzymes also act as deconiugases removing SUMO molecules from target protein. Figure adapted after [134].*

addition to SUMO maturation, SENP enzymes are involved in removing SUMO moieties from target proteins.
In contrast with ubiquitin system, the sumoylation pathway relies on a single E2 conjugating enzyme, Ubc9. Many proteins possess SUMO E3 ligase activity and appear to function in a similar manner as RING-domain ubiquitin E3 ligases. They do not directly receive SUMO through a thioester linkage, but act as scaffolds bringing SUMO-loaded Ubc9 into contact with the substrate protein, or holding the SUMO–Ubc9 thioester in a conformation, facilitating SUMO transfer. However, Ubc9 is cable of directly recognizing and sumoylating the substrates in vitro in absence of an E3 ligase. Nevertheless, the presence of E3 ligase enhances the process of SUMO conjugation. Thus, substrate specificity in the SUMO pathway is still under investigation.

7.1. SUMO

SUMO belongs to a family of ubiquitin-like molecules that include ubiquitin, SUMO, Nedd8 and others. There are four genes encoding SUMO molecules in mammalian cells, SUMO1-4. SUMO2 and SUMO3 differ from each other only by three N-terminal residues and are often referred to as SUMO2/3. They are ubiquitously expressed, while SUMO1, which shares only 50% sequence homology with SUMO2/3, has a limited distribution and occurs mainly in the nucleus. The role of SUMO4 is unknown and it is not sure whether endogenous SUMO4 can be attached to proteins. SUMO2/3 are able to make chains through internal lysine residues [135]. Although SUMO1 chains have been reported in vitro [136], in vivo it appears that SUMO1 may act as chain terminator on SUMO2/3 polymers [137].

7.2. Role of sumoylation at the organism level

The sumoylation pathway is essential for the normal function of all eukaryotic cells. In yeast Saccharomyces cerevisiae and Schizosaccharomyces pompe that contain only one SUMO protein (Smt3 and Pmt3, respectively) disruption of SUMO pathway cause severe

growth impairment, but is not lethal to the cells [138, 139]. Whether SUMO molecules can compensate for each other's deficiency is not yet clear. Initial studies showed that mouse embryos, lacking SUMO1, die during embryonic development or soon after birth [140]. However, recent studies show that mice lacking SUMO1 are viable and display an apparently normal phenotype [141, 142] suggesting that SUMO2/3 can compensate for the lack of SUMO1. Nevertheless, a functional sumoylation pathway is necessary for normal development as mouse embryos with disrupted Ubc9 function exhibit defects in nuclear architecture as well as in mitotic chromosome organization, and die at early stages of embryonic development [143].

7.3. Consequences of protein modification with SUMO

Consequences of sumoylation are not easily predictable as it depends on the function of target protein. Attachment of SUMO moiety may alter protein localization, activity and stability, and usually occurs by three main, non-mutually exclusive, mechanisms (Figure 11) [144]. First, attachment of SUMO molecule can mask a binding site for a protein that interacts with a target. Second, SUMO can increase the number of binding sites on its target. Other proteins can interact non-covalently with SUMO or may bind to a newly created domain at the SUMO-substrate interface. Finally, sumoylation can create conformational changes within the substrate, if the modified protein contains a second, non-covalent binding site for SUMO (SUMO-interacting motif – SIM). This may induce a change in the protein activity.The first report that SUMO1 functions as a covalent protein modifier described sumoylation of the nuclear pore protein RanGTPase activating protein, RanGAP1 [145, 146]. In this case, SUMO attachment is responsible for targeting RanGAP1 to nuclear pore.

The majority of SUMO targets have been described in the nucleus, where many transcription factors are subject to sumoylation. Only in few cases sumoylation induces transcriptional activity, like in the case of the orphan receptor RORα [147]. Sumoylation can cause direct or indirect transcriptional repression. In some cases, addition of SUMO causes intranuclear relocalization. Translocation into a particular subnuclear domain, such as PML bodies or nuclear

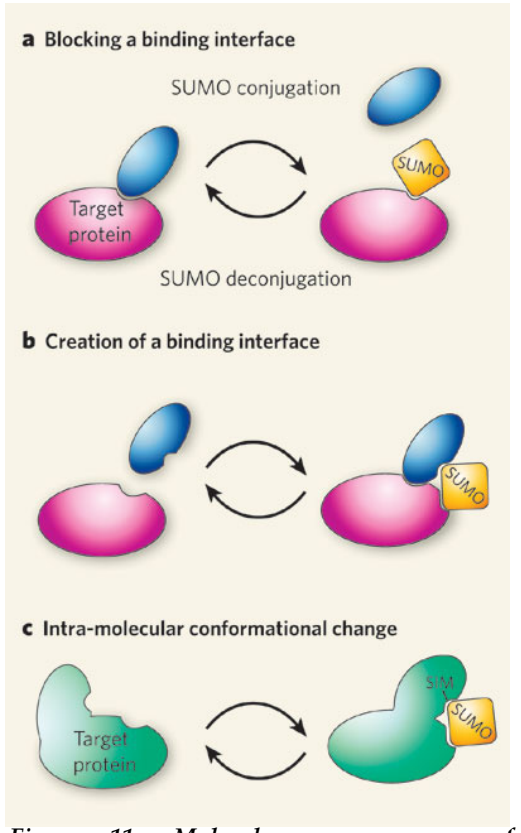

Figure 11. Molecular consequences of sumoylation. a. *Attachment of SUMO may block the interacting surface for binding partners.* ***b.*** *Attachment of SUMO may create a new binding surface for binding partners.* ***c.*** *Attachment of SUMO may induce a conformational change within the substrate protein if the target contains SUMO-interaction motif (SIM). Figure adapted after [134].*

speckles, has been reported for liver receptor homologue LRH-1, steroidogenic factor SF-1, and testicular receptor TR2 [148-151]. Sumoylation may also directly modulate or inhibit the DNA-binding ability of proteins. This has been characterized in case of SF-1, where DNA-binding and sumoylation at sites, adjacent to the DNA-binding domain, occurred mutually exclusive [152].

There is a growing body of evidence for an important role of sumoylation in the cytoplasm. For instance, modification with SUMO has been implicated in plasma membrane receptor endocytosis. Sumoylation of the glutamate receptor subunit 6 (GluR6) is induced in response to kainite, and this modification appears to be a prerequisite for kainite-induced endocytosis of the receptor [153]. Arrestin-3 is subjected to stimulus-dependent sumoylation, and this modification is necessary to promote G protein-coupled receptor (GPCR) endocytosis [154].

Additionally, sumoylation is involved in the regulation of nuclear import and export. Sumoylation of CtBP1 [155], of the IκB kinase regulator NEMO [156] or of the IGF-1 receptor [157] are required for their nuclear translocation. Modification of nuclear pull of actin inhibits its export from the nucleus [158]. On the other hand, nuclear sumoylation of Dictyostelium Mek1 is responsible for its movement to the cytoplasm [159]. A similar situation holds for the TEL protein, where a mutation in an acceptor lysine increases the levels of proteins in the nucleus [160].

7.4 Sumo consensus sites

Mapping acceptor lysine residues allowed identification of SUMO consensus sites. Many proteins contain the consensus motif ΨKxE, where Ψ is a large hydrophobic residue [161]. This motif is recognized by E2 SUMO-conjugating enzyme Ubc9 [162]. These residues directly interact with Ubc9 and, thus, play a critical role in regulating the stability of the interactions between Ubc9 and the substrate [162]. Analysis of structural data show that the consensus motif adopts extended conformation, where the acceptor lysine fits into a hydrophobic groove on Ubc9. Additionally, electrostatic and hydrogen bond interactions between Ubc9 and substrate residues that flank the lysine residue facilitate the recognition of the consensus motif [163, 164].

Apart from canonical SUMO consensus motif, longer sequences that include both SUMO consensus site and additional residues have been identified in some SUMO substrates. These include phosphorylation-dependent sumoylation motifs (PDSM) and negatively charged amino-acid-dependent sumoylation motifs (NDSM). PDSM contains classical SUMO consensus motif, followed by a serine that is subject to phosphorylation, ΨKxExxSP [165]. Phosphorylation of the serine residue enhances sumoylation as is also the case for heat shock factor-1 (HSF-1) [166], Smad nuclear interacting protein-1 (SNIP-1) [165], myocyte-specific enhancer factor 2A (MEF2A) [167] and others. Both PDSM and NDSM are thought to share the same basic mechanism. In the NDSM ΨKxExxEEEE motif, a negative charge, next to the basic SUMO consensus motif, enhances sumoylation [168]. Additionally, recent studies identified new motifs for SUMO conjugation [169]. These include inverted consensus motifs [ED]xKx[≠ED] and motifs with and N-terminal hydrophobic cluster ΨΨΨKxE.

8. Interconnections between different protein modifications

8.1. Phosphorylation and sumoylation

Protein phosphorylation controls multiple cellular pathways and it emerges as a regulator of sumoylation. Protein phosphorylation can both increase and inhibit sumoylation of substrate proteins.

8.1.1. Inhibition of sumoylation by phosphorylation

Phosphorylation-dependent regulation of substrate sumoylation was first shown for the nuclear protein PML, where chemically induced hyperphosphorylation of PML significantly decreased its sumoylation [170]. Similarly phosphorylation represses the sumoylation of the transcription factor Elk1 [171].

8.1.2. Enhancement of sumoylation by phosphorylation

Phosphorylation can enhance sumoylation, especially when phosphorylation site is downstream of the acceptor lysine residues within the phosphorylation-dependent SUMO motif (PDSM) [166, 172]. For example, phosphorylation of heat shock factor 1 (HSF1) is required for its sumoylation [165].

8.1.3. Sumoylation of the phosphorylation machinery

Sumoylation can regulate phosphorylation dynamics through modification of the phosphorylation machinery. Many kinases have been reported to be a substrate for sumoylation. In case of glycogen synthase kinase 3β (GSK3β), it has been suggested that sumoylation within the kinase domain decreases its kinase activity and stability, leading to increased cell survival [173]. Also in case of mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2), lack of sumoylation leads to increased kinase activity and prolonged phosphorylation of HSP27, which promotes endothelial cell actin filament organization and migration [174]. Sumoylation activates autophosphorylation of focal adhesion kinase (FAK) [175]. In response to DNA damage, homeodomain-interacting protein kinase 2 (HIPK2) becomes activated and phosphorylates the E3 SUMO ligase Pc2. This, in turn, stimulates Pc2 to increase sumoylation of HIPK2 that enhances the ability of kinase to mediate transcriptional repression [176]. Sumoylation of extracellular-signal-regulated kinase 5 (ERK2) inhibits its transcriptional potential but not kinase activity [177]. Also, phosphatases can be subjected to sumoylation. Sumoylation of protein tyrosine phosphatase 1B (PTP1B) reduces its catalytic activity [178].

8.2. Sumoylation and ubiquitylation

More and more data are reporting a cross talk between the ubiquitin and sumoylation pathways. Schimmel and colleagues [179] have shown that endogenous SUMO2/3 conjugates accumulate in response to proteasome inhibitors. Additionally, ubiquitylated proteins accumulate in purified SUMO2 conjugates. They also show that a subset of SUMO-2-conjugated proteins is subsequently ubiquitylated and degraded by the proteasome. They conclude that SUMO2/3 conjugation and the ubiquitin-proteasome system are tightly integrated and act in a cooperative manner. Apart from that, many proteins have been reported to be a substrate for both ubiquitylation and sumoylation,

sometimes competing for the same lysine residues. Sumoylation and ubiquitylation can either act antagonistically or in concert to regulate protein function and fate.

8.2.1. Acting antagonistically

Antagonism between ubiquitylation and sumoylation was first reported for the NFκB (nuclear factor κB) regulator, IκBα (inhibitory κBα) [180]. Ubiquitylation of Lys21 and Lys22 targets IκBα for proteasomal degradation. However, sumoylation, at the same Lys21 residue, protected the protein from proteasome mediated proteolysis. Additionally, the data suggest that ubiquitin and SUMO do not compete directly. Ubiquitin modification requires phosphorylation of serines S32 and S36, while phosphorylation inhibits sumoylation. Thus, the actual competition may occur between sumoylation and phosphorylation.

Proliferating cell nuclear antigen (PCNA) can be monoubiquitylated, modified with K-63-linked polyubiquitin chain or sumoylated on the same conserved lysine residue (Lys164) [181]. All these modifications occur in S phase and lead to different outcome. Ubiquitylation occurs after DNA damage. Monoubiquitylation enhances the interaction between PCNA and polymerases facilitating translesion synthesis – DNA synthesis across the damaged site [182, 183]. Polyubiquitylation is necessary for an error-free DNA-damage-tolerance pathway and sumoylation attracts the anti-recombinogenic helicase Srs2 to inhibit unwanted recombination during DNA synthesis [184, 185].

8.2.2. Acting in concert

SUMO and ubiquitin were also reported to act sequentially in the regulation of another component of the NFκB pathway, NEMO (NFκB essential modulator), a regulatory subunit for IKK (IκB kinase) [156]. Attachment of SUMO1 causes nuclear translocation of NEMO, where it becomes ubiquitylated in a phosphorylation-dependent manner on the same lysine residues. Thus, sumoylation facilitates its phosphorylation, which in turn stimulates its ubiquitylation. Modification with ubiquitin induces NEMO translocation to the cytoplasm, where it associates with another IKK subunit and forms an active kinase that can take part in the regulation of IκBα degradation.

8.2.3. SUMO-targeted ubiquitin ligases

Recently the discovery of SUMO-targeted ubiquitin ligases shed new light onto the interaction between sumoylation and ubiquitylation in the regulation of protein degradation. Protein PML can be modified both by SUMO1 and SUMO2/3 chains. Modification with SUMO1 is necessary for its localization to nuclear structures, called PML nuclear bodies [170]. However, attachment of SUMO2/3 recruits SUMO-targeted ubiquitin ligase RNF4 (ring finger protein 4) that ubiquitylates SUMO2/3 chains, which targets the whole complex to proteasomal degradation [186-188].

Outline of this thesis

GHR and Jak2 act in concert in order to trigger signaling cascades via MAPK/STAT5 that regulate many important processes in the cell. Disturbances in GHR signaling have been strongly associated with cancer. Apart from that, aberrant Jak2 activity has been implicated in myeloproliferative diseases as well as tumorigenesis. Thus, understanding how the receptor and the kinase are being regulated is crucial for curing those conditions. Posttranslational modifications are a powerful tool to regulate the function of proteins at postsynthetic level. Both GHR and Jak2 are subjected to phosphorylation and ubiquitylation. In this thesis we analyzed how posttranslational modifications regulate GHR and Jak2 function.

In **chapter 2** we analyze how a novel Jak2 posttranslational modification – sumoylation - regulates its localization and function. We provide evidence that Jak2 is modified by SUMO2/3 up to high molecular weight on multiple lysine residues. Analysis of Jak2 mutants shows that sumoylation of kinase depends on the presence of an active catalytic center and is stimulated both by GH and multiple stresses. Interestingly, phosphorylated kinase is monosumoylated by SUMO2/3, which may represent a regulatory mechanism that allow its association with the receptor. Additionally, we provide evidence that sumoylation facilitates Jak2 translocation to the nucleus. In **chapter 3** we analyze GHR complexes at early stages of endocytosis with a novel method, blue native gel electrophoresis, followed by second dimension SDS-PAGE (2D BN/SDS-PAGE). We were able to identify two main endocytosis-competent GHR complexes at the plasma membrane, corresponding in sizes to GHR tetramers and octamers. Upon GH stimulation, there is a transition from smaller to bigger complexes that are subject to posttranslational modifications: phosphorylation and ubiquitylation. We were able to confirm previous results showing that GHR association with both Jak2 and TrCP2 are transient. Our data indicate that GH binding is sufficient for complex transition. Moreover, we confirm that both K48- and K63-linked ubiquitylation is involved in GHR endocytosis. In **chapter 4** we explain the optimization of the 2D BN/SDS-PAGE to analyze membrane receptor signaling. We describe in more details the application of the method on two important signaling systems: GHR as a prototype class I receptor and Wnt-βcatenin signaling pathways. We show that GHR complexes, stimulated with STREP-GH, can be eluted in native conditions and analyzed on 2D BN/SDS-PAGE. Additionally, we show for the first time that analysis of posttranslational modifications is possible with this method. In **chapter 5** we show that the sumoylation machinery is involved in GHR trafficking. Both SUMO2/3 overexpression and Ubc9 depletion result in intracellular accumulation of the GHR. SUMO3 overexpression does not affect GHR endocytosis nor causes it increased targeting to the recycling pathway. In addition, we show that synthesis of GHR is not increased. Our data suggests that the mechanism of receptor accumulation is specific for GHR. We provide evidence that GHR segregating at the level of MVBs is affected by exogenously expressed SUMO2/3, which leads to inhibited degradation of the receptor. The data show that ESCRT proteins can be potential substrates of sumoylation machinery. Moreover, SUMO3 overexpression causes increased GHR targeting to the nucleus.

The findings of this thesis represent a significant progress in the understanding of how posttranslational modifications regulate GHR and Jak2 function. For the first time, we report and analyze a role of the sumoylation system in both Jak2 function and GHR

trafficking. Additionally, we optimize BN/SDS-PAGE for studying GHR complexes and its posttranslational modifications that contribute to understanding the mechanism of GHR endocytosis. Our findings can be applied to design new therapeutic strategies for patients with myeloproliferative diseases and GHR/Jak2 related malignancies.

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CHAPTER 2

Regulation of Jak2 function via modification with SUMO2/3

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Summary

Jak2 kinase regulates the signal transduction of many cytokine receptors. Growth hormone (GH) binding to its receptor triggers Jak2, which leads to activation of the STAT pathway and activation of genes involved in growth and metabolism. We discovered that Jak2 is sumoylated on multiple lysine residues by SUMO2/3 chains. Analysis of Jak2 mutants revealed that sumoylation of Jak2 depends on the presence of an active catalytic site. Both GH stimulation and multiple other environmental stressors increase the modification with SUMO chains. Additionally, we provide evidence that phosphorylated kinase is monosumoylated, which may represent a direct mechanism of regulating Jak2 activity. The majority of nuclear Jak2 is highly sumoylated. We hypothesise that modification with SUMO provide for regulatory functions in the activity of Jak2. Nuclear translocation may be one important mechanism.

Introduction

Jak2 kinase belongs to the family of Janus kinases (Jaks) that are necessary for cytokine signaling. They associate with oligomeric cytokine receptors that lack intrinsic kinase activity and, upon cytokine addition, transphosphorylate themselves and the accompanying receptor, allowing the signaling cascade to proceed. This leads to a variety of biological responses and is associated with many important biological processes in hematopoiesis and immune system regulation. Cytokine receptors, associated with Jak2, include the prolactin, erythropoietin (Epo) and growth hormone receptor (GHR). In this study we use GHR as a model to study Jak2 function. Upon growth hormone (GH) stimulation Jak2 triggers signaling cascades via STAT5/MAPK.

Jak2 signaling is implicated in various biological processes, including cell cycle progression, apoptosis, mitotic recombination and alteration of heterochromatin. The most common somatic alteration of Jak2 is a gain-of-function mutation of V617F, associated with human myeloproliferative diseases [1]. The diverse roles of Jak2 in normal and leukemic hematopoiesis are believed to be restricted to cytoplasmic events. However, Dawson and coworkers reported that Jak2 can translocate to the nucleus, where it phosphorylates histone H3, which leads to activation of gene transcription [2]. The mechanism of this translocation is still unknown as Jak2 lacks either typical nuclear localization or nuclear export signals.

In addition to being tyrosine phosphorylated, Jak2 is also subjected to other posttranslational modifications, such as ubiquitylation. Several studies have analyzed Jak2 being ubiquitylated and degraded by the proteasome [3-5]. Ali et al [6] showed that

the kinase is modified in response to prolactin stimulation, which leads to its degradation. They also showed that association of Jak2 with SOCS-1 is important for this event. Haan and coworkers analyzed the stability of the V617F Jak2 mutant and showed that it is much less stable than the wild type Jak2. Its degradation is both phosphorylation- and proteasome-dependent [7].

Sumoylation of proteins is an essential process within the cells that alters protein-protein interactions. This can lead to changes in protein localization, stability and activity (reviewed in [8]). The human genome encodes four SUMO proteins: SUMO1-SUMO4. SUMO2 and SUMO3 are 97% identical and can contribute to polysumo chains. SUMO1 occurs only as a mono-substituent and is less ubiquitously expressed than SUMO2 and SUMO3. The role of SUMO4 is unknown. Sumoylation begins with the activation of a SUMO molecule by the sumo-activating enzymes SAE1 or SAE2 (SUMO E1). Next, activated SUMO molecules are transferred to the specific SUMO-conjugating enzyme, Ubc9 (SUMO E2), and finally attached to lysine residues in targeted proteins. A distinguishing feature of sumoylation is that only a small fraction of protein is modified at any given time.

Many proteins that are modified by SUMO contain the consensus motif ΨKX(D/E) where Ψ is a large hydrophobic residue. This motif is recognized by SUMO E2 Ubc9 [9]. However, the presence of such a motif does not imply that the protein is subjected to modification. Additionally, SUMO modification can also occur on residues located outside this motif and an increasing number of studies report other possible SUMO consensus motifs [10]. Other modifications like phosphorylation can enhance sumoylation, especially, when they include phosphorylation sites downstream of acceptor lysine residues within the phosphorylation-dependent SUMO motif (PDSM) [11, 12]. However, there are reports showing that phosphorylation can also inhibit sumoylation [13, 14].

Many substrates are preferentially modified by either SUMO1 or SUMO2/3. However, proteins, like in p53 or pRB, are modified by both SUMO1 and SUMO2/3 [15], sometimes competing for the same lysines. In case of PML, modification with SUMO1 is required for its localization to PML nuclear bodies, while addition of SUMO2/3 chains causes the recruitment of the SUMO-binding ubiquitin ligase RNF4 that ubiquitylates SUMO chains leading to PML degradation via the proteasome [16-18].

Depending on the target protein, sumoylation can occur in the cytoplasm or the nucleus. For several proteins like CtBP1 [19] or the IκB kinase regulator NEMO [20], sumoylation is required for nuclear translocation. For the IGF-1 receptor, it was shown that stimulation with IGF causes receptor modification with SUMO1, and its subsequent translocation to the nucleus [21]. However, lack of sumoylation did not affect the kinase dependent signaling. Additionally, there is evidence that SUMO modification could also regulate nuclear export of some substrates. For example, nuclear sumoylation of Dictyostelium Mek1 is responsible for its movement to the cytoplasm [22], while mutation of sumo-acceptor lysine of TEL protein increases its level in the nucleus [23].

In this study, we show that Jak2 is modified by SUMO2/3 up to high molecular weight. Both GH and extracellular stresses, like elevated temperature or serum starvation stimulate Jak2 sumoylation. Sumoylation and phosphorylation of Jak2 appear to be connected. The kinase inactive mutant, K882E, shows substantially less sumoylation signal, while the constitutively active Jak2 V617F mutant has increased sumoylation signal. Furthermore, we provide evidence that sumoylation of Jak2 facilitates its translocation to the nucleus.

Additionally, we demonstrate that, in response to cytokine stimulation, phosphorylated Jak2 can be monosumoylated by SUMO2/3. We propose that sumoylation provides important regulatory mechanisms controlling both Jak2 activity and location.

Materials and Methods

Reagents

Mouse monoclonal antibody against Jak2 was purchased from Invitrogen (AHO1352), anti-SUMO2/3 from Abcam (ab81371), anti-HA tag 12CA5 antibody was from Babco (Richmond CA), against phosphorylated tyrosines (pY) from Millipore (clone 4G10), anti-polyubiquitin (FK2) from Enzo Life Sciences, Inc., anti-KDEL from Merck (10C3), anti-GAPDH from Millipore (MAB374) anti anti-actin from MP Biomedicals Inc (clone C4). Rabbit polyclonal antibody against FLAG tag was purchased from Sigma, anti-EE1 from BD Biosciences, San Diego, CA and anti-Ubc9 from Santa Cruz (sc-10759). Rabbit polyclonal anti-GHR B antibody used for western blot detections and anti-GHR T antibody used for immunoprecipitations were previously described elsewhere [24, 25]. Rabbit polyclonal anti-Jak2 antibody raised against a synthetic peptide corresponding to the hinge region (amino acids 758–777) between domains 1 and 2 of murine Jak2 as previously described in [26], was used for immunoprecipitations. Rabbit monoclonal anti-histone H3 antibody was from Cell Signaling (D1H2). Rabbit monoclonal antibody used for blots and for immunoprecipitations targeted against phosphorylated Y1007 and Y1008 of Jak2 (pJak2) was purchased from Abcam (ab32101). The secondary antibodies Alexa Fluor 680 and IR 800-conjugated goat anti mouse and anti-rabbit IgGs were obtained from Molecular Probes. Beads coupled to antibody against FLAG tag M2 were obtained from Sigma. ProteinA beads were purchased from RepliGen Corporation. Ni-NTA beads were from Qiagen. Human GH was kindly provided by Eli Lilly & Co. Research Labs (Indianapolis, IN). Culture media, fetal calf serum (FCS), L-glutamine and antibiotics for tissue culture were purchased from Invitrogen.

Plasmids

The FLAG-Jak2 mouse construct was a kind gift from Prof. Carter-Su. The pcDNA3 HA-SUMO1, SUMO2 and SUMO3 and pcDNA3 His-SUMO1 and SUMO2 plasmids were kind gifts from Frauke Melchior. FLAG-Jak2 truncations (1-842, 1-525 and 1-280) were constructed by the introduction of a stop-codon using the Quickchange mutagenesis kit from Stratagene. To generate plasmids encoding FLAG-Jak2 K882E, Y1007F, V617F, K167R, K630R, K991R, K991R/K1011R, K912R, K914R and K912R/K914R mutants, a pair of mutagenic primers with a point mismatch at the desired site were designed for each mutation (Supplementary Table 1) and used to generate mutated sequences on the FLAG-Jak2 plasmid as a template with Site Directed Mutagenesis kit from Stratagene. The mutations were confirmed by DNA sequencing (Macrogen, Korea).

Cell culture, transient transfections and gene silencing

The cell line Hek293 was cultured under standard conditions in DMEM high glucose containing 10% fetal bovine serum. The Hek293 cells stably expressing the wild type GHR (Hek-wt GHR) were grown in the same medium supplemented with 0.6 mg/ml Geneticin (G418; Gibco). Transfections were performed with Fugene (Roche). Cells were plated to 60% confluency (106 cells in 6 cm dish format) 24 hours before the transfections, and then transfected with 2 μg of DNA and 6 μl of Fugene. Cells were silenced for 48 hours with Lipofectamine2000 according to standard conditions. siRNA used for Ubc9 silencing was purchased from Ambion (s14591).

SDS-PAGE and western blotting

Proteins were solubilized in sample buffer and electrophoresed on denaturing SDS-polyacrylamide

gels. The proteins were transferred to Immobilon-FL polyvinylidenedifluoride membranes (Millipore) and analyzed by western blotting with the indicated antibodies. Blots were washed and incubated with fluorescent secondary antibodies (Molecular Probes). Reactive bands were detected with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, Nebraska).

Cell lysis and immunoprecipitations

Cells were washed once with PBSa and harvested in denaturing lysis buffer (1 mM EDTA, 1% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa). The lysates were boiled for 6 min, five times sheared with a 25G needle, boiled again for 3 min, vortexed and clarified for 5 min at 16,100g. Supernatants were diluted to a final concentration of 0.5% SDS with Immunomix special (2% TritonX-100, 1 mM EDTA, 1% BSA, 0.5% DOC, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa). Each sample was incubated with polyclonal anti-Jak2 antibody for 2 h before adding ProteinA beads for 45 min in an end-over-end rotator at 4°C. Beads were washed two times with immunomix (1% TritonX-100, 0.1% SDS, 1 mM EDTA, 1% BSA, 0.5% DOC, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa) and two times with 0.1 concentration of PBSa and boiled with sample loading buffer for 5 min. To assess the phosphorylation state of Jak2, cells were grown under basal serum conditions or serum starved for 1-2 h before stimulation with 180 ng/ml of human GH. For coimmunoprecipitation experiments cells were lysed in non-denaturing lysis buffer (1% Triton, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa). Subsequently, the same amount of immunomix was added (1% TritonX-100, 0.5% DOC, 1% BSA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa) and GHR was immunoprecipitated with anti-T antibody as described above.

Subcellular fractionations

The high salt nuclear fraction protocol was performed as followed. Hek293 cells in a 10-cm dish were scraped into 1 ml of ice-cold PBS and centrifugated for 5 min at 16000g. Supernatant was discarded and cells were resuspended in 200 μl of buffer 1 (25 mM HEPES pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 1mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4) and lysed by adding 200 μl of buffer 2 (buffer 1 supplemented with 1% NP-40) and incubating for 15 min end-over-end at 4°C. Nuclei were pelleted at 500g for 5 min and washed once with buffer 3 (1:1 mixture of buffer 1 and 2). The cytoplasmic fraction was collected and immunoprecipitation was performed by adding 500 μl of buffer (2% TritionX-100, 1% SDS, 1 mM EDTA, 1% BSA, 0.5% DOC, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF, 1 mM Na3VO4 in PBSa) followed by incubation with appropriate antibody as described above. To the nuclear fraction 500 μl of denaturing lysis buffer was added and the procedure was performed as described in the Cell lysis and immunoprecipitations section.

Results

Jak2 is modified by SUMO2/3

In order to determine whether Jak2 is sumoylated, Hek293 cells were transiently transfected with Flag-Jak2 and HA-tagged SUMO1, SUMO2 or SUMO3, and analyzed on Western blot (Fig. 1A). Cells transfected with either SUMO2 or SUMO3 showed a clear high molecular weight signal, indicating extensive SUMO modification. The fact that sumoylated Jak2 is hardly detectable with anti Jak2 antibody (data not shown) indicates that only a small fraction was sumoylated. In Fig. 1B we used preimmune serum to

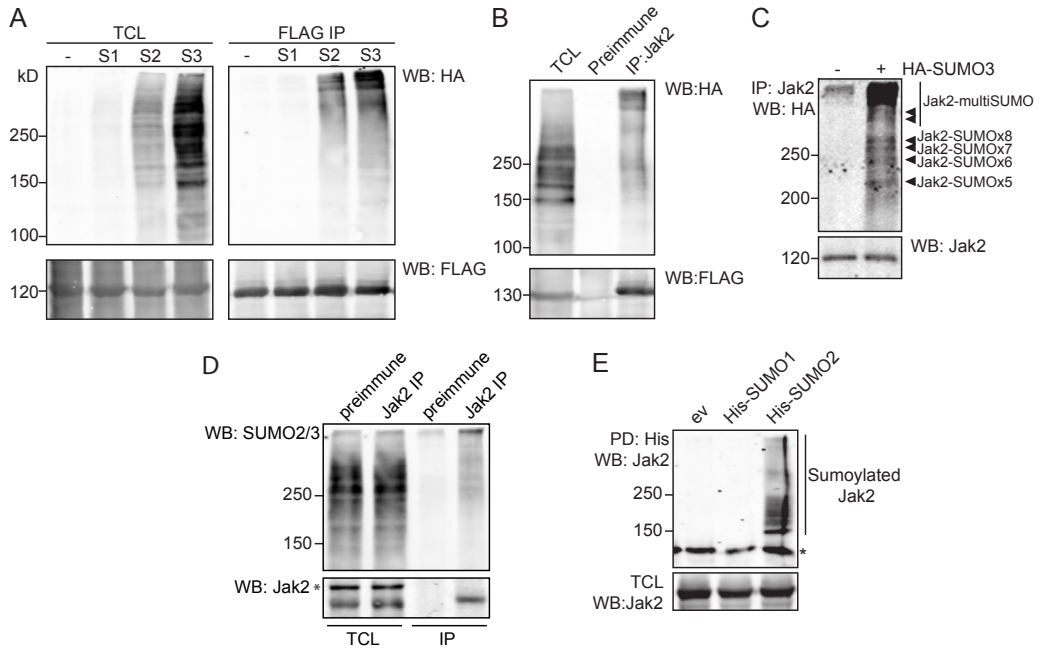

Figure 1. Jak2 is sumoylated by SUMO2/3.

A. *Hek293 cells were transfected with FLAG-Jak2 and with HA-SUMO1 (S1), HA-SUMO2 (S2), HA-SUMO3 (S3) or no SUMO (-). Cells were lysed in denaturing conditions and anti-FLAG immunoprecipitations were performed. The blot was detected with antibodies against HA and FLAG.* **B.** *Hek293 cells were transfected with FLAG-Jak2 and HA-SUMO3. Cells were lysed in denaturing conditions and immunoprecipitations were performed with pre-immune or anti-Jak2 antibodies.* **C.** *Hek293 cells were transfected with HA-SUMO3 and endogenous Jak2 was immunoprecipitated in denaturing conditions. Western blot was analyzed with anti-HA and anti-Jak2 antibodies.* **D.** *Anti-Jak2 immunoprecipitations were performed in denaturing conditions and the blot was developed with anti-Jak2 and anti-SUMO2/3 antibodies. The blot represents three independent experiments. Star indicates background band.* **E.** *Hek293 cells were transfected with FLAG-Jak2, His-SUMO1 and His-SUMO2. Subsequently, anti-His pull down on Ni-beads was performed in denaturing conditions. The blot was analyzed with antibody against Jak2. Star indicates Jak2 unspecifically bound to the beads.*

demonstrate that the SUMO signal on Jak2 is specific.

Next, we asked whether endogenous Jak2 is also modified by SUMO2/3. Hek293 cells were transiently transfected with HA-SUMO3 and endogenous Jak2 was immunoprecipitated. The most abundant sumoylated Jak2 migrated as high-molecular weight species, while clear bands, probably representing Jak2 molecules with 5-8 SUMO moieties, were visible (Fig. 1C). In Fig. 1D we show that sumoylated endogenous Jak2 was also detectable with anti-SUMO antibodies. Furthermore, we detected high molecular Jak2 signal in a pull-down of all sumoylated proteins. To confirm the experiments, Hek293 cells were transiently transfected with Flag-Jak2 and His-SUMO1 or His-SUMO2 and anti-His pull down was performed in denaturing conditions using Ni-beads. As seen in Fig. 1E, clear bands corresponding to sumoylated Jak2 were visible. A star marks Jak2, non-specifically bound to Ni-beads.

Together, our data show that at steady state a small portion of Jak2 is modified specifically by SUMO2/3 chains, up to high molecular weights.

Jak2 sumoylation requires an intact kinase domain

To determine the mechanism of sumoylation, we analyzed several Jak2 truncations (truncated after amino acid 280, 542 and 842; Fig. 2A) as substrates for sumoylation (Fig. 2B). Only wild type Jak2 was modified (Fig. 2B). This indicates that sumoylation requires the presence of the kinase domain.

To test whether sumoylation depends on an intact catalytic activity, we analyzed the sumoylation state of three mutants: K882E in the ATP binding pocket lacking kinase activity, Y1007F inhibiting full kinase activation, and V617F, most common in chronic myeloproliferative neoplasms, constitutively active that still depends on a scaffold protein to bind and signal (Fig. 2A) [27]. Fig. 2C shows the summary of several separate experiments. The two mutants lacking kinase activity (truncated at amino acid 842 and K882E) have substantially decreased sumoylation signals, while the constitutively active V617F mutant showed a 50% increased sumoylation signal, comparing to wild type. Interestingly, the Y1007F mutant contains an almost normal sumoylation signal. Together, these results show that sumoylation of Jak2 depends on presence of active catalytic center.

In an effort to identify functional sumoylation sites we mutated several lysine residues within or near sumoylation consensus sites within the kinase domain. In several cases the mutants lost kinase activity, probably due to structural changes caused by the mutations (data not shown). All the kinase-inactive mutants showed a decreased sumoylation signal, supporting our hypothesis that Jak2 sumoylation depends on an active catalytic centre. On the other hand, mutations K991R and K1011R (within SUMO consensus sites assessed by the SUMOplotTM prediction program; www.abgent.com/tools/sumoplot) and K912R and K914R had decreased sumoylation signal, but normal kinase activity as inferred from the degree of autophosphorylation measured with anti-pY antibodies. This indicates that phosphorylation is not sufficient for sumoylation. Additionally, mutations within SUMO consensus motifs at K167R, localised in the FERM domain, and K630R, localised in the pseudokinase domain also showed lower SUMO signal (Fig. 2D). In Fig. 2A the mutations in Jak2 used in this study are depicted. All single or double mutants have decreased sumoylation signal (Fig. 2D). Since one or two single mutations reduce sumoylation of Jak2 up to 50%, we hypothesize that Jak2 is sumoylated on multiple sites. Jak2 contains ~80 lysine residues and SUMO2/3, in contrast to SUMO1, is able to make chains. Since attachment of each SUMO moiety increases the molecular weight of a protein by 20kDa, modification of many lysine residues with SUMO chains would result in very high molecular weight signal. Therefore, our data indicate that Jak2 is modified by SUMO chains on multiple lysines.

Both GH-induced activation and stressors stimulate Jak2 sumoylation

To further analyze the relation between phosphorylation and sumoylation we used GHR as a model system. GHR-expressing Hek293 cells were stimulated with GH and endogenous Jak2 was analyzed in denaturing conditions. Fig. 3A shows that a short GH stimulus caused a 40% increase in Jak2 sumoylation. To analyze the relation between phosphorylation and sumoylation further we treated the cells with the general kinase inhibitor, staurosporin. As seen in Fig. 3B GH did not stimulate Jak2 sumoylation in the presence of staurosporin. However, treatment with staurosporin alone caused a

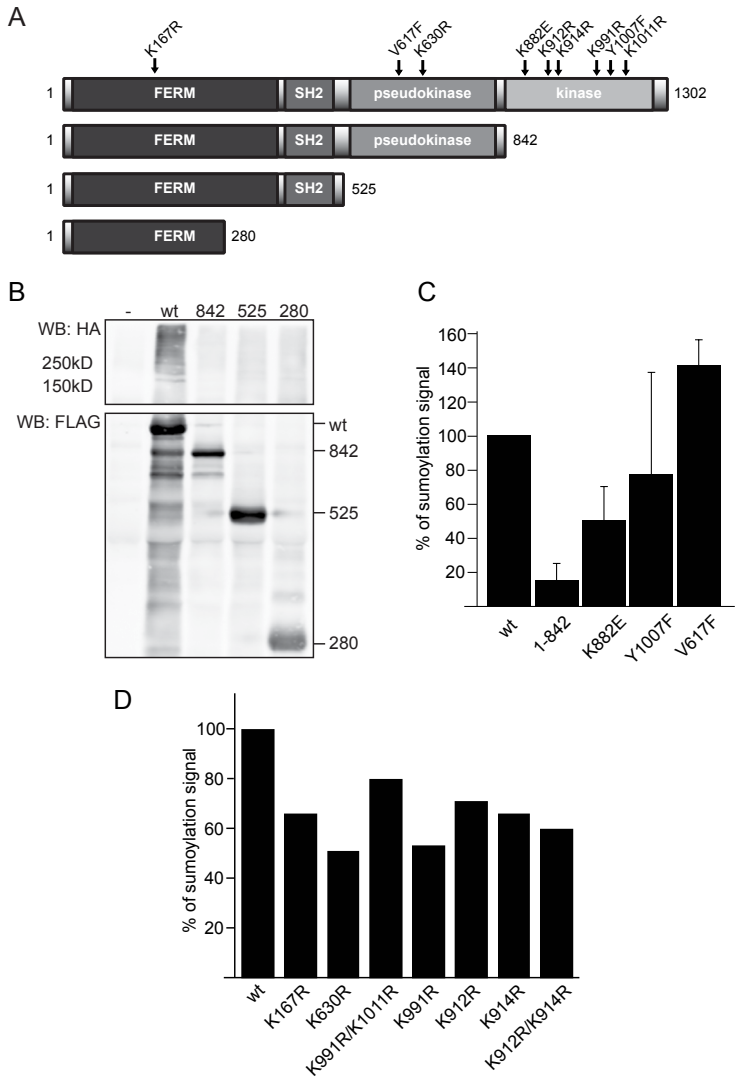

Figure 2. Jak2 sumoylation and phosphorylation signals are connected.

A. *Structure of Jak2. Wild type Jak2 consists of several different domains. The FERM domain is responsible for receptor binding, SH2 domain for protein-protein interactions and pseudokinase domain is an inhibitory domain for the kinase domain. Three Jak2 truncations were used: truncated after 842, lacking kinase domain, after 525 lacking both pseudokinase and kinase domain and after 280, consisting only of part of the the FERM domain. Different mutations used in this study are marked.* **B.** *Hek293 cells were transfected with HA-SUMO3 and different FLAG-Jak2 truncations (wt, 842, 525 and 280), lysed in denaturing conditions and anti-FLAG immunoprecipitations were performed. The blot was analyzed with antibodies against HA and FLAG.* **C.** *Hek293 cells were transiently transfected with different FLAG-Jak2 constructs as indicated. Anti-FLAG immunoprecipitations were performed in denaturing conditions. Graph is based on 15 different experiments. It is expressed as a ratio of sumoylation signal and Jak2 signal.* **D.** *Graph representing several different experiments is showing relative sumoylation signal of different Jak2 lysine to arginine mutants. Hek293 cells were transiently transfected with FLAG-Jak2 constructs and anti-FLAG immunoprecipitations in denaturing conditions were performed. Sumoylation signal was divided by FLAG signal and plotted on graph as relative sumoylation signal. Wt, wild type.*

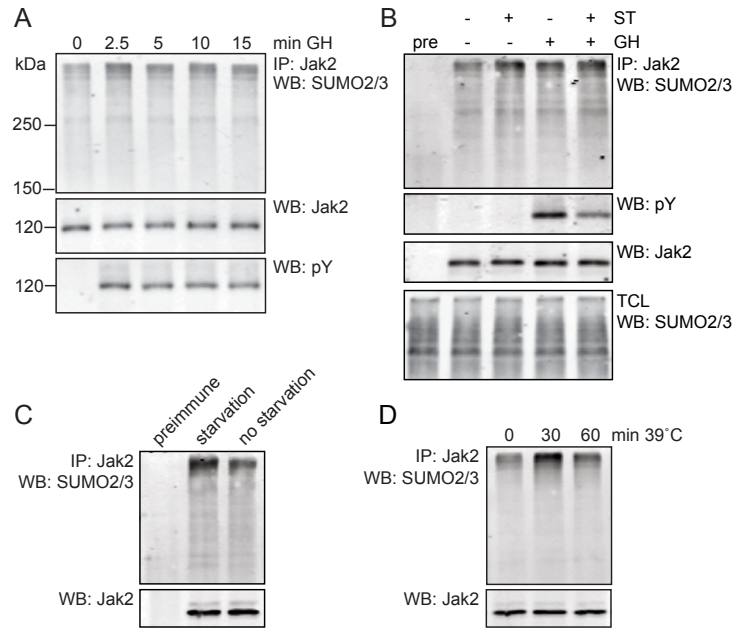

Figure 3. GH and stressor effect on Jak2 sumoylation.

A. *Hek293 cells were stimulated for different times with 180 ng/ml GH, and anti-Jak2 immunoprecipitations were performed in denaturing conditions. The blot was analyzed with antibodies against SUMO2/3, Jak2 and pY. The figure represents three independent experiments.* **B.** *Hek293 cells were pretreated for 4 h with staurosporin (ST) before GH stimulation for 2.5 min. Next, Jak2 immunoprecipitations in denaturing conditions were performed and phosphorylation of Jak2 was analyzed with anti phosphotyrosine antibody (pY).* **C.** *Hek293 cells were subjected to 4 h serum starvation, lysed in denaturing conditions and anti-Jak2 immunoprecipitations were performed. Western blot was analyzed with antibodies against SUMO2/3 and Jak2. The figure represents three independent experiments.* **D.** *Hek293 cells were subjected to 39ºC for 0, 30 or 60 min, lysed in denaturing conditions and anti-Jak2 immunoprecipitations were performed. The blot was analyzed with antibodies against SUMO2/3 and Jak2. The figure represents two independent experiments.*

substantial increase in Jak2 sumoylation, independent of GH. Therefore, we reasoned that general stressors might induce Jak2 sumoylation. As seen in Fig. 3C, starvation caused a 40% increase in Jak2 sumoylation. To further test the hypothesis that stress induces Jak2 sumoylation we exposed cells to elevated temperature. As seen in Fig. 3D, incubation at 39ºC increased Jak2 sumoylation more than 100%. Thus, while there is a basic level of sumoylated Jak2 in the cells, both cytokine receptor activation and several stressors increase Jak2 sumoylation.

Is there a relationship between Jak2 phosphorylation, sumoylation and ubiquitylation?

It has been shown before that stimulation with ligands like GH and prolactin increases Jak2 ubiquitylation leading to its degradation via proteasome [6, 28]. As sumoylation and ubiquitylation are often connected, we addressed the question whether GH stimulation increases Jak2 ubiquitylation. Indeed, as seen in Fig. 4A, incubation with GH increased ubiquitylation of Jak2. As sumoylation of Jak2 depends on the presence of an active ATP binding pocket we used the Jak2 K882E mutant to determine whether sumoylation and ubiquitylation are connected. Cells were transfected with empty vector, Flag-Jak2

or Flag-Jak2 K882E and analyzed with antibodies against ubiquitin. As seen in Fig. 4B, ubiquitylation of Jak2 wt was enhanced by GH. However, the ubiquitylation of kinase inactive mutant was not up regulated in response to cytokine stimulation. Previous results have shown that a kinase inactive mutant has a decreased sumoylation signal. This suggests that phosphorylation enhances not only sumoylation but also ubiquitylation. Additionally, ubiquitylation of Jak2 occurs later than high molecular weight sumoylation (compare Fig 3A). Therefore, we propose that ubiquitylation of Jak2 is enhanced by its sumoylation, which in turn is enhanced by kinase phosphorylation. Since ubiquitylation leads to proteasomal degradation, we tested Jak2 modification with SUMO when the proteasome was inhibited. As can be seen in Fig. 4C MG132 did not prevent sumoylation of Jak2. On the contrary, inhibition of proteasome caused accumulation of both endogenous and overexpressed sumoylated Jak2. That was especially seen for endogenous Jak2,

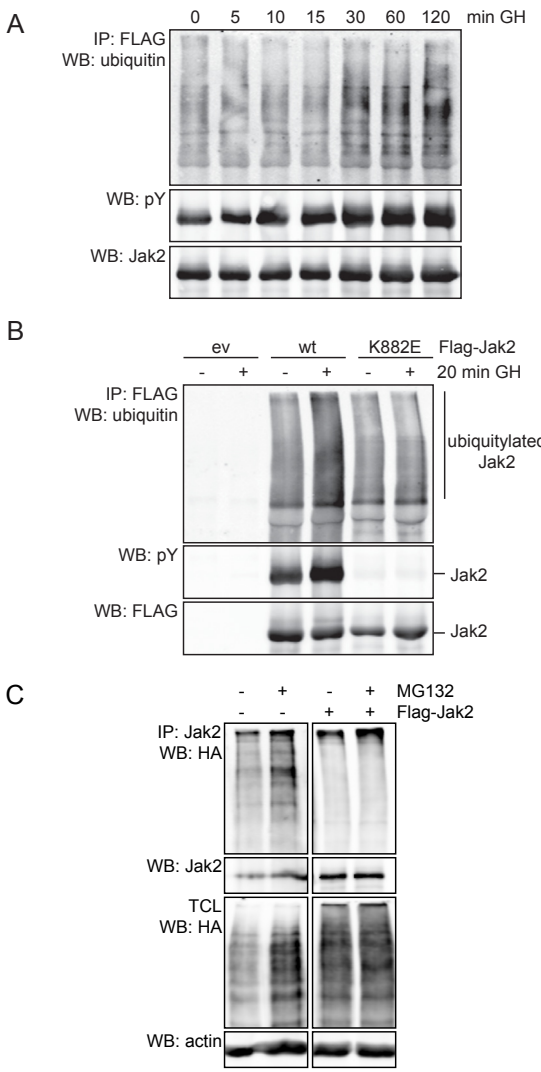

Figure 4. Cross talk between Jak2 phosphorylation, sumoylation and ubiquitylation.

A. *GHR expressing Hek293 cells were transiently transfected with FLAG-Jak2 and stimulated with GH as indicated. Anti-FLAG immunoprecipitations were performed in denaturing conditions. Western blot was analyzed with anti-ubiquitin, anti-pY and anti-FLAG antibodies.* **B.** *GHR expressing Hek293 cells were transiently transfected with FLAG-Jak2, wild type and K882E. Anti-FLAG immunoprecipitations were performed in denaturing conditions. The blot was analyzed with anti-ubiquitin, anti-pY and anti-FLAG antibodies.* **C.** *Hek293 cell were transiently transfected with HA-SUMO3 and without or with FLAG-Jak2. Cells were treated with 20 μM MG132 for 4 h prior to lysis in denaturing conditions. The blot was analyzed with antibodies against HA and Jak2.*

which may be explained by partial phosphorylation of overexpressed Jak2. However, the accumulation was not robust which may be due to incomplete proteasomal inhibition. Jak2 is a very stable protein and 4 h of treatment may not be enough to see an effect. Nevertheless, the data support previous results.

Our data suggest a relationship between Jak2 phosphorylation, sumoylation and ubiquitylation. However, full elucidation of how these modifications together regulate Jak2 function requires further studies.

Sumoylation facilitates Jak2 translocation to the nucleus

Sumoylation of a target protein can induce change in its localization. It can both be a prerequisite for nuclear translocation like in the case of CtBP1 [19], NEMO [20] and IGF-1 receptor [21] or be required for nuclear export [22, 23, 29]. As Jak2 has a major function in the cytosol, we considered the first possibility. To examine whether Jak2 is detectable in the nucleus we fractionated cells and analyzed the fractions for Jak2 (Supplementary Fig. 1B). The majority of Jak2 was present in the cytoplasmic fraction (85%) and only 15% in the nuclear fraction (Fig. 5A). Next, we tested whether sumoylated Jak2 is present in the nucleus. As can be seen in Fig. 5B, endogenous sumoylated Jak2 is clearly present in the nuclear fraction with only a relative small amount in the cytosolic fraction. The difference in ratios of sumoylated Jak2 vs unmodified Jak2 between the two cell fractions is striking, considered the limited transfer efficiency of high-molecular protein complexes during

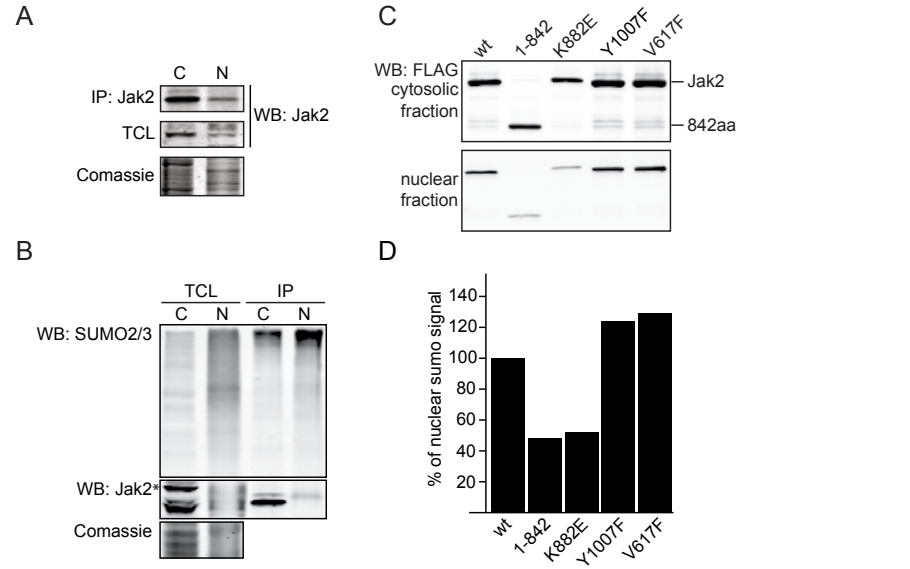

Figure 5. Sumoylation of Jak2 and its translocation into the nucleus.

A. *Cells were fractionated and Jak2 was immunoprecipitated from cytoplasmic and nuclear fractions. The blot was analyzed with anti-Jak2 antibody.* **B.** *Cytoplasmic and nuclear fractions from Hek293 cells were analyzed both in the total fractions and after immunoprecipitation with anti-Jak2 in denaturing conditions. The blot was analyzed with anti-SUMO2/3 and anti-Jak2 antibodies. Star indicates background band.* **C.** *Hek293 cells were transiently transfected with FLAG-Jak2 wt, 1-842 truncation, K882E and V617F. Cytoplasmic-nuclear fractionation was performed and the western blot was detected with anti-FLAG antibody.* **D.** *Graph representing results from part C. Data are expressed as percentage of nuclear fraction in comparison to wt Jak2. Wt, wild type.*

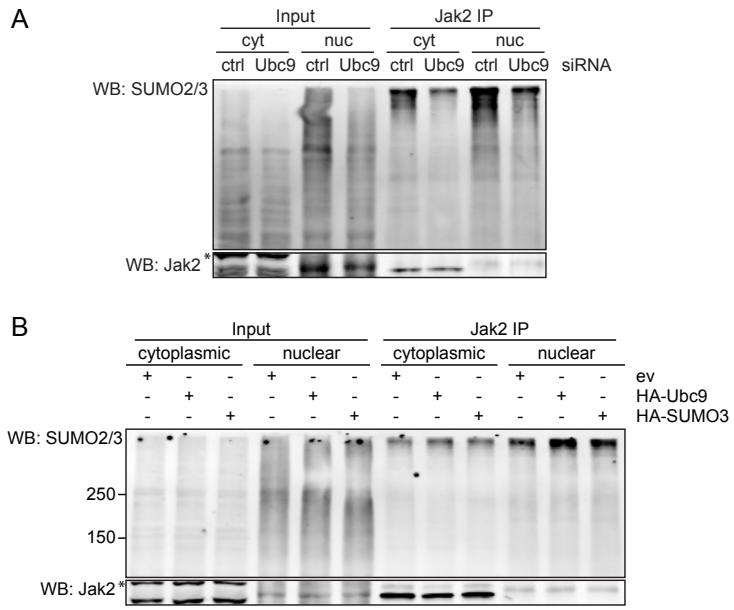

Figure 6. Effect of both Ubc9 silencing and Ubc9/SUMO3 overexpression on Jak2 in the nucleus.
A. *Hek293 cells were silenced for 48 h with control siRNA and Ubc9-specific siRNA. Subsequently, cells were separated in cytosolic and nuclear fractions. Anti-Jak2 immunoprecipitations in denaturing conditions were performed on both fractions. The blot was analyzed with antibodies against SUMO2/3 and Jak2. Star indicates a background band.* **B.** *Hek293 cells were transiently transfected with HA-Ubc9 or HA-SUMO3 and cytosolic/nuclear fractionation was performed. Subsequently anti-Jak2 immunoprecipitations in denaturing conditions were performed on both fractions. The blot was analyzed with antibody against SUMO2/3 and Jak2. The star indicates a background band.*

the electrophoretic transfer. This shows that high molecular weight sumoylated Jak2 accumulates in the nucleus.
Since Jak2 sumoylation depends on its activity (Fig. 2C) we asked whether there is a relation between Jak2's enzyme activity and its cellular distribution. Cells were transfected with different FLAG tagged Jak2 mutants, cytosolic and nuclear fractions were isolated, and analyzed with anti-FLAG antibodies (Fig. 5C). In Fig. 5D Jak2 mutants were expressed relative to wild type Jak2. Kinase inactive species showed a 50% decreased nuclear localization, while both the Y1007F and the V617F mutants showed a 20% increase in nuclear localization comparing to wild type Jak2. Thus, there is a clear correlation between sumoylation and nuclear translocation of Jak2.
To further examine the sumoylation of Jak2 in the nucleus, we silenced Ubc9 for 48 h and analyzed Jak2 in cytosolic and nuclear fractions under denaturing conditions. Fig. 6A (Supplementary Fig. 1A) shows that the amount of sumoylated Jak2 decreased to 50% in both fractions. Also, a 15% decrease occurred in the level of unmodified Jak2 in the nuclear fraction. Next we transfected the cells with HA-SUMO3 and HA-Ubc9 constructs and analyzed sumoylation state of endogenous Jak2 in the nucleus. Again, as expected, we observed respectively a 15% and 35% increase in high molecular weight Jak2 in the nuclear fraction compared to normal conditions indicating that higher cellular concentrations of either factor drives both sumoylation and nuclear translocation (Fig. 6B).

In conclusion, we show that Jak2 is present in the nucleus, almost exclusively in its sumoylated state. Our data provide the first evidence that sumoylation might be responsible for Jak2 translocation to the nucleus.

Phosphorylated Jak2 is monosumoylated
Since GH stimulates sumoylation of Jak2, we asked whether sumoylated Jak2 is associated with the GHR. Cells were transiently transfected with Flag-Jak2 and HA-SUMO3 and GHR immunoprecipitation was performed in non-denaturing conditions. As seen in Fig. 7A a limited amount of Jak2 could be coimmunoprecipitated with GHR. However, no high molecular weight HA-positive signal was detectable. This suggests that polysumoylated Jak2 is not present on GHR in steady state conditions. Next, we asked whether Jak2 sumoylation is required for its kinase activity. Hek293 cells expressing GHR were transiently transfected with Flag-Jak2 and HA-SUMO3 and silenced for the expression of the SUMO conjugase, Ubc9 for 48 h. As seen in Fig. 7B (Supplementary

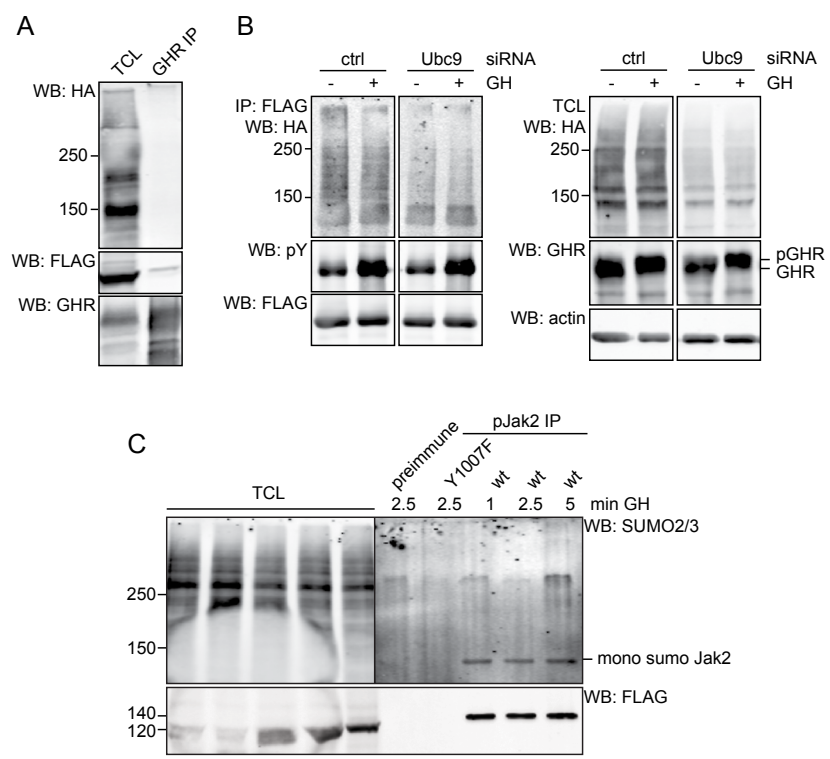

Figure 7. Phosphorylated Jak2 is modified with mono SUMO2/3.
A. *GHR expressing Hek293 were transiently transfected with FLAG-Jak2 and HA-SUMO3. Anti-GHR immunoprecipitations were performed. The blot was analyzed with antibodies against HA, FLAG and GHR.* **B.** *GHR expressing Hek293 cells were transfected with HA-SUMO3 and FLAG-Jak2. Ubc9 was silenced for 48 hours. Anti-FLAG immunoprecipitations were performed in denaturing conditions. The blot was detected with antibodies against HA, GHR and FLAG. Phosphorylation of Jak2 was detected with anti-pY antibody. Actin staining was used as loading control.* **C.** *GHR expressing Hek293 cells were transfected with empty vector, FLAG-Jak2 Y1007F or FLAG-Jak2, stimulated for different times with 180 ng/ml GH and lysed in denaturing conditions. Phosphorylated Jak2 was immunoprecipitated with anti-pJak2 antibody. The blot was detected with anti-SUMO2/3 and anti-FLAG antibodies and is representative of three experiments.*

fig. 1A) Ubc9 silencing substantially reduced Jak2 sumoylation. However, it had no effect on GH-induced phosphorylation of Jak2. Additionally, GHR shift caused by its phosphorylation was not affected. This suggests that Jak2 sumoylation is not required for its phosphorylation and occurs downstream of phosphorylation.

To address, whether phosphorylated Jak2 is sumoylated, we transiently transfected Hek293 cells with Flag-Jak2 or Flag-Jak2 Y1007F mutant and stimulated the cells with GH for 1, 2.5 and 5 min. Subsequently, phosphorylated Jak2 was immunoprecipitated in denaturing conditions with an antibody against phosphorylated Y1007 and Y1008. Previously, it has been shown that this mutation precludes full kinase activity of Jak2 [30]. As can be seen in Fig. 7C phosphorylated Jak2 appears 20 kDa higher than non-phosphorylated Jak2. Addition of one sumo molecule increases the size of a protein approximately 20 kDa. And indeed, antibody against SUMO2/3 also recognizes the band on 140 kDa. This suggests that phosphorylated Jak2 is monosumoylated. In our case we analyze Jak2 phosphorylation and sumoylation only on Jak2 that detached from the GHR. The finding that mono-sumoylated Jak2 can be immunoprecipitated by antibodies specific for 1007pY, 1008pY indicates that activated Jak2 is a substrate for sumoylation.

Discussion

In this study we present evidence that Jak2 is modified by SUMO2/3 chains up to high molecular weight on multiple lysine residues. This modification is induced both by cytokine stimulation and by stressors. It depends on the presence of an active catalytic site in Jak2. In addition, we show that phosphorylated Jak2 can be monosumoylated by SUMO2/3. Our data indicate that there is a relation between phosphorylation, sumoylation and ubiquitylation of Jak2. Importantly, we provide evidence that sumoylation of Jak2 regulates nuclear translocation of Jak2.

The majority of sumoylated Jak2 migrates as high-molecular weight species in the gel. Usually, sumoylation of proteins generates smaller conjugates. In addition, we observe ladder characteristic for 5-8 SUMO moieties (Fig. 1C). Analysis of Jak2 lysine mutants (Fig. 2D) indicate that Jak2 is modified on several lysine residues, probably by SUMO2/3 chains. However, multiple mono-sumoylation remains possible. As Jak2 contains ~80 lysines, sumoylation of only a few would already result in the observed high molecular weight signal. Whether other modifications, such as ubiquitylation, contribute to the high molecular weight appearance remains to be determined.

Our results indicate that Jak2 sumoylation depends on the presence of an active catalytic site. Mutants lacking the kinase domain or harboring an inactive catalytic site (842stop and K882E, respectively) show a strongly decreased sumoylation signal. However, mutation of the activating tyrosine Y1007 does not prevent sumoylation. This indicates that the conformation of Jak2 is a critical factor for substrate recognition by the sumoylation machinery.

It has been shown before that Jak2 can translocate to the nucleus via a yet unknown mechanism, where it activates gene transcription [2]. For SUMO1 it has been well documented that sumoylation can be a prerequisite for nuclear translocation [19, 20]. However, the SUMO2/3 modification has not been implicated in this process. Our results show that Jak2 in the nucleus exists mainly in its sumoylated state. Additionally, we show that nuclear abundance increase with stress-induced sumoylation of Jak2

(Supplementary Fig. 1C and D). This is in line with our finding that kinase inactive mutants K882E and truncation after amino acid 842 translocate less, while constitutively active V617F is more abundant in the nuclear fraction (Fig. 6C). This is in agreement with the finding that Jak2 V617F mutant is more abundant in nucleus of hematopoietic cells [31]. It cannot be fully excluded that phosphorylation counts for this effect. However, analysis of kinase inactive Y1007F mutant that is normally sumoylated and translocates to the nucleus like the wt kinase points toward sumoylation being prerequisite for nuclear translocation. We also show that upon Ubc9 silencing there is less and upon Ubc9 or SUMO3 overexpression more sumoylated Jak2 in the nuclear fraction (Fig. 7A and B). Together, our data suggest that Jak2 sumoylation and nuclear translocation are connected and point toward sumoylation being a prerequisite for nuclear translocation. Jak2 translocation to the nucleus makes cytoplasmic pool of kinase smaller which could serve as a mechanism of inhibiting cytokine signaling. Additionally Jak2 translocation may be important for transmission of certain signals to the nucleus. We cannot fully exclude possibility that sumoylation is preventing Jak2 export from the nucleus like it is in case of nuclear pull of actin [29]. However that would imply that sumoylation of Jak2 takes place both in cytoplasm and nucleus and that physiological relevance of modification in those two compartments is different.

Serum starvation, heat shock and staurosporin treatment all increase the sumoylation of Jak2. These results show that Jak2 is sumoylated in response to stress. The general increase in SUMO2/3 modification of proteins in response to various different stresses has been reported before [32-37]. Although it seems to be a general phenomenon, specific proteins can be differently modified [38]. This common cellular response of enhanced SUMO conjugation suggests that Jak2 sumoylation might constitute a protective response. It is not yet clear why sumoylation of Jak2 would be important for a cell in such a situation. A possible scenario is that since Jak2 is an important player in activating signaling cascade leading to activation of gene transcription and cell proliferation, the tight regulation of its activity is crucial for cells. The kinase is a very stable protein with half-life more than 12 hours. Therefore sumoylation may serve as regulatory mechanism to control Jak2-induced anabolic activity in order for the cells to cope with the stressor first. Our data suggest that sumoylated Jak2 is not binding GHR therefore cannot take part in initiation of signal transduction. Keeping Jak2 in such a state would be beneficial for the cell under stress condition especially that unregulated active Jak2 leads to cancer transformation.

Additionally, we show that GH stimulation increases polysumoylation. This event probably depends on Jak2 phosphorylation. The observation that constitutively active Jak2 V617F mutant shows higher SUMO modification signal than wild type and Y1007F mutant strengthens this notion. There are several reports showing that protein phosphorylation can either enhance [12] or inhibit [13, 14] substrate sumoylation. PDSMs have been identified where phosphorylation on downstream serine residue is prerequisite for sumoylation [39-41]. Jak2 is phosphorylated mainly on tyrosines. The kinase has 49 tyrosines out of which 16 are predicted to be subject of phosphorylation [30, 42-48]. Recently, a cross talk between sumoylation and tyrosine phosphorylation have been shown in a screen [49]. It is possible that the phosphorylation-dependent sumoylation in case of tyrosines is different than for serines and the relevant residues remain to be determined.

Without any stimuli small pool of Jak2 is subjected to sumoylation. It has been shown that

Jak2 is constitutively phosphorylated on S523 that leads to inhibition of kinase activity [45, 50, 51]. This serine does not lie within PDSM motif. However, it would be interesting to test whether phosphorylation on this serine is connected with Jak2 sumoylation under basal conditions.

In our study we also provide evidence that phosphorylated Jak2 is monosumoylated by SUMO2/3. However it remains to be determined whether monosumoylated Jak2 is present already on GHR or monosumoylation appears after phosphorylation event. We have some data (data not shown) indicating that 140 kDa Jak2 exists in the cells independent of cytokine stimulation but only in cells expressing GHR. That would suggest that monosumoylation of Jak2 is regulating Jak2 accessibility for the receptor. Additionally, monosumoylation could serve as a general mechanism regulating Janus kinase family members binding to corresponding receptors possibly via mechanism affecting kinase conformation. Since GH stimulation increase Jak2 polysumoylation, mono SUMO can act as a platform for the sumo chains elongation and subsequent nuclear translocation.

The results indicate that there is a relationship between Jak2 phosphorylation, sumoylation and ubiquitylation. Many proteins have been shown to be a substrate for both SUMO and ubiquitin, often at the same lysine residues. Two modifications can act antagonistically [52] or in concert [20] to regulate the substrate protein. The most common role of protein ubiquitylation is targeting substrates to proteasomal degradation (reviewed in [53, 54]). In the case of PML it has been shown that sumoylation recruits SUMO-dependent ubiquitin ligase RNF4 which ubiquitylates SUMO chains targeting the whole complex to proteasome degradation [17]. However, in the case of Jak2 RNF4 is not the ligase responsible for its ubiquitylation (data not shown). Other data are showing that all three modifications can act in concert. For example, sumoylation of NEMO facilitates its phosphorylation in the nucleus which leads to ubiquitylation, nuclear export and activation of NEMO to full kinase in the cytoplasm [20]. Ungureanu et al [4] and Ali et al [6] provide evidence that polyubiquitylation of Jak2 increases upon cytokine stimulation and is further enhanced after treatment with proteasomal inhibitors. In addition, they show that this modification depends on Jak2 phosphorylation. Cytokine stimulation does not increase ubiquitylation of kinase inactive K882E Jak2 mutant. They conclude that phosphorylation of Jak2 serves as a signal for the ubiquitylation reaction and for the degradation of Jak2 through the proteasome pathway. Our results showing that cytokine stimulated ubiquitylation of Jak2 depends on its kinase potential corroborate aforementioned findings (Fig. 5A). Additionally we show that sumoylation may be an intermediate step further stimulating Jak2 ubiquitylation (Fig. 5B). However, our data show that K882E mutant is not binding GHR. Thus, it cannot be excluded that receptor binding is necessary for GH induced Jak2 ubiquitylation. As Jak2 is a long-lived protein, and cytokine stimulation does not seem to affect its half-life, further studies are needed to fully elucidate how those modifications together regulate Jak2 function.

The data presented here demonstrate a new posttranslational modification of Jak2 kinase that regulates its localization and presumably function. Additionally, we provide data pointing towards a relationship between other modifications of Jak2: phosphorylation and ubiquitylation. A future challenge is to investigate the interplay between those modifications in regulating the function of Jak2 in health and disease. Jak2 have been implicated in myeloproliferative disorders as well as in cancer development and progression. Since mainly nuclear pull of Jak2 acts as oncogene, interfering with nuclear

translocation would be beneficial in treatment of cancer. Thus our discovery may be valuable for the development of new therapeutic strategies.

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Supplementary data

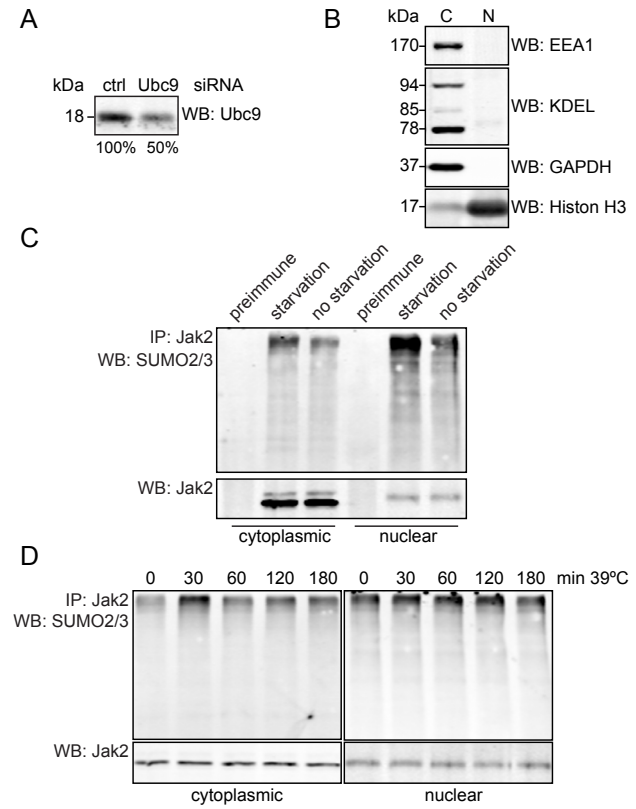

Figure S1. **A.** *Effect of Ubc9 silencing. Cells were silenced with either control (ctrl) siRNA or Ubc9-specific siRNA. The amount of Ubc9 was accessed with anti-Ubc9 antibody.* **B.** *Fractionation controls were tested with different antibodies: endosomes – EEA1, ER – KDEL, cytoplasm – GAPDH, nucleus – histonH3.* **C.** *Cells were serum starved for 4 h and cytoplasmic/nuclear fractionation followed by Jak2 immunoprecipitations in denaturing conditions was performed. The blot was analyzed with antibody against SUMO2/3 and Jak2.* **D.** *Cells were subjected to 39ºC for different time periods and cytoplasmic/nuclear fractionation followed by Jak2 immunoprecipitations in denaturing conditions was performed. The blot was analyzed with antibodies against SUMO2/3 and Jak2.*

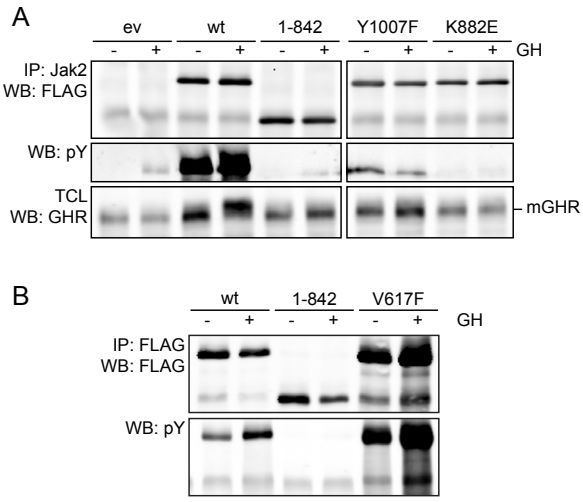

Figure S2. Phosphorylation status of Jak2 mutants.

A. *and* **B.** *Hek293 were transiently transfected with different FLAG-Jak2 plasmids (wt, 1-842, Y1007F, K882E, V617F) and anti-FLAG immunoprecipitations were performed in denaturing conditions. Phosphorylation of Jak2 mutants were assessed with anti-pY antibody.*

Table S1. Primers used for mutagenesis of FLAG-Jak2 plasmid.

K882E	F 5'-GGGGAGGTGGTCGCTGTAGAAAAGCTTCAGCATAG-3' R 5'-CTATGCTGAAGCTTTTCTACAGCGACCACCTCCCC-3'
Y1007F	F 5'-CCGCAGGACAAAGAATTCTACAAAGTAAAGGAGCC-3' R 5'-GGCTCCTTTACTTTGTAGAATTCTTTGTCCTGCGG-3'
V617F	F 5'-GAATTATGGTGTCTGTTTCTGTGGAGAGGAGAAC-3' R 5'-GTTCTCCTCTCCACAGAAACAGACACCATAATTC-3'
K167R	F 5'-CACGGATGGATAAGGGTACCTGTGACTC-3' R 5'-GAGTCACAGGTACCCTTATCCATCCGTG-3'
K630R	F 5'- GGTTCAAGAATTTGTAAGATTTGGATCACTGG – 3' R 5'- CCAGTGATCCAAATCTTACAAATTCTTGAACC – 3'
K912R	F 5'-CAACATCGTCAGGTACAAGGGAGTGTGC-3' R 5'-GCACACTCCCTTGTACCTGACGATGTTG-3'
K914R	F 5'- GCATGACAACATCGTCAAGTACCGCGGAGTGTGCTACAGTGC-3' R 5'-GCACTGTAGCACACTCCGCGGTACTTGACGATGTTGTCATGC-3'
K991R	F 5'-GGAGAACGAGAACAGAGTT CGA ATTGGAGATTTTGGGTTAACC-3' R 5'-GGTTAACCCAAAATCTCCAAT TCG AACTCTGTTCTCGTTCTCC-3'
K1011R	F 5'-GCCACAAGACAAAGAATACTATAAAGTACGTGAACCTGGTGAAAGTCCC-3' R 5'-GGGACTTTCACCAGGTTCACGTACTTTATAGTATTCTTTGTCTTGTGGC-3'
K912R/ K914R	F 5'-GCATGACAACATCGTCAGGTACCGCGGAGTGTGCTACAGTGC-3' R 5'-GCACTGTAGCACACTCCGCGGTACCTGACGATGTTGTCATGC-3'
K991R/ K1011R	Mutagenesis of K991R was performed on plasmid K1011R with K991R primers

CHAPTER 3

Analysis of GHR complexes at early stages of endocytosis

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Summary

Growth Hormone (GH) is a major regulator of growth and metabolism. GH acts via growth hormone receptor (GHR) that belongs to the class I cytokine receptors. Members of this family lack intrinsic kinase activity. To signal via the MAPK/STAT5 cascade the receptor needs Jak2 kinase. To be endocytosed and degraded both ubiquitylation by the SCF^{TrCP2} ubiquitin ligase and proteasomal activity are necessary. In order to analyze GHR complexes at early stages of signaling and endocytosis we utilized Blue Native Gel Electrophoresis (BN-PAGE) followed by second dimension SDS-PAGE (2D BN/SDS-PAGE) that allows analysis of multiprotein complexes. This method allowed us to identify two main complexes (size ~500kDa and 900kDa) of GHR present on the plasma membrane. Upon GH stimulation there is a shift from ~500kDa complex to 900kDa complex. GHR molecules within 900kDa complex are both phosphorylated and ubiquitylated. Surprisingly neither Jak2 nor βTrCP2 seem to be present in those complexes. Instead, they are in higher molecular weight complexes of GHR in a transient way. This suggests that GHR is present on the plasma membrane as a tetramer that can dimerize upon GH binding.

Introduction

Growth Hormone Receptor (GHR) is a key regulator of postnatal growth and has important actions on lipid and carbohydrate metabolism. It belongs to the class I cytokine receptors that among others include prolactin and erythropoietin receptors [1]. Members of this family lack intrinsic kinase activity. Therefore, they rely on Janus kinase family (Jaks) of non-receptor tyrosine kinases to initiate signaling [2]. Binding of Growth Hormone (GH) to GHR initiates conformational change that leads to full activation of kinase. Jak2 transphosphorylates itself and the receptor, which initiates signaling cascade mainly via STAT5/MAPK pathway.

Previously, we identified SCF$^{\beta TrCP2}$ ubiquitin ligase complex necessary for GHR endocytosis [3]. TrCP2 directly ubiquitylates the receptor (Thesis Almeida da Silva) that is subsequently internalized via clathrin-coated pits and degraded in the lysosomes [4]. GHR endocytosis depends on a functional ubiquitin system but ubiquitylation of the receptor itself is not necessary for this event [5]. Recently we have shown that another E3 ubiquitin ligase CHIP is required for GHR endocytosis (Slotman et al., submitted).

GHR is synthesized in the ER as a precursor. Here, high-mannose oligosaccharides are added and the receptor dimerizes. Next, the receptor travels to the Golgi complex where it becomes complex glycosylated and, as a mature receptor dimer, appears on the plasma membrane [6]. GHR is constitutively endocytosed in absence of GH. However,

binding of GH stimulates GHR internalization. This leads to the following sequence of events: Jak2 becomes active, phosphorylates itself and the receptor and dissociates from the GHR [7]. Next, the two ubiquitin ligases, SCF$^{\beta TrCP2}$ and CHIP/Ubc13, assemble on the receptor, and, finally, ubiquitylation of yet unidentified substrates initiate clathrin-mediated endocytosis. The relative abundance of GHR on the plasma membrane is a key determinant of cellular GH sensitivity. Thus, an understanding of receptor stability on the cell surface is critical for understanding GH action.

Multiprotein complexes (MPC) play crucial roles in nearly all cell biological processes. Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful method to analyze those complexes in native conditions [8, 9]. In this method a dye Coomassie Brilliant Blue is used that binds non-specifically to proteins without disturbing the complexes. Thus, complexes gain negative charge and can be separated according to masses on native gel. Combining BN-PAGE with second dimension SDS-PAGE (2D BN/SDS-PAGE) allows separation and analysis of proteins within the complexes according to their size, subunit composition and relative abundance.

In this study we utilized 2D BN/SDS-PAGE followed by Western blotting to study GHR at early stages of endocytosis. We show that there are mainly two GHR complexes present on the plasma membrane. Additionally, we provide evidence that receptors within the bigger complex are subjected to posttranslational modifications: phosphorylation and ubiquitylation. Using wild type and mutant GHRs we show the dynamics of the Jak2 signalosome at the cell surface and after endocytosis by analyzing GHR together with two main binding partners Jak2 and βTrCP2.

Materials and methods

Cells, plasmids, antibodies and reagents

Hek293 TR cells were described elsewhere. Hek293 cells expressing GHR were previously described (van Kerkhof et al., 2011). The FLAG-Jak2 mouse construct was a kind gift from Prof. Carter-Su. The pcDNA HA-ubiquitin, HA-ubiquitin K48R and HA-ubiquitin K63R were generous gifts of Tomoki Chiba, Tokyo. The rabbit wild type and truncated (399 and 399Kless) GHR cDNAs in pcDNA3 have been described before [5]. GHR Yless construct was described in Joyce Putter's PhD thesis. TrCP2 cDNA in pcDNA3 expressing the FLAG-tagged proteins were generous gifts of Tomoki Chiba (Tokyo Metropolitan Institute of Medical Science). Mouse monoclonal antibody against phosphorylated tyrosines (pY) was from Millipore (clone 4G10), anti-HA tag 12CA5 antibody was from Babco (Richmond CA), anti-polyubiquitin (FK2) from Enzo and antibody against FLAG tag (M2) was purchased from Sigma. Rabbit polyclonal anti-GHR B antibody was previously described elsewhere [5, 10]. Rabbit monoclonal antibody targeted against phosphorylated Y1007 and Y1008 of Jak2 (pJak2) was purchased from abcam (ab32101). The secondary antibodies Alexa Fluor 680 and IR 800-conjugated goat anti mouse and anti-rabbit IgGs were obtained from Molecular Probes. GH-(his-TEV-STREP3-his) referred in article as Strep-GH was made by U-Protein Express BV (Utrecht, The Netherlands). Culture media, fetal calf serum (FCS), L-glutamine and antibiotics for tissue culture were purchased from Invitrogen.

Cell culture and transient transfections

Hek 293 cells were cultured under standard conditions in DMEM high glucose containing 10% fetal bovine serum. GHR-expressing Hek293 cells (Hek-wt GHR) were grown in the same medium supplemented with 0.6 mg/ml Geneticin (G418; Gibco). Transfections were performed with

Fugene (Roche) according to standard conditions or with calcium phosphate method. For calcium phosphate-mediated transfections cells were grown in 10 cm dishes to 60% confluence 48 h before transfections. Next, 10 μg of DNA was mixed with 62 μl of $CaCl_2$ and TE buffer up to 500 μl in total. Solution was added drop wise to 500 μl of 2x HBS buffer (280 mM NaCl, 1.5 mM $Na_2HPO_4.H_20$, 50 mM HEPES, 10 mM KCl, 12 mM glucose). After 30 min the mixture was added to cells. After 16 h the medium was replaced by fresh medium.

STREP-GH pull down and elution for BN-PAGE

Cells were stimulated with 180 ng/ml of Strep-GH for 5-15min in a CO_2 incubator at 37ºC. Next, the cells were washed 4 times with ice-cold PBS. Cells were lysed for 30 min in lysis buffer containing 1% Triton-X100, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 1 mM Na_3VO_4 and 15 mM N-ethylmaleimide. Cells were scraped and the lysates were clarified by centrifugation for 5 min in 4ºC. Supernatants were incubated with Strep-tactin beads for 2 h at 4ºC. Complexes were eluted with buffer containing 2.5 mM desthiobiotin, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 0.5mM Na_3VO_4, and 15 mM NEM (3 times 30 min). Eluates were combined and filtered in Microcon Centrifugal Filter Devices (Millipore) and washed once with BN base buffer containing 15mM NEM in order to decrease amount of salt in the sample. Next, sample was loaded on 1st dimension BN-PAGE the same day.

Preparation of total cell lysates for BN-PAGE

Cells were washed 4 times with ice-cold PBS and lysed for 30 min in lysis buffer containing 1% Triton-X100, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 1 mM Na_3VO_4 and 15 mM N-ethylmaleimide. Cells were scraped and the lysates were clarified by centrifugation for 5 min in 4ºC. Supernatants were filtered in Microcon Centrifugal Filter Devices (Millipore) and washed once with BN base buffer containing 15mM NEM in order to decrease amount of salt in the sample. Next, sample was loaded on 1st dimension BN-PAGE the same day.

2D BN/SDS-PAGE, transfer and western blotting

NativePAGE Bis-Tris Gel System was purchased from Invitrogen. Samples were loaded into dry wells on 3%-12% gels and run for 16 h in 4ºC at 2mA. Native Protein Markers (Invitrogen) were used to standardize complex sizes. After first dimension BN-PAGE, gel slices were prepared for every sample and put into 15 ml tubes containing 10 ml sample buffer (12.5 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue) and 2.5% β-mercaptoethanol. Slices were incubated for 30 min at 70ºC in the water bath. Next, the gel slices were loaded on 1,5 mm-thick SDS-PAGE gels. Gels were run at 10 mA per each gel. Gel slice with the marker was stained with Coomassie brillant blue R250. Gels were wet transferred to Immobilon-FL polyvinylidenedifluoride membrane (Millipore) with 0.8 mA per Criterion system for 60 min. Blots were immunostained with the indicated primary antibodies followed by Alexa Fluor 680, Alexa-800 IRDye conjugated anti-mouse or anti-rabbit antibodies. Detection was performed with an Odyssey system (LI-COR Biosciences).

Proteinase K treatment of cells

To degrade the extracellular domain of the GHR cells were washed twice with ice-cold PBS and treated with proteinase K (0.5 mg/ml) in PBS/1 mM EDTA for 30 min on ice and transferred to tubes. The digestion was terminated with PBS with 2 mM phenylmethylsulfonyl fluoride (PMSF) and the detached cells were centrifuged for 5 min at 300g at 4 °C, washed three times with PBS containing 1 mM PMSF and lysed according to *Preparation of total cell lysates for BN-PAGE* section.

Results

There are mainly two complexes present on the plasma membrane

We analyzed GHR complexes from GHR-expressing Hek293, both unstimulated and after stimulation and isolation with Strep-GH by 2D BN/SDS-PAGE (Fig. 1A). In this method, Coomassie G250 is used that unspecifically binds to all proteins without denaturation, which enables separation of intact protein complexes according to their masses. Several distinct GHR complexes can be seen in total cell lysate sample corresponding to both mature and immature GHR complexes reflecting the steady state situation in the cell (Fig. 1A, panel 1). Immature GHR complexes start at ~100 kDa, which probably correspond to monomeric immature receptor molecules (spot 1). Spot 2 presumably contains dimeric folding intermediates. Based on our previous studies that show that dimerization and folding occurs within 1-2 minutes after termination of translation [6], it is likely that the majority of immature complexes that ranges from ~200 kDa up to a few thousands kDa represent properly folded multimeric, high-mannose GHRs, ready to leave the ER. The discrete spot 3 represents mature GHR of an estimated size of a tetramer. At steady state, without GH, the majority of mature GHR is in complexes ranging from ~500-2000 kDa (spot 4). Previously, we have shown that at steady state, the majority of mature GHR resides at the cell surface [11]. In Fig. 1A, panel 2, the cells were stimulated with Strep-GH for 10 min, GHRs were isolated on streptavidin beads, eluted with desthiobiotin, and analyzed on 2D BN/SDS-PAGE. Under these conditions the majority of the GH-bound receptor has entered the cell via clathrin-mediated endocytosis [12]. Two main GHR complexes can be distinguished, a 500 kDa (spot 5) and a 900kDa (spot 7). Minor complexes migrated between the two main complexes (spot 6) and at ~1200 kDa (spot 8). In order to confirm that at steady state the mature complexes are indeed present on the cell surface, we treated wild type (GHR wt) and endocytosis-defective GHR F327A mutant cells with proteinase K on ice (depicted in supplementary Fig. 1A). GHR F327A mutant has a crucial phenylalanine within UbE motif, required for endocytosis, mutated to alanine which precludes endocytosis [3]. Proteinase K removes the extracellular part of GHR, thereby leaving the transmembrane and cytosolic domains intact. While the ER species remained intact, in both cell lines the mature receptor complexes shifted to lower molecular weights, almost completely (Fig. 1B, dashed boxes). Remarkably, the shift in the first dimension was minor, indicating that the composition of the complexes remained the same. We conclude that at steady state most of the GHR complexes reside at the plasma membrane and that the extracellular domain has no part in the composition of the complexes.

Covalent posttranslational modifications of the GHR complexes

Our previous studies have shown that GH stimulation accelerates GHR endocytosis [7]. Upon GH binding the GHR is phosphorylated by Jak2 and ubiquitylated by the $SCF^{\beta TrCP2}$ ubiquitin ligase. Therefore, we asked what happens to the 4 complexes, isolated via Strep-GH depicted in Fig. 1A, panel 2. Analyses with antibodies recognizing phosphorylated tyrosines (pY) or ubiquitin (ubi) shows that only proteins present or derived from spot 8 were either phosphorylated or ubiquitylated. Because the molecular mass of Jak2 is close to that of GHR, we cannot exclude that the pY signal partially originates from Jak2, although our previous finding that phosphorylated Jak2 detaches from its receptor renders

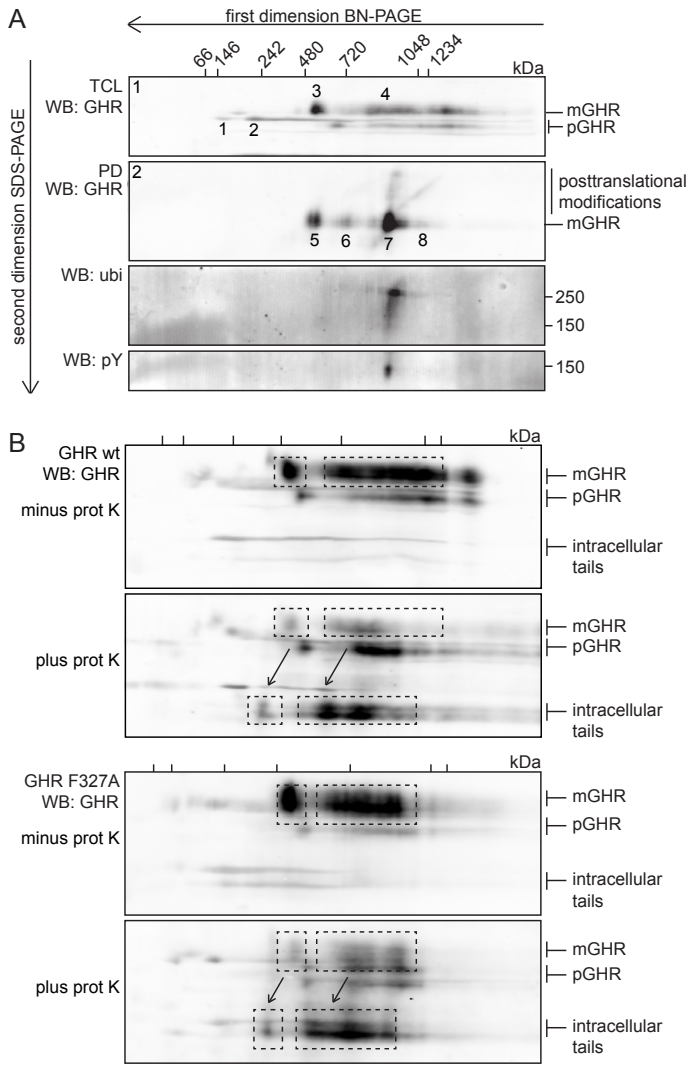

Figure 1. Analysis of GHR complexes present inside the cell and on the cell surface. *All samples were lysed in native conditions and subjected to 2D BN/SDS-PAGE. Gels were immunoblotted and analyzed with specific antibodies.* **A.** *GHR-expressing Hek293 cells were either stimulated for 10 min with Strep-GH, and GHR complexes were pulled down and eluted in native conditions (lower panel - PD) or total cell lysates were analyzed (upper panel - TCL).* **B.** *Hek293 cells expressing GHR wt (upper part) or GHR F327A mutant (lower part) were pre-treated or not with proteinase K. Dashed boxes are explained in the text.*

this unlikely [7]. The smear originating at the GHR position upwards (spot 8) very likely represents ubiquitylated GHR as both staining patterns overlap. The nature of diagonal modification is unknown. However, as its molecular weight increases equally in both dimensions it may represent multimeric, covalently linked GHRs that are insensitive to reducing conditions [13]. As signal for phosphorylation and ubiquitylation only partially overlap in lower parts of blot, ubiquitylated receptor high up in the gel seems not to be phosphorylated. According to Putters et al. [7] phosphorylation of the receptor by Jak2 precedes its ubiquitylation. This suggests that the receptor is first phosphorylated, then ubiquitylated. Our results are consistent with that finding.

GHR is subjected to dynamic changes upon GH binding

As we concluded from the distribution of mature GHR complexes in the lysate at steady state (Fig. 1A), except for spot 3, GHR occurs in multiple complexes ranging from 500 to

more than 2000 kDa. In order to analyze the effect of GH binding and receptor activation we compared GHR complexes at the plasma membrane, with the situation after stimulation for different periods of times. In Fig. 2A Strep-GH was allowed to bind for 2 h on ice, the complexes were isolated and analyzed for GHR, ubiquitin and pY. The differences with the steady state situation are striking. While spot 5 kept its appearance, upon GH binding, the steady state distribution, seen in Fig. 1A, spot 4, completely changed into a single GHR complex of 900 kDa (spot 7 from Fig. 1A). Thus, GH binding to GHRs at 0ºC seems to induce complexes of uniform size. Obviously, this complex showed neither phosphorylation nor ubiquitylation (data not shown). Next, we incubated the cells with Strep-GH for 5, 10 and 15 min (Fig. 2B-D) and analyzed the complexes. GH treatment caused increasing ubiquitylation that was maximal after 10 min, while pY labeling was induced and the signal remained steady over the incubation period. Remarkably, the 500 kDa complex depicted in dashed boxes (spot 5 from Fig. 1A) gradually disappeared.

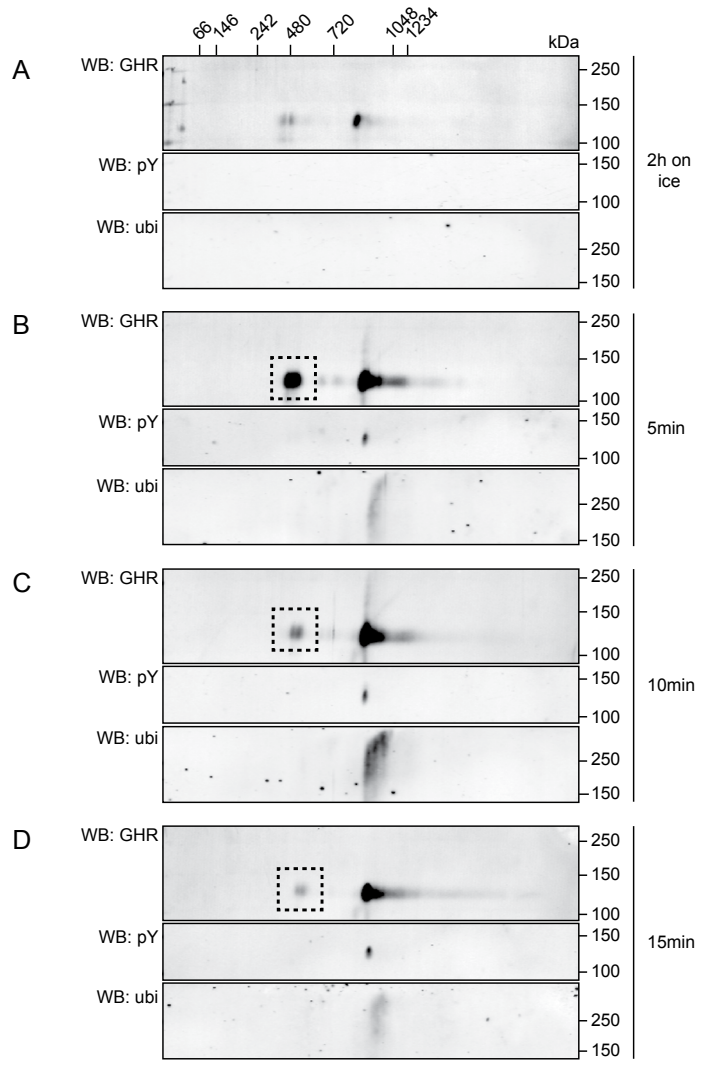

Figure 2. GH stimulation induces GHR complex shift. *GHR-expressing Hek293 cells were kept on ice for 2 h (**A**) or stimulated for 5 min (**B**), 10 min (**C**) or 15 min (**D**) in 37˚C, with Strep-GH. GHR complexes were pulled down and eluted in native conditions. Samples were subjected to 2D BN/SDS-PAGE. Gels were immunoblotted and analyzed with specific antibodies. Dashed boxes are explained in the text.*

Since in total cell lysates, treated for 0, 5 and 10 min with Strep-GH, 900kDa complex increased in size (Supplementary figure 1B, dashed boxes), we conclude that 500 kDa complex is converted into the 900 kDa complex. As the majority of GHRs enters the cells within 10 min of GH addition, most of the GHR complexes, depicted in Fig. 2C and 2D, must have entered the cells. Whether this also applies for the ubiquitylated species, remains to be determined.

Analysis of GHR-βTrCP2 complexes

The $SCF^{\beta TrCP2}$ ubiquitin ligase complex is responsible for ubiquitylation and subsequent endocytosis of the GHR. The ligase acts as a complex of βTrCP2, Skp1, Cul1 (modified with Nedd8), Rbx1, UbcH7 as E2, with a total molecular mass of approximately 200 kDa. Based on structural studies it might act as a dimer [14-16]. A substrate binding component of the ligase, βTrCP2, binds to the UbE motif of the GHR [3]. To investigate whether it binds as a dimer to the dimeric tails of the GHR, we transfected GHR-expressing Hek293 cells with FLAG-TrCP2, and analyzed the GHR complexes on 2D BN/SDS-PAGE. Fig. 3A shows the complexes in steady state in total cell lysates: The majority of βTrCP2 migrated in complexes of molecular masses of more than 10^6. As we overexpressed only one factor of the SCF E3 it is unlikely that the βTrCP2 label is in SCF complexes. However, as a presumed dimer, it might associate with many different substrates both in the cytosol and in the nucleus, explaining the heterogeneity of the βTrCP2 containing complexes. Analysis of GHR complexes showed that βTrCP2 did not occur in the 900 kDa complex and only colocalized with receptors in complexes of molecular weight above 1000 kDa (Fig. 3B). This suggests that GHR associate with SCF complex transiently creating large complexes. Apparently, ubiquitylation leads to dissociation from the receptor complex. This would explain why ubiquitylated GHR is only present in the 900 kDa complex.

In order to further analyze the mechanism of GHR ubiquitylation, we used our GHR truncation mutant that has all lysine residues mutated into arginines (GHR399Kless, supplementary Fig. 1A) [5]. As a control, we used GHR truncated after amino acid 399 (GHR399, Supplementary Fig. 1A). GHR complexes were isolated from transiently transfected Hek293 cells after 10 min of incubation with Strep-GH. GHR399 and 399Kless-containing complexes showed similar patterns as complexes containing full length GHR (Fig. 3C compared to Fig. 1A). Interestingly, the size of the complexes in the first dimension was approximately half the size as full-length GHR, 250 and 500-kDa, respectively. While the 500 kDa GHR399 complexes are clearly ubiquitylated, the ubiquitylated state of the 250-kDa complex is less clear. Since 399Kless is not ubiquitylated the residual ubiquitylation signal probably comes from other associated molecules, e.g. Jak2. In the GHR complexes above 500 kDa (continuous boxes) GHR399Kless is much more abundant than GHR399. The mutant cannot be ubiquitylated but it can still associate with βTrCP2 via the intact UbE motif. That would imply that Kless binding to $SCF^{\beta TrCP2}$ is prolonged supporting previous results. Additionally, above the 399 but also 399Kless 550kDa complex, there is a clear diagonal smear (Fig. 4C, dashed boxes) corresponding to unknown GHR modification. As mentioned before, this smear may represent multimeric, covalently linked GHRs that are insensitive to reducing conditions [13]. However, it may also be novel yet to be identified GHR posttranslational modification. Since 399Kless does not have lysines to be modified this modification is lysine-independent.

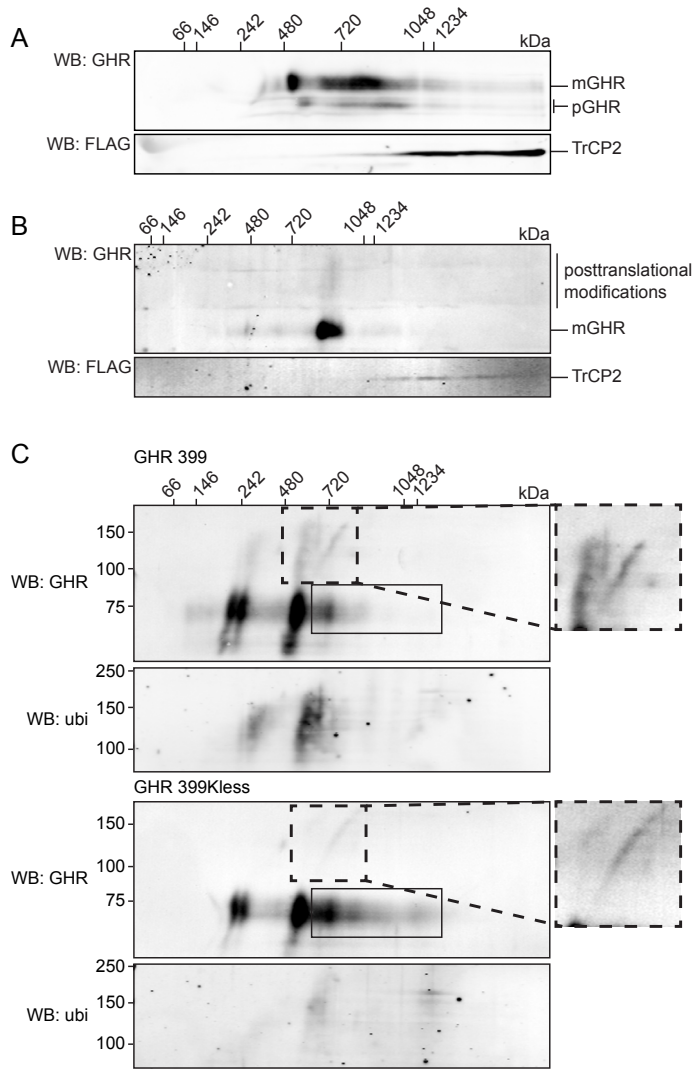

Figure 3. Analysis of GHR-βTrCP2 complexes.
All samples were lysed in native conditions and subjected to 2D BN/SDS-PAGE. Gels were blotted and analyzed with specific antibodies. **A.** *GHR-expressing Hek293 cells were transiently transfected with FLAG-βTrCP2.* **B.** *GHR-expressing Hek293 cells were transiently transfected with FLAG-βTrCP2 and stimulated for 15 min with Strep-GH. GHR complexes were pulled down and eluted in native conditions.* **C.** *Hek293 were transfected with GHR truncated after amino acid 399 (GHR 399) or truncation with all lysines mutated to arginines (GHR 399Kless). Cells were stimulated for 10 min with Strep-GH. GHR complexes were pulled down and eluted in native conditions. Continuous boxes show GHR complexes and dashed boxes show modifications of GHR (explanation in text).*

Both K48 and K63 linked ubiquitylation are involved in GHR endocytosis

It has been shown before that GHR is subjected to 48K-linked ubiquitin chains [7]. Therefore, we analyzed the effects of overexpressing two important ubiquitin mutants: ubiquitin K48R that precludes the formation of K48 poly-ubiquitin chains and ubiquitin K63R that prevents K63-linked ubiquitylation. GHR-expressing Hek293 were transiently transfected with wild type HA-ubiquitin, K48R, and K63R mutants. Cells were stimulated for 10 min with Strep-GH, and the isolated complexes were analyzed on 2D BN/SDS-PAGE. Analysis with anti-GHR antibody shows that receptor can be modified by both types of chains as overexpression of K48 mutant caused a clear reduction in polyubiquitylation of a slightly bigger complex than the 900 kDa complex, while, if ubiquitin K63R was overexpressed, ubiquitylation of the 900 kDa complex itself was diminished (Fig. 4). Assuming that both polyubiquitin chains originate from GHR, we conclude that both K48 and K63 linked polyubiquitin chains can be attached to GHR.

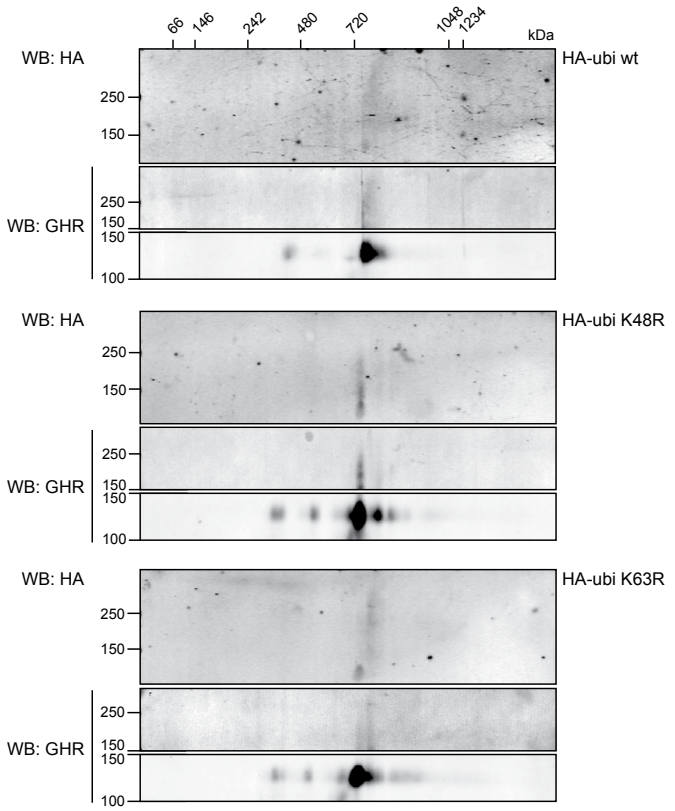

Figure 4. Both K48- and K63-linked ubiquitylation is involved in GHR endocytosis.

GHR expressing Hek293 cells were transiently transfected with HA-ubiquitin wt, K48R or K63R DNA. Cells were stimulated for 10 min with Strep-GH. GHR complexes were pulled down and eluted in native conditions. Samples were subjected to 2D BN/SDS-PAGE. Gels were Western blotted and analyzed with specific antibodies.

Although GHR, devoid of lysine residues, still requires an active ubiquitylation system for its endocytosis, our data imply that this process needs both a K48- and a K63 ubiquitin-specific ligase. This is in line with our recent finding that, in addition to $SCF^{\beta TrCP2}$ also CHIP and Ubc13 are necessary for GHR endocytosis (Slotman et al., submitted).

Analysis of GHR-Jak2 complexes

GHR does not have intrinsic kinase activity. Instead, it conducts signaling via association with the non-receptor tyrosine kinase, Jak2. To analyze the involvement of Jak2 in the GHR complexes GHR-expressing Hek293 cell were transiently transfected with FLAG-Jak2, and total cell lysates as well as GHR complexes were analyzed on 2D BN/SDS-PAGE. As seen in Fig. 5A, Jak2 mostly occurred in high molecular weight complexes partly overlapping with the 900-kDa GHR complexes (Fig. 5A, dashed box). However, in GHR complexes isolated with Strep-GH Jak2 was not detectable (Fig. 5B). If the complexes were analyzed in one dimension in the presence of SDS a small amount of Jak2 was associated with GHR both after incubation with Strep-GH at 0º and 37ºC (Fig. 5C). Previously, we showed that, upon GH stimulation, Jak2 detaches from the GHR due to phosphorylation on Y119 in Jak2 [7]. This predicts that only a small amount of Jak2 is bound to GHR at any given time confirming that the interaction between GHR and Jak2 is a transient event. We conclude that, given the size of Jak2, it is unlikely that Jak2 is part of the 900 kDa GHR complex. We hypothesize that Jak2 and $SCF^{\beta TrCP2}$ are

transiently present in the GHR receptor complexes above 1000 kDa and that the resulting ubiquitylated and phosphorylated GHR molecules are in the 900 kDa complexes.

To further investigate the association between GHR and Jak2, we co-transfected cells with FLAG-Jak2 and either wild type GHR or a mutant GHR that had all tyrosines mutated into phenylalanines (GHR-Yless, Supplementary Fig. 1A) (Thesis Putters). The rationale for this experiment came from the assumption that Jak2 detaches from phosphorylated GHRs. Cells were stimulated for 15 min with Strep-GH, and the GHR complexes were isolated and analyzed. In case of wild type GHR, only the 900 kDa GHR complex was detectable because the transition from small to big already took place (Fig. 5D, dashed boxes; compare to Fig. 2). In case of Yless, the transition was clearly blocked, suggesting that not only GH binding (as shown in Fig. 2B) but also Jak2-driven phosphorylation of the receptor is necessary for this event. Obviously, the pY staining is different. While

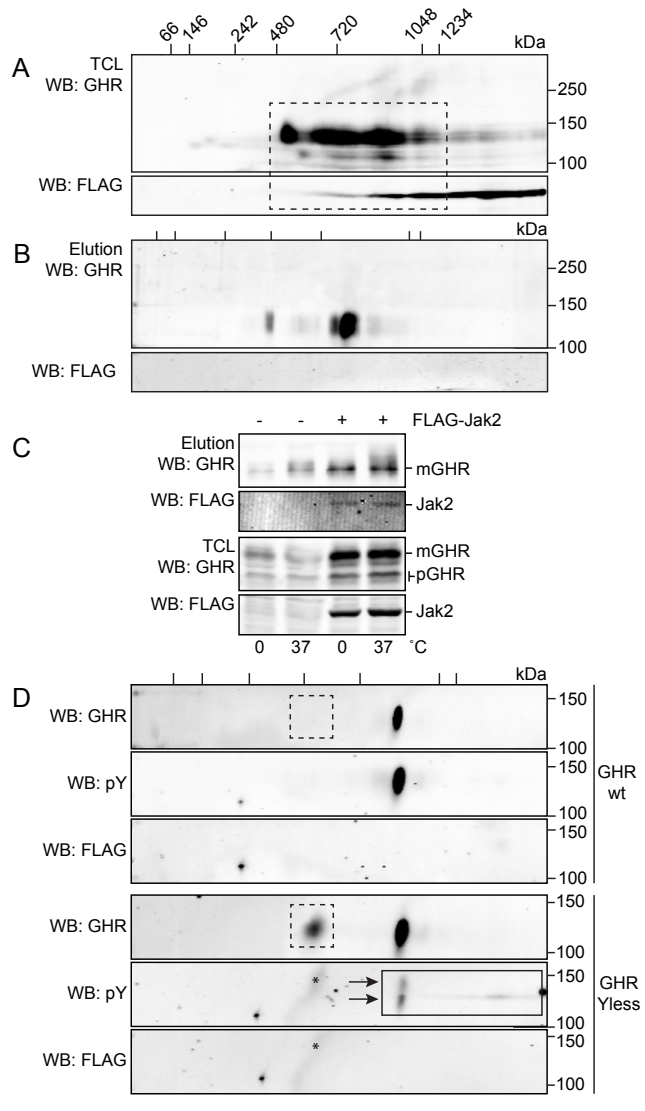

Figure 5. Analysis of GHR-Jak2 complexes.

A. *GHR-expressing Hek293 cells were transiently transfected with FLAG-Jak2.* **B.** *GHR-expressing Hek293 cells were transiently transfected with FLAG-Jak2 and stimulated for 15 min with Strep-GH.* **C.** *GHR-expressing Hek293 cells were transiently transfected with FLAG-Jak2, kept on ice for 2 h (0˚C lanes) or stimulated for 15 min in 37˚C (37˚C lanes) with Strep-GH and the complexes were subjected to 1D SDS-PAGE and immunoblotting.* **D.** *Hek293 cells were transfected with GHR wild type or GHR Yless mutant that has all the lysines mutated to phenylalanines and stimulated for 15 min with Strep-GH. Samples from parts* **A**, **B** *and* **D** *were lysed in native conditions, the complexes were isolated and subjected to 2D BN/SDS-PAGE. Gels were immnunoblotted and analyzed with specific antibodies. Continuous and dashed boxes are explained in the text. Stars indicate background smear.*

the wild type 900 kDa GHR complex is phosphorylated as in Fig. 2, the Yless mutant cannot be phosphorylated. All the pY label must be assigned to co-eluting factors. As the pY label runs exactly at 120 kDa, the band (continuous box, lower arrow) presumably represents Jak2. The Strep-GH pulled fraction, stained with anti-pY, contained a second band in the 900 kDa complex of approximately 140 kDa (continuous box, upper arrow). Based on our study on Jak2 sumoylation this pY-positive band may represent mono-sumoylated Jak2 (Sedek and Strous, in preparation). The pY-positive smear in complexes above 1500 kDa complexes contained a protein of the size of Jak2 (120 kDa, continuous box, lower arrow). The results indicate that not only phosphorylation of Y119 of Jak2 is necessary for its detachment from GHR but also phosphorylation of the receptor itself takes part in that event.

Discussion

In this study we analyzed GHR complexes at the early stages of endocytosis with a novel method, 2D BN/SDS-PAGE. We have identified two main GHR complexes size around 500 kDa and 900 kDa. Upon GH stimulation, there is a phosphorylation-dependent transition from 500 kDa to 900 kDa complexes. Additionally, we show that both Jak2 and TrCP2 are only present in very high molecular weight complexes (> 10^6 kDa), confirming that the interaction between those proteins and GHR is transient. The 900 kDa complex is both ubiquitylated and phosphorylated. Therefore, we conclude that the 500 kDa complex represents GHR tetramers, while the 900 kDa complex contains octameric GHR with no ancillary proteins attached. This conclusion is confirmed by the finding that a C-terminally truncated GHR yields comparable complexes as depicted in Fig. 3C.

It has been shown before that GHR on the plasma membrane appears as a dimer [6]. In unstimulated cells GHR complexes range from 100 kDa to more than 1000 kDa (Fig. 1A). Mature GHR complexes represent receptors trafficking from plasma membrane to lysosomes. Immature GHR complexes represent receptors trafficking from ER to plasma membrane. As can be seen there are many different transient GHR complexes both mature and immature. Stimulation and isolation with Strep-GH allowed us to elute GHR complexes in native conditions from different locations and stages of signaling. Analysis of dynamic changes on GHR composition upon GH stimulation revealed that there is a transition from 500 kDa complex to 900 kDa complex while endocytosis progresses (Fig. 2). That suggests that both GH binding and Jak2 activity are required for catalyzing the dimerization of tetrameric GHR complexes. At the same time, the heterogeneous high molecular weight (> 10^6 kDa) complexes disappeared (Supplementary Fig. 1B). As the isolated complexes only contain discrete 500 and 900 kDa complexes, even after pull-down from cells kept on ice, at first sight, the formation of the 900 kDa-complex does not require kinase activity. However, dimerization of the 500 kDa complex depends on tyrosine phosphorylation, as is anticipated from the experiment in Fig. 4D, where we show that a mutant GHR in which all cytosolic tyrosine residues were replaced by phenylalanine was unable to dimerize. Thus, mechanistically, GH binding and tyrosine phosphorylation are in the same line. It is unclear how these two events are involved in the dimerization of the 500 kDa complex.

At present, the cellular location of the complexes remains uncertain. Fig. 2A, together with the results of Fig. 1B in which endocytosis-deficient GHRs were used, suggests that

complex formation and dimerization occurs at the cell surface. If cells are incubated with cy3-GH or ^{125}I-GH, 50% of the label is internalized within 15 min [5, 12]. Therefore, it is likely that most of the GHR complexes, isolated after incubation with Strep-GH for 5-15 min, are inside the cells at the moment of cell lysis. In the absence of GH, GHR is constitutively endocytosed and uses identical ubiquitylation factors and routes as GH-bound GHR [7]. The experiments with proteinase K clearly show that most of the GHRs, present in the various GHR complexes, reside at the cell surface. It remains to be determined in which configuration complexes that are devoid of GH are endocytosed.

The mode of Jak2 action on cytokine receptors remains enigmatic. Our data show that Jak2 occurs in large complexes, ranging from 700 up to several thousand kDa. However, even at high expression levels of both the GHR and FLAG-Jak2, we were unable to pull-down Jak2-containing wild type GHR complexes. As both the GHR and Jak2 were abundantly phosphorylated, it indicates that the Jak2-GHR interaction is transient. Previously, we reported, in a γ2A cell line expressing both low amounts of Jak2 and a mutant GHR, in which all cytosolic tyrosine residues were replaced by phenylalanine, (GHR Yless) that mutation of all tyrosines did not affect GH induced GHR endocytosis [7]. To explore this further, we analyzed these complexes on 2D BN/SDS-PAGE. The immediate observation in this experiment was that complex dimerization did not happen. As we reasoned above, dimerized complexes are most likely endocytosis-competent. As GHR Yless shows decreased dimerization, and at the same time, this mutant endocytose normally, it is likely that also the 500-kDa GHR complex can endocytose. Additionally the data suggest that GHR phosphorylation is important for the transition event.

Interestingly, analysis of GHR Yless complexes with anti-pY antibody revealed a band corresponding in size to Jak2 (Fig. 5D). This result indicates that phosphorylated Jak2 can still bind to 900 kDa and bigger complexes. It has been shown before that phosphorylated Jak2 detaches from the receptor [7] and phosphorylation of Jak2 Y119 is important for this event. However our study imply that phosphorylation of the receptor itself is also important for kinase detachment from GHR. Binding of Jak2 to GHR has been shown to block receptor internalization [7, 17]. However, GHR Yless is normally endocytosed [7]. That would mean that attachment of phosphorylated Jak2 in this case is not blocking the progression of endocytosis. Additionally, the GHRYless complex does not increase in size due to pJak2 binding. It is possible that there are only few Jak2 molecules still binding which goes in line with the fact that Jak2 itself is under detection. This indicates that only one (phosphorylated) Jak2 molecules binds to the octameric GHR receptor complex.

The experiment with GHRYless revealed another striking result: The 900-kDa GHR complex contained two pY-positive factors in the size of Jak2. The size difference is approximately 20 kDa, indicative of either di-ubiquitin or mono-sumo. We have shown before that phosphorylated Jak2 is monosumoylated (Sedek and Strous, in preparation). However whether monosumoylated Jak2 is present on the receptor was not resolved yet. Whether the upper band indeed corresponds to monosumoylated Jak2 in complex with octameric GHR remains to be determined.

Analysis of 399Kless mutant revealed that GHR complexes above 500 kDa (900 kDa in case of full length) are more abundant (Fig. 3C, red boxes). 399Kless mutant cannot be ubiquitylated. However the truncation is able to bind βTrCP2 because it contains UbE motif. Therefore, we speculate that in case of the lysine-less mutant the association with $SCF^{\beta TrCP2}$ components is prolonged. Apart from that, analysis of both βTrCP2-GHR and

Jak2-GHR complexes shows that the interaction with the GHR via Box1 for Jak2 and via the UbE motif for βTrCP2 does not elucidate whether both molecules can bind at the same time. Inferred from the resulting posttranslational modifications, only the octameric GHR complex can serve as a substrate for both the kinase and ubiquitin ligase. The action of ubiquitylating enzymes has been a major subject in the group for many years. K48-specific ubiquitylation by $SCF^{\beta TrCP}$ is required for GHR endocytosis [3]. Recently, we discovered that also K63 ubiquitylation occurred, presumably by the activity of Ubc13 in collaboration with the ubiquitin ligase, CHIP (Slotman et al., submitted). The experiment of Fig. 4 reveals that the two types of ubiquitin chains occur in different octameric GHR complexes. Assuming that the exogenously expressed ubiquitin mutants dominated endogenous ubiquitin, overexpression of K48 mutant caused a clear reduction in length of polyubiquitin chains confirming previous results that GHR is modified with K48 lined ubiquitin chains [7]. The K63-linked complexes were slightly bigger than the K48-containing complexes. As the latter bear shorter ubiquitin chains, the ubiquitin load of the GHRs may contribute to their complex sizes. The experiment does not allow conclusions, whether both ubiquitin chain types are present on the same GHR molecules, neither is it clear whether GHR can be provided with K63 linked ubiquitin chains *in vivo* but we have showed that both TrCP2 and CHIP are able to ubiquitylate GHR *in vitro* (Thesis Nespital). Exact involvement of K63 linked chains in GHR endocytosis progression remains to be determined in the future.

In this study we showed that the GHR resides at the plasma membrane both as a tetrameric and an octameric complex. Upon GH binding there is a transition from a smaller to larger complex, the latter being subjected to phosphorylation and ubiquitylation. We speculate that this is due to dimerization of the GHR tetramer. However, neither Jak2 nor βTrCP2 are present in those complexes confirming that interaction between GHR and the enzymes is transient. Additionally, the data show that GHR presumably together with GH are sufficient to cause the multimerization of GHR. Therefore, use of 2D BN/SDS-PAGE allowed us to acquire detailed insight into the activity and arrangement of GHR complexes at early stages of endocytosis.

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Supplementary data

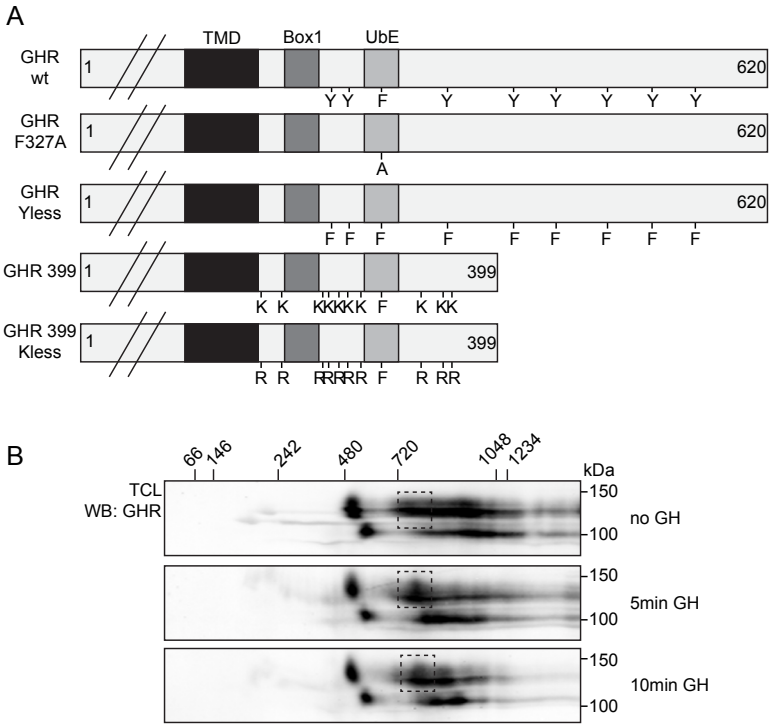

Figure S1. **A.** *Schematic representation of GHR wild type and mutants used in this study. GHR F327A has a crucial phenylalanine within UbE motif mutated to alanine. GHR Yless has all tyrosines mutated to phenylalanines. GHR 399 Kless has all lysines mutated to arginines. TMD – transmembrane domain, Box1 –Jak2 binding site, UbE – βTrCP2 binding site.* **B.** *GHR-expressing Hek293 cells were transiently transfected with FLAG-Jak2 and stimulated for 0, 5 or 10 min with Strep-GH. Cells were lysed in native conditions and subjected to 2D BN/SDS-PAGE. Gels were Western blotted and analyzed with specific antibodies.*

CHAPTER 4

Blue native gel electrophoresis:
A powerful tool for the analysis of posttranslational modifications of growth hormone receptor and Wnt-related protein complexes

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Summary

The definition of structure and function of proteins, genes, membranes and organelles have increased our knowledge of cellular life enormously. However, it has become clear that numerous protein complexes accomplish the real work in the cell. They are dynamic in composition and versatile in function. Therefore, there is growing demand for effective techniques that allow study of the functions of these complex machineries at the highest sensitivity. Functional studies of protein complexes derived from both the cytosol and cellular membrane fractions are essential to understand signaling events both from the cell surface and within the cells. The potential of Blue Native Electrophoresis is illustrated here by applying the technique on two important signaling systems: The growth hormone receptor (GHR), a prototype of class I cytokine receptor and the Wnt/β-catenin signaling pathway. Our experiments show that combining powerful isolation techniques combined with various experimental conditions allow in depth study of signaling and membrane transport systems.

Introduction

Functional studies of protein complexes derived from both the cytosol and membrane fractions are essential to understand signaling events both from the cell surface and within the cells. There are many ways to investigate protein complexes, each of which has its own advantages. Powerful screening tools like the yeast two-hybrid system enabling the identification of low-affinity protein-protein interactions, co-immunoprecipitations to probe conformation-specific structures using specific antibodies, BRET/FRET fluorescence studies for functional studies *in vivo*, all have contributed to our knowledge about the function of protein complexes. Critical parameters include sensitivity, specificity, composition and dynamics. Two-dimensional blue native electrophoresis (BN-PAGE) is a powerful separation method to study protein complexes in native state [1] and meets these parameters to a great extent. It allows the determination of the size, the relative abundance and the subunit composition of multi-protein complexes. Depending on the conditions, even fragile enzyme complexes or supercomplexes combining different enzymatic activities migrate as structural entities [2-4]. An important advantage over other techniques to study native protein complexes is that BN-PAGE allows the analysis of extremely hydrophobic protein complexes, like membrane complexes. BN-PAGE is based on the mild reagent Coomassie G250, which is non-denaturing and preserves the structure of the protein complexes. It binds nonspecifically to all proteins, gives them a negative charge and allows the separation of the complexes on a polyacrylamide gel [5]. BN-PAGE can be combined with SDS-PAGE in a two dimensional manner (2D BN/SDS-PAGE). In the first dimension BN-PAGE separates the native complexes. Second

dimension SDS-PAGE allows the separation and identification of the individual proteins of the complex. Combined with specific and powerful detection methods using antibodies, complex composition and posttranslational modifications such as phosphorylation, ubiquitylation, and sumoylation can be measured in a quantitative way as a function of time following signals and stressors. Gene silencing and expression of dominant negative factors increase the potential of the BN-PAGE to solve basic questions in cell biology. In addition, integral protein complexes can be affinity purified by using the combination of antibodies and specific protein-tags that allow gentle elution. Best known are anti-flag tag and anti-phosphotyrosine antibodies that can be detached using flag peptides and phenylphospate, respectively [4].

Among others, BN-PAGE analyses were previously applied on the complexity of aquaporin 4 [6], the AMPA receptor, that revealed stargazin as an AMPA receptor auxiliary subunit [7], presinilin-associated factors [8], high-molecular-mass complexes of the insulin receptor substrates that modulate the insulin/insulin-like growth factor-I receptor tyrosine kinases [9], glucocorticoid receptor interacting proteins [10], oxidative phosphorylation enzymes in Parkinson's disease [11], various mitochondrial complexes [2, 12] and photosynthetic complexes [13]. The potential of BN-PAGE challenged us to develop methods to monitor changes in the quantity and subunit composition of protein complexes involved in two important signaling systems: The growth hormone receptor (GHR), prototype of class I cytokine receptors [14-16] and the Wnt/β-catenin signaling pathway, critical in tissue patterning, development and adult tissue homeostasis, and frequently misregulated in cancer [17, 18]. Our experiments illustrate signaling and trafficking events of the GH receptor as well as the distribution and implications of β-catenin pools at the plasma membrane and in the cytoplasm.

Materials and Methods

Cells, DNA constructs and materials

DNA constructs: GH-(his-TEV-STREP3-his)C DNA was made by U-Protein Express BV (Utrecht, The Netherlands). Hek293 cells expressing GHR were previously described (van Kerkhof et al., 2011). TrCP2 cDNA in pcDNA3, expressing the FLAG-tagged proteins, were generous gifts of Tomoki Chiba (Tokyo Metropolitan Institute of Medical Science). Full-length rabbit GHRcDNA in pcDNA3 was described [19]Utrecht University, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Antibody (mAb 5) against the extracellular domain of the GHR was from Agen (Acacia Ridge, Australia), goat anti-mouse IgG Alexa680 was from Molecular Probes, Inc., and goat anti-rabbit IgG IRDye800 was from Rockland Immunochemicals Inc. (Gilbertsville, PA). Mouse monoclonal anti-FLAG (M2) was from Sigma. The polyclonal anti-βTrCP2 serum was raised in rabbits against a GST fusion proteins of residues 1–68 of βTrCP2α as described [20]. Culture media, fetal calf serum, L-glutamine, and antibiotics for tissue culture were purchased from Invitrogen.

STREP-tagged GH production

Hek293E cells were transfected in 1 L, 10^6 cells/mL, with GH-(his-TEV-STREP3-his)C DNA in 100% FreeStyle medium containing 0.9% primatone, 0.02% FCS, 1.35 ml/mg/cm polyethylenimine (PEI). After 3 days the medium was harvested by centrifugation (12 min, 1000g) and a 200 µl sample was stored (sample 1). The supernatant was concentrated using Quixstand apparatus with a 10 kDa hollow fiber cartridge to about 250 ml. Next, the concentrated medium was diafiltrated against 1 L 25 mM Tris-HCl, 500 mM NaCl, pH 8.2. The concentrated and diafiltrated medium (170 ml) was

filtered over a glass syringe filter and a sample was stored for analysis (sample 2). GH-(his-TEV-STREP3-his)C was bound to about 10 ml Strep-tactin Sepharose (GE) (3 h, 11ºC). STREP-tagged GH was affinity purified on Streptactin-sepharose beads. The beads were equilibrated in 25 mM Tris-HCl, 500 mM NaCl, pH 8.2. After binding the beads were packed into an empty XK16 column that was connected to an Akta-Explorer. The column was washed with equilibration buffer, and with PBS, GH-(his-TEV-STREP3-his)C was eluted with elution buffer (2.5 mM desthiobiotin in PBS). The fractions were stored at 4ºC. Peak integration yields 6.5 mg GH-(his-TEV-STREP3-his)C. Fractions A4-A8 (Fig. S1A) were pooled and used in the experiments.

Cell culture and transfections

HEK293 cells were cultured in 4.5 g/L glucose (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 0.1 mg/ml streptomycin (both Invitrogen). Media for culturing HEK293 cell stably expressing GHR were supplemented with 0.6 mg/ml Geneticin (G418; Gibco). Cells were transfected according to the calcium phosphate method or FuGene 6 (Roche, Applied Sciences, Almere, The Netherlands) as indicated by the supplier, for 24 or 48 hours.

Lysis method for detergent selection

It is very important in the BN-PAGE method to avoid salts because they precipitate in the presence of Coomassie Brilliant Blue. Instead of salt aminocaproic acid is used. To optimize detergent conditions and lysis time periods, the cells were lysed in the appropriate detergent (0.2% Triton-X100, 0.5% digitonin, 0.3 n-dodecylmaltoside or 0.3% Brij-96) in BN base buffer (20 mM Bis-Tris, pH 7.0, 0.5 M 6-aminocaproic acid, 20 mM NaCl, 2 mM EDTA, 10% glycerol,10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4). Samples were clarified by centrifugation (total cell lysate) and analyzed on SDS-PAGE. For biotin-GH pull down cells were lysed in the appropriate detergent containing BN base buffer. Pull downs were made on Streptavidin beads. Beads were boiled in sample buffer and loaded on SDS-PAGE.

Cell lysis, immunoprecipitation and biotin-GH pull down

For routine analysis cell lysates were prepared by washing the cells with cold PBS, after which the cells were lysed with 1% Triton-X100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mMPMSF in PBS for 15 min. The lysates were centrifuged for 5 min and the supernatants were used for immunoprecipitations by incubation with the appropriate antibody for 2 h followed by incubation of 45 min with protein A-agarose beads. Biotin-GH pull down was performed by incubating the lysates for 1 h with biotin-GH, followed by a 30-min incubation with streptavidin agarose beads. Lysates, immunoprecipitates and pull down samples were subjected to SDS-PAGE and western blotting, after which the membranes were detected with the indicated antibodies. Western blots were analyzed using an Odyssey infrared imaging system.

Biotin-GH pull down and elution for BN-PAGE

Cell lysates were prepared by washing the cells two times with cold PBS, after which the cells were lysed in buffer containing 0.2% Triton-X100 or 0.3% Brij-96 in BN lysis buffer (20 mM Tris-HCl pH 7.0, NaCl 137 mM, 2 mM EDTA, 10% glycerol) for 60 min on ice. Cells were scraped and lysates were centrifuged for 30 min at 13200 rpm in an Eppendorf centrifuge at 4ºC. Supernatants were collected and incubated with 200 ng/ml biotin-GH for 60 min. Subsequently, monomeric avidin agarose resin beads were added and samples were incubated for 90 min in 4ºC. Beads were washed 4 times with BN base buffer. Next, beads were incubated for 2.5 h with 150 μl of 2 mM D-biotin in BN base buffer. Eluates were filtered in Microcon Centrifugal Filter Devices (Millipore), and samples were immediately loaded on a first-dimension BN-PAGE gel.

STREP-GH pull down and elution for BN-PAGE

Cells were stimulated with 180 ng/ml of STREP-GH for 5-15min in a CO_2 incubator at 37ºC. Next, the

cells were washed 4 times with ice-cold PBS. Cells were lysed for 30 min in lysis buffer containing 1% Triton-X100, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 1 mM Na_3VO_4 and 15 mM N-ethylmaleimide. Cells were scraped and the lysates were clarified by centrifugation for 5 min in 4ºC. Supernatants were incubated with Strep-tactin beads for 2 h at 4ºC. Complexes were eluted with buffer containing 2.5 mM desthiobiotin, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 0.5mM Na_3VO_4, and 15 mM NEM (3 times 30 min). Eluates were combined and filtered in Microcon Centrifugal Filter Devices (Millipore) and washed once with BN base buffer containing 15 mM NEM in order to decrease amount of salt in the sample. Next, sample was loaded on the 1st dimension BN-PAGE the same day.

First dimension BN-PAGE

Native PAGE Bis-Tris Gel System was purchased from Invitrogen. Samples were loaded dry on 3%-12% gels and run for 16 h in 4ºC on 2mA. Native Protein Markers (Invitrogen) were used to standardize complex sizes.

Preparation of gel slices for second dimension SDS-PAGE

After first dimension BN-PAGE, gel slices were prepared for every sample and put into 15 ml tubes containing 10 ml sample buffer (12.5 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue) and 2,5% β-mercaptoethanol. Slices were incubated for 30 min in 70ºC in the water bath. Next, the gel slices were loaded on 15 mm-thick SDS-PAGE gels. Gels were run at 10 mA per each gel. Gel slice with the marker was stained with Coomassie brillant blue R250.

Transfer and western blotting

Gels were wet transferred to Immobilon-FL polyvinylidenedifluoride membrane (Millipore) with 0.8 mA per Criterion system for 60 min. Blots were immunostained with the indicated primary antibodies followed by Alexa Fluor 680, Alexa-800 IRDye conjugated anti-mouse or anti-rabbit antibodies. Detection was performed with an Odyssey system (LI-COR Biosciences).

Results

Detergent optimization and sample preparation

Solubilization of complexes requires the use of detergents and since the extraction efficiency can vary from one protein complex to another the best detergent must be selected, empirically. Finding suitable detergents for the solubilization of different protein complexes is a key for a reliable application of BN-PAGE in the investigation of membrane protein complexes. Detergents create a hydrophobic environment and stabilize membrane proteins. Non-ionic detergents tend to be best for multi-protein complex stability. They contain uncharged, hydrophilic head groups and break lipid-lipid and lipid-protein interactions, while keeping protein-protein interactions intact. Therefore, they are considered as non-denaturant and are widely used in the isolation of membrane proteins in their biologically active form.

As most of our protein complexes contain at least one integral membrane protein, we optimized the solubilization of the GHR, a type I membrane protein [14]. Four non-ionic detergents were tested: Triton-X100, Brij-96, digitonin and n-dodecylmaltoside. For this test we utilized biotin-GH (bGH) to isolate GHR from the solubilized cells, transfected the cells with a βTrCP2-expressing vector to be able to probe the GHR-βTrCP2 interaction [16], and visualized the protein on Western blot with anti-GHR antibody (mAb5) and anti-

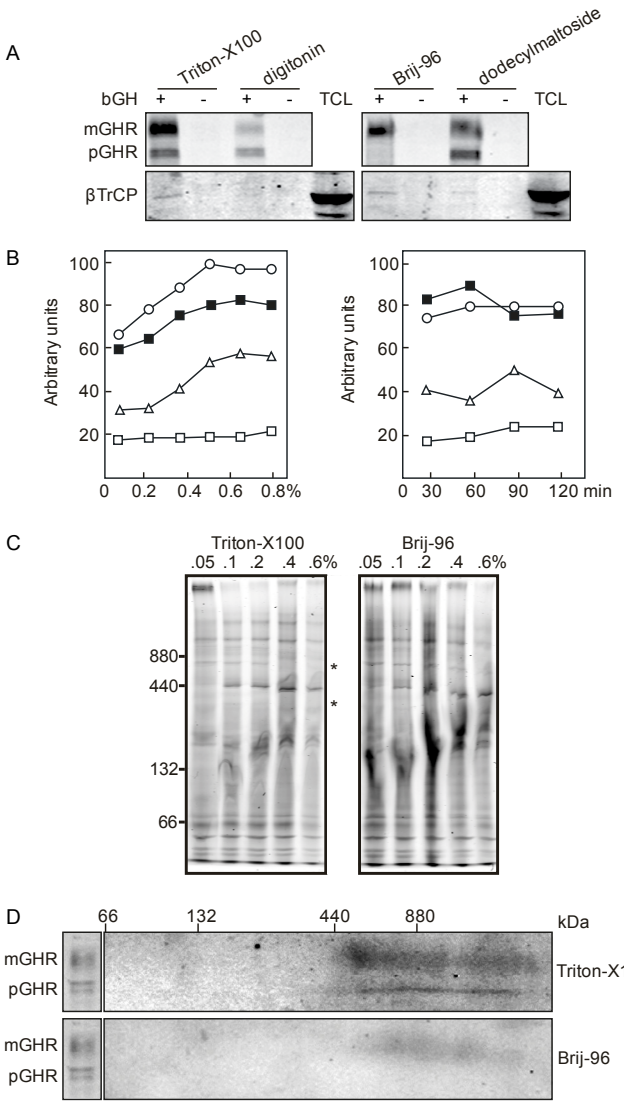

Figure. 1. Protein complex solubilization and integrity. **A.** *Detergent solubilization of GHR-βTrCP2 complexes. GHR expressing Hek293 cells were transiently transfected with a βTrCP2-expressing DNA construct and lysed after 2 days in 0.2% Triton-X100, 0.5% digitonin, 0.3% Brij-96 or 0.3% n-dodecylmaltoside, as indicated. Clarified lysates were incubated with biotinylated GH (bGH), the proteins were isolated with streptavidin-agarose beads and analyzed on western blot using anti-GHR antibody and anti-βTrCP2 antibody (lower panel). TCL, total cell lysate.* **B.** *GHR-expressing Hek293 cells were lysed for 60 min in increasing concentrations of different detergents, as indicated (left panel). In the left panel the lysis time was varied, as indicated. The amounts of mature GHR were quantified on western blot and plotted in arbitrary units. ○, n-dodecylmaltoside; ■, Triton-X100; Δ, Brij-96; □, digitonin.* **C.** *One-dimension BN-PAGE of cell extracts. Cells were lysed at increasing concentrations of detergent as indicated, for 60 min. After centrifugation the lysates were analyzed on a BN-PAGE gel and the gel was stained with Coomassie Brillant Blue. *, indicate desintegrating complexes, due to high detergent concentration. Native Protein Markers (Invitrogen) were used as a ladder to standardize complex sizes.* **D.** *Cells were lysed in either 0.2% Triton-X100 or 0.3% Brij-96 for 60 min and subjected to 2D BN/SDS-PAGE. The blots were analyzed with an antibody against GHR (mAb5). The gel calibration was as in Fig. 1C. All data in this figure are representative of three independent experiments.*

βTrCP2 antiserum. n-Dodecylmaltoside and Triton-X100 solubilized both mature (cell surface) and immature (ER) form of the GHR equally well, Brij-96 mainly solubilized the mature form of GHR and digitonin appeared to preferentially solubilize the immature GHR form, although the cell lysis itself appeared to be less efficient with this detergent (Fig. 1A). These results demonstrate the necessity to optimize solubilization for each complex. Most likely, the differences in GHR solubilization are due to the differences in lipid/protein composition of plasma and ER membranes.

The next question was to determine which detergent preserves the integrity of a GHR complex the best. As a first indication we analyzed the effect of the four detergents on the interaction between GHR and the F-box protein βTrCP2. This protein is a subunit of the $SCF^{\beta TrCP}$ E3 ligase complex and is known to bind specifically to the ubiquitin-dependent endocytosis (UbE) motif that is required for GHR internalization and degradation in lysosomes [15]. GHR-containing complexes were isolated with biotinylated GH (bGH) from cell lysates, the proteins were run on SDS-PAGE and visualized on Western blot with anti-GHR (mAb5) and anti-βTrCP2 antibody (Fig. 1A, lower panel). We conclude that Triton-X100 and Brij-96 were the most efficient detergents to solubilize and maintain GHR-βTrCP2 complexes. n-Dodecylmaltoside solubilized lower amounts of the GHR-βTrCP2 complex. In samples solubilized by digitonin βTrCP2 was under detection level, which was probably due to less effective solubilization of the mature form of the receptor. Since Triton-X100 solubilized more mature GHR than Brij-96, it seems to be the best detergent in preserving GHR complex. We then optimized detergent concentration and lysis time. As seen in Fig. 2B, the lowest concentrations that solubilized the highest amount of GHR were considered the best and were further used (0.2% Triton-X100, 0.3% Brij-96, 0.5% digitonin and 0.3% n-dodecylmaltoside). For all experiments 60 min lysis time was used. Further experiments were carried out with both Brij-96 and Triton-X100.

First dimension BN-PAGE offers the possibility to monitor both solubilization efficiency and complex integrity. Cell lysates prepared at increasing concentrations of either Triton-X100 or Brij-96 were subjected to first dimension BN-PAGE and the gradient gel was stained with Coomassie Brilliant Blue (Fig. 1C). Insoluble complexes were either discarded during centrifugation or did not enter the gel. Bands that disappeared at increasing detergent concentrations were regarded disintegrating protein complexes (arrows). We conclude that Triton-X100 and Brij-96 at 12 and 24 mg/mL, respectively, are optimal concentrations and were used in the experiments.

To analyze GHR-containing protein complexes we applied two-dimensional BN/SDS-PAGE on total cell lysates and immunoblotted for GHR. In line with results in Fig. 1A, Brij96 solubilized only mature form of GHR (Fig. 1D). Whether this was due to poor solubilization of the endoplasmic reticulum (ER) or whether the precursor GHR is insoluble in the detergent is unclear. All GHR complexes migrated as complexes of more than 440 kDa. The GHR form dimers, immediately after synthesis in the ER [21]. We did not detect GHR monomers in our analysis. Dimeric GHR is expected to migrate at 260 kDa. On immunoblots GHR complexes can be detected with a molecular weight of 450850, and > 900 kDa for both detergent extractions. Whether this is due to the occurrence of multimeric GHR complexes or to the association of other factors like Jak2, $SCF^{\beta TrCP2}$, or CHIP remains to be investigated [15, 22]. The fact that the distribution of ER-derived GHR is roughly identical to that of the mature GHR renders the first possibility more likely.

To analyze GHR complexes present on the plasma membrane we used STREP-GH, which allowed much more effective elution of GHR-containing complexes in native conditions than bGH (data not shown). For STREP-GH pull down we tested different concentrations of Triton-X100 (0.3%, 0.5% and 1%), different lysis times (30 and 60 min) and different amounts of glycerol (0%, 5% and 10%) in the buffer. We did not observe any differences in the composition of GHR complexes after 2D BN/SDS-PAGE (data not shown). This is consistent with results shown in Fig. 1B. Thus, although proper detergent, concentration of detergent and time of lysis should be optimized empirically, they also allow some flexibility. Additionally, comparing results from Fig. 1D and Fig. 2A it can be seen that while mature GHR complexes exhibit similar pattern, immature GHR complexes are slightly different. Immature GHR complexes smaller than 440kDa are hardly visible probably because they are under detection level. Self-made native gradient gels were used in experiment from Fig. 1D and precast native gels were used in experiment from Fig. 2A. Therefore, in our situation precast gels have better resolution that self-made gels.

BN Complex analyses

To illustrate the potential of 2D BN/SDS-PAGE we analyzed complexes containing β-catenin as well as the GHR. GHR, a prototypic cytokine receptor that is involved in growth and regulation of metabolism, dimerizes in the ER, and travels via the Golgi complex to the plasma membrane, where it is endocytosed to be degraded in lysosomes. During its relative short life time (roughly 50 min in the ER, and 50 min at the cell surface) it undergoes several posttranslational modifications. In the Golgi complex its 5 high mannose N-linked oligosaccharide chains are converted to complex type. At the cell surface, upon GH binding, 79 tyrosine residues are phosphorylated by Jak2. Finally, at the cell surface and in endosomes, the GHR is ubiquitylated by the ubiquitin ligase $SCF^{\beta TrCP2}$ as a first step in endocytosis and transport to lysosomes, respectively. Most of these events can be visualized on 2D BN/SDS-PAGE. In Fig. 2A, lower panel, total cell lysate of GHR-expressing cells was analyzed. Early biosynthetic stages are characterized by discrete sharp bands and smears (indicated 1-3), while "mature", complex-glycosylated species have a more diffuse appearance due to their complex glycosylation (indicated 4, 5). Previously, we described dimerization of the GHR in the ER as a very efficient process that even after short radioactive pulse labeling was impossible to visualize [23, 24]. Surprisingly, 2D BN/SDS-PAGE shows 2 spots (location 1) that correspond to monomeric GHR polypeptides: they behave in both electrophoretic dimensions as monomeric species, and they amount less that 1% related to the total of ER species (1, 2, 3 and 4). The status of smear 2 is less clear as it probably contains (oligomeric) folding intermediates. The discrete spot at 3 is striking as its apparent molecular mass is approximately 4-fold the molecular mass of monomeric GHR. Smear 4 very likely represents well-folded, oligomeric GHR ready to leave the ER. As this smear has exactly the same distribution as mature GHR (in position 6) these entities probably represent a collection of complexes with a similar array of proteins attached. Previously, we have shown that at steady state the majority of mature GHR resides at the cell surface [25]. Spot 5 contains mature receptors. With its discrete appearance of approximately 500 kDa it again might represent tetrameric GHRs. Together, we hypothesize that GHR complexes leave the Golgi complexes as tetramers complexed to various ancillary proteins such as chaperones, Jak2, βTrCP2, CHIP, phosphatases, and other signaling factors.

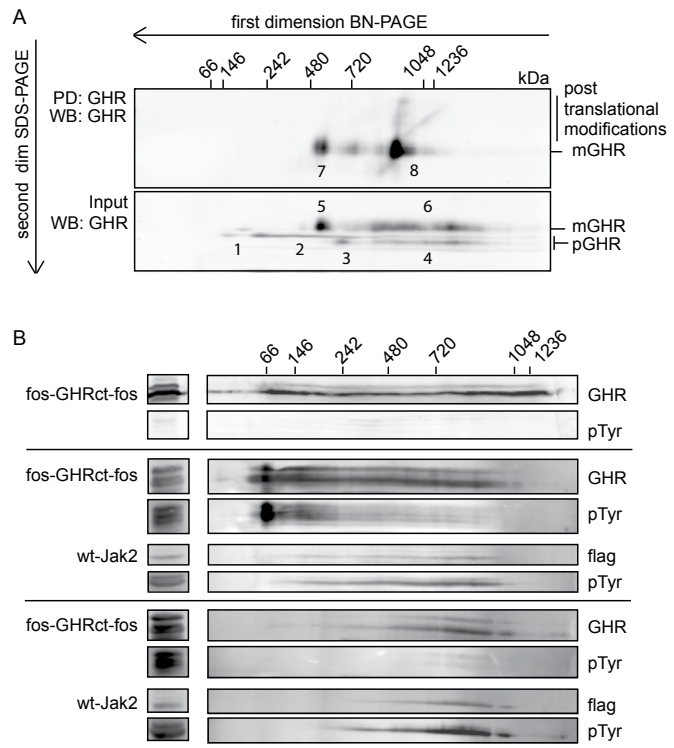

Figure 2. 2D BN/SDS-PAGE analyses of GHR and β-catenin protein complexes. **A**. *Comparison of total GHR complexes existing in the cell (lower panel) and mature GHR complexes (upper panel). Hek293 expressing GHR were lysed, the complexes were isolated on Strep-tactin beads, eluted with 2.5 mM desthiobiotin and subjected to 2D BN/SDS-PAGE followed by Western Blot. Upper sample was stimulated for 10 min with STREP-GH, isolated on and elution in native conditions. GHR was visualized with anti GHR antibody.* **B.** *Complex formation of Jak2 co-expressed with GHR cytosolic tails (fos-GHRct-fos). Double zippered cytosolic tail-GHR constructs were co-expressed with Jak2 in HEK293-TR cells. 2D BN/SDS-PAGE was carried out, the left lane shows sample only in SDS-PAGE. HekTR cell lysate containing fos-GHRct-fos with (middle and lower sections) or without (upper section) Jak2. In the lower section anti-Flag-IP was performed from the cell lysate, the complexes were eluted with Flag peptides and analyzed.* **C.** *Distribution of β-catenin over different multi-protein complexes. Hek293T cells were lysed in BNE base buffer containing 0.1% Triton-X100. Lysates were subjected to 2D BN/SDS-PAGE and immunoblotted for the indicated proteins.*

Next, we analyzed GHR complexes at the cell surface. To isolate complexes for BN-PAGE, affinity purification is the method of choice as it can be applied to small samples in a variety of experimental conditions. The key issue is to elute the complexes from the affinity support without disturbing the integrity of the complexes. Until now affinity-based purifications have not been widely utilized because it requires (over)expression of exogenous (tagged) proteins. In this study we have used flag-tagged proteins that can be eluted with flag-peptides (below). For the analysis of GHRs at the cell surface we have used strep-tagged GH. This methodology can easily be combined with primary cells and systems. Cells were incubated with STREP-GH for 15 min to obtain saturating binding and to initiate signal transduction and endocytosis, the cells were lysed and the complexes were purified and analyzed on 2D BN/SDS-PAGE (Fig. 2A, upper panel). The majority of the GHRs were present in two spots (7 and 8) with two discrete minor spots in between. Except for spot 7 the complexity of the GHR changed dramatically. Incubation with STREP-GH caused redistribution and appearance of a complex of approximately 900 kDa. This complex also contained polyubiquitylated GHR as is inferred from the appearance of a "plume" that slightly inclines to higher molecular weight complexes as can be expected from a heavily ubiquitylated protein. Immunblotting with anti-ubiquitin antibodies confirmed this (not shown). A second GHR-positive streak emanated in an angle of approximately 45º towards heavier complexes. As protein complexes are fully denatured in the second dimension this material must present covalently-linked posttranslational modified GHR of yet unidentified nature. As it has a continuous appearance and the molecular size increases by almost 200 kDa, the modification must consist of the addition of multiple molecules, bigger than ubiquitin. Together, we conclude that BN-PAGE is a powerful tool to analyze complex biological processes in the cell including signaling receptors.

In a second application based on our GHR research we sought to study GHR-specific signal transduction *in vitro*. This requires soluble, dimeric polypeptides derived from the cytosolic tails from the GHR. We provided the cytosolic tails with fos-zippers both N- and C-terminally. Theoretically, such a zippered dimer possesses 2 binding sites for Jak2 through their canonical box-1 motifs, as they are present in all cytokine receptor. Important questions are: What is the stoichiometry of double fos-zippered GHR cytosolic tail protein with Jak2, how does Jak2 interact with this construct, and how does phosphorylation affect their binding? BN-PAGE provides a powerful tool to answer these questions and characterize this *in vitro* system. We co-expressed the double zippered GHR construct with wt-Jak2 in HEK293-TR cells and analyzed the protein complexes on two-dimensional BN/SDS-PAGE. In the first dimension the proteins remain in complexes, while in the second dimension, in the presence of SDS, the proteins that constitute the complexes can be analyzed. Fig. 2B, upper section, shows the analysis of the lysate, if the fos-zippered GHR was expressed without exogenous Jak2. The tails migrated in a broad range from the monomeric 70 kDa to more than 1.3 MDa. Staining with anti-phosphotyrosine antibodies confirmed that endogenous Jak2 was not able to act on these tails. Interestingly, coexpression with Jak2 brought about the appearance of monomers and increased dimeric receptor tails (70-140 kDa) at the expense of high molecular weight complexes (Fig. 2B, middle section). Mainly the lower molecular masses (70 – 250 kDa) were phosphorylated, probably by the interaction with Jak2. Jak2 was detected in the range of monomeric molecular mass (130 kDa) up to 1 MDa, the

majority being in complexes of around 1 MD. Staining with anti-pY showed that all species were tyrosine-phosphorylated. To investigate the interaction with the cytosolic tails, Jak2 was immunoprecipitated via its flag-tag and eluted with flag-peptide (Fig. 2B, lower section). If we compare this western blot with the 2D BN/SDS-PAGE blots of the total lysate, it is clear that only in higher molecular masses (0.7-1.0 MDa) GHR tails and Jak2 form complexes, in which only Jak2 is phosphorylated, suggesting that GHR phosphorylation by Jak2 causes complex dissociation.

The Wnt-β-catenin system is another important signaling pathway that we analyzed. β-catenin is a component of E-cadherin-based adherens junctions and is a key factor in the Wnt/β-catenin signal-transduction pathway. Unstimulated cells keep the intracellular levels of β-catenin very low by efficiently degrading β-catenin through the ubiquitin-proteasome system. β-catenin is targeted for degradation by its N-terminal phosphorylation in the Axin1-based destruction-complex. The degradation of β-catenin is blocked upon Wnt stimulation, which leads to its intracellular accumulation and allows transcriptional activation of target genes in the nucleus. In contrast to the intracellular forms, E-cadherin bound β-catenin stably associates with adhesive complexes at the plasma membrane and is protected from degradation.

The different characteristics of the membrane bound adhesive-complexes and the soluble destruction-complex makes the analysis of the total cellular β-catenin complexes difficult. 2D BN/SDS-PAGE allows the separation of both membrane and cytoplasmic complexes. Hereby, we are able to study both complexes in a single analysis. Using 0.1% of Triton-X100 during cell lysis solubilizes both complexes while retaining their assembly. 2D BN/SDS-PAGE enabled us to immunoblot several specific proteins. E-cadherin resides in complexes of approximately 700-1300kD, whereas the major fraction of Axin1 forms complexes of 250-500kD (Fig. 2C). β-catenin co-migrated with both proteins. Because of this difference in the size of the adhesive complexes and the destruction-complex we can determine the properties of β-catenin in either complex. Using antibodies that specifically recognize the N-terminally unphosphorylated form of β-catenin we were able to show that this form of β-catenin is only present in the E-cadherin based complexes at steady-state. Unphosphorylated β-catenin perfectly co-migrated with E-cadherin while none co-migrated with Axin1.

Discussion

Analysis of transient protein complexes remains a challenge. Unlike stable protein complexes such as proteasomes transient protein complexes do not appear as dots on BN-PAGE but as smear in almost all first dimension gels. In such situation second dimension SDS-PAGE is necessary to separate proteins within the complexes. Also since proteins span almost the whole gel, one has to be very careful when analyzing the results. Two proteins running in the same size range do not necessarily exist in the same complex. These can be two separate complexes accidentally having in the same size. Therefore, either additional co-immunoprecipitation assays should be performed to confirm true interactions, or protein complexes should be analyzed that has been reported before to exist in the same complex. In the context of this study we analyzed GHR/βTrCP2, GHR/Jak2, β-catenin/E-catherins and β-catenin/Axin complexes, complexes that indeed are known to respond to changing growth and stress conditions.

Elution of specific protein complexes under native conditions for BN-PAGE has not been reported before. We optimized the method of analyzing the early stages of GHR endocytosis by stimulating the cells with STREP-GH which triggers signaling cascade and subsequent pull down of STREP-GH/GHR complexes in combination with elution in native conditions. We were able to distinguish several GHR complexes present on the plasma membrane and also show the dynamics of those complexes upon different GH stimulation time (data not shown).

For the first time BN-PAGE followed by second dimension SDS-PAGE was used to analyze posttranslational modifications of proteins. In the case of GHR it has been known that it is subjected to ubiquitylation. However, with applying BN-PAGE followed by 2D SDS-PAGE, we were not only able to identify a specific complex that is subjected to this modification (Fig 1A number 8) but also to identify a novel modification of GHR of unknown nature. In standard SDS-PAGE both posttranslational modifications run in the same size range, and are thus are impossible to distinguish. BN-PAGE combined with SDS-PAGE provided a new insight into posttranslational modification of GHR. Additionally, we have also been able to show that GHR within the same complex is also subjected to phosphorylation (data not shown). To sum up BN-PAGE can be efficiently used to specifically identify complexes subjected to posttranslational modifications.

Several limitations of the BN-PAGE method were experienced. Proteins provided with Hemagglutinin (HA) or Flag tags can be released by specific peptides under physiological conditions. Use of the His6 tag is problematic as it requires imidazole, ion chelators or unphysiological pH conditions. All these methods work only with exogenously expressed proteins that may disturb protein complexes by depleting binding partners from low-affinity complexes. Despite the latter disadvantage, the use of a STREP-tagged protein provides an elegant solution due to the mild conditions to isolate the complexes from Strep-tactin beads. In general, the popular biotin-streptavidin system requires high concentrations of biotin to detach the complexes that are also not favorable for the integrity of complexes. On the other hand, specific anti-phosphotyrosine antibodies that can be detached with phenylphospate are excellent tools to study the details of signal transduction without exogenous protein expression [4]. As the analytical power of the system is high, most disadvantages can be overcome by using additional protocols such as time course studies (ligand binding and uptake or pulse-chase metabolic labeling), gene silencing, or overexpression of dominant-negative factors.

The analysis of β-catenin complexes with 2D BN/SDS-PAGE demonstrates another important potential of the technique for the analysis of complex signaling systems. The high abundance of β-catenin in membrane-tethered adhesive complexes overrules the β-catenin pool, which is involved in the Wnt/β-catenin signaling pathway, when analyzed by conventional SDS-PAGE. This makes the analysis of the signaling form difficult. As we show in Fig. 2C, BN-PAGE segregates the E-cadherin bound pool of β-catenin from the Wnt involved forms of β-catenin. Hereby, we can analyze β-catenin in either system in one single analysis without the need of other methods to enrich specific β-catenin pools. BN-PAGE provides a promising tool to study the interplay between the signaling and adhesive functions of β-catenin.

By using BN-PAGE, protein complexes can be analyzed in different perspectives. Analysis of the total lysates of cells shows the migration of protein complexes at their specific molecular mass, but make it impossible to distinguish whether a protein

is bound in different complexes of the same size. Here, we demonstrate that protein immunoprecipitation and subsequent blue native gel analysis allows the determination of all complexes in which the precipitated protein is bound. Comparing complex sizes in total extracts and after isolation shows that complex purification does not change complex size. This renders BN-PAGE a legitimate method that enables SILAC and other quantitative approaches. Furthermore, western blot analysis with the Odyssey® Infrared Imaging System allows two color detection of gels as shown in the GHR cytosolic tail/Jak2 complex analysis (Fig. 2B), allowing simultaneous analyses of different posttranslational modifications on the same proteins. In our blots it could be precisely determined which proteins in the BN gel are phosphorylated. Here, GHR and Jak2 antibodies were used in one channel and an anti-phosphotyrosine antibody in the other one. In the overlay, the phosphorylated parts of the proteins become visible.

In conclusion, BN-PAGE, together with pulldown experiments and the additional use of two channels western blot detection system, allows a very specific determination of protein complex composition in all states of posttranslational modification. Despite all these advantages, this method requires certain assumptions. In 2D BN/SDS-PAGE proteins are distributed over large areas in two different gels - native and denaturing. Thus a certain protein expression level is necessary, depending on the quality of the antibody. In addition, similar to immunoprecipitation, it is absolutely necessary to know the potential interaction partners in the protein complex that is being analyzed. Therefore, in analytical approaches this method does not allow the identification of proteins in cellular complexes if they are not known or at least suspected to be part of the complex. Furthermore, 2D BN/SDS-PAGE does not allow a precise determination of complex masses, since the protein marker run in the first dimension it cannot be applied on the second dimension. Nevertheless, knowing the possible binding partners and its molecular mass together with the approximate size of the proteins enables a unique analysis of protein complexes. In combination with mass spectrometric analysis the beauty of the system is clear.

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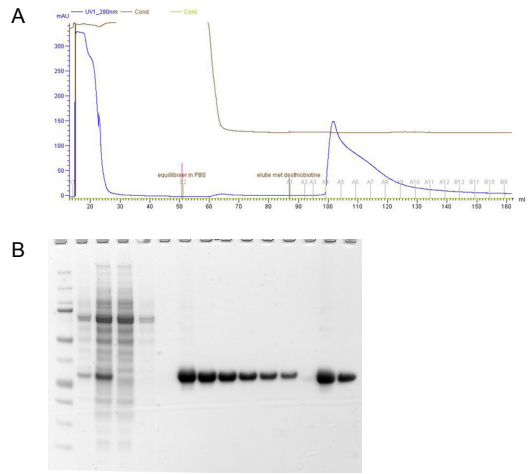
Supplementary data



Supplementary Fig. 1. Strep-tagged-GH production. **A**. *Strep-tractin purification. Strep-tagged GH was eluted with PBS and 2.5 mM desthiobiotin. Fractions A4-A8 were pooled and used in the experiments. Peak integration yields 6.5 mg GH-(his-TEV-STREP3-his)C.* **B**. *NuPage analysis of fractions from the Strep-tactin purification of GH-(his-TEV-STREP3-his)C. 4 12% MES gel, Only the reduced samples were 10 minutes incubated at 70ºC. Lanes: 1, Mw marker; 2, culture supernatant; 3, concentrated supernatant; 4, unbound; 5, X1; 6, A3; 7, A4; 8, A5; 9, A6; 10, A7; 11, A8, 12, A9; 14, A4 Non-reduced; 15, A7 Non-reduced.*

CHAPTER 5

SUMO2/3 regulates GHR trafficking

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Summary

Growth Hormone (GH) is a main regulator of growth and metabolism. GH acts via growth hormone receptor (GHR), and belongs to the class I cytokine receptors that utilize Jak2 tyrosine kinase in order to perform signaling cascade via STAT/MAPK pathway. Regulation of GHR availability on the plasma membrane is crucial for GH sensitivity of cells. In our study we show that the sumoylation pathway is important for GHR trafficking. Receptor itself does not seem to be modified by SUMO. However, both overexpression of exogenous SUMO2/3 and silencing of the SUMO conjugase, Ubc9, cause accumulation of GHR inside the cells, while endocytosis and synthesis of GHR are not affected. Additionally, the GHR accumulation is independent of Jak2 kinase that, previously, has been shown to be a substrate for the sumoylation machinery. Our data show that degradation of GHR is inhibited, probably due to interference with sorting at the level of multi vesicular bodies (MVBs). Moreover, preliminary data suggest that exogenously expressed SUMO2/3 increase targeting of GHR to the nucleus.

Introduction

Growth hormone (GH) is a main regulator of postnatal growth and an important metabolic regulator. It acts via the growth hormone receptor (GHR), which is a class I cytokine receptor that lacks intrinsic kinase activity. In order to trigger the signaling cascade, GHR needs Jak2, a member of the Janus family of cytosolic kinases to initiate the signal transduction. Upon GH stimulation Jak2 autophosphorylates itself and the receptor, which creates recognition sites for signaling proteins with Src homology 2 (SH2) or other phosphotyrosine binding domains. Members of the signal transducers and activators of transcription (STAT) family undergo phosphorylation by Jak2, which stimulates their dimerization and translocation to the nucleus, where they act as transcription factors.

GHR molecules are synthetized in the endoplasmic reticulum (ER) where they dimerize, and travel through the Golgi apparatus towards the plasma membrane [1]. The GHRs undergo complex glycosylation in the Golgi. Mature GHRs on the plasma membrane are subjected to clathrin-mediated endocytosis, either constitutively, or GH –induced [2]. GHR endocytosis is ubiquitin system-dependent [3] and its degradation takes place in the lysosome. Upon GH binding, GHR becomes ubiquitylated by the SCFβTrCP2 ubiquitin-ligase [4] (da Silva Almeida et al., to be published). Recently, the CHIP ubiquitin ligase together with the ubiquitin conjugase, Ubc13, has been shown to be necessary for GHR internalization (Slotman et al., submitted). Additionally, Tsg101 and Hrs, components of the ESCRT system, as well as βTrCP2, CHIP and Ubc13 have been implicated in GHR sorting at the level of MVB [5].

Using immunohistochemical and other approaches, Waters and colleagues demonstrated the presence of GHR in the nuclei of several different cells and tissues [6-11]. Additionally,

they suggested that GHR translocation to the nucleus is implicated in carcinogenesis [12]. The translocation mechanism is largely unknown. However, it may resemble the route taken by bacterial toxins [13]. In case of epidermal growth factor receptor (EGFR) it has been proposed that upon EGF stimulation it travels to the ER, where it associates with Sec61β. This component of the Sec61 translocon inserts secretory and transmembrane proteins into the ER during protein synthesis [14, 15]. Since the translocon is bidirectional, it can retrotranslocate misfolded proteins from the ER to the cytosol for degradation. In the cytoplasm, EGFR would associate with importin β and translocate to the nucleus. Wang and colleagues propose a slightly different scenario, where EGFR in ER associates with Sec61β, but remains membrane-bound [16]. Importin β would then regulate the translocation of the EGFR via the nuclear pore from the outer nuclear membrane to the inner nuclear membrane and only there the receptor becomes detached from the membrane. In addition to EGFR family members [17-19], also fibroblast growth factor receptors (FGFR) 1, 2 and 3 [20-24], prolactin receptor [25, 26], the interferon-γ (IFN-γ) receptor [27, 28], the insulin receptor (IR) [29], and the vascular endothelium growth factor (VEGF) receptor fetal liver kinase 1 [Flk1, also known as kinase insert domain receptor (KDR)] [30, 31] can accumulate in nuclei. Occasionally, full length receptors or proteolytically cleaved fragments, with or without their corresponding ligands can be found in the nucleus.

Sumoylation is connected to nuclear transport and cell stress [32-40]. It is a reversible process that involves covalent attachment of the small ubiquitin-related modifier, SUMO. There are four different SUMO molecules in mammalian cells. SUMO1 is expressed less ubiquitously than SUMO2 and SUMO3 that share high homology and ability to make poly-SUMO chains. The role of SUMO4 is unknown. Attachment of a SUMO moiety influence protein-protein interactions and can alter subcellular localization, stability or activity of a substrate (reviewed in [41]). The SUMO conjugation machinery consists of the SUMO-activating enzymes, SAE1 or SAE2 (acting analogously as ubiquitin E1), a unique SUMO conjugating enzyme, Ubc9 and several SUMO ubiquitin ligases. Attachment of one or more SUMO moieties often regulates subcellular localization of a substrate protein. CtBP1 [32] and the IκB kinase regulator, NEMO [33], require sumoylation for nuclear translocation. IGF stimulation regulates IGF-1 receptor modification with SUMO and its subsequent translocation to the nucleus [40]. Additionally, sumoylation have been implicated in nuclear export. Dictyostelium Mek1 become modified in the nucleus, which leads to its movement to the cytoplasm [42] and mutation of SUMO-acceptor lysine of the TEL protein causes its accumulation in the nucleus [43].

In addition to its role in nuclear translocation sumoylation has been implicated in plasma membrane receptor endocytosis. Sumoylation of the glutamate receptor subunit 6 (GluR6) is induced in response to kainite, and this modification appears to be a prerequisite for kainite-induced endocytosis of the receptor [44]. Wyatt and colleagues have shown that arrestin-3 is subjected to stimulus-dependent sumoylation and that this modification is necessary to promote G protein-coupled receptor (GPCR) endocytosis [45]. So far only one protein belonging to the general endocytosis machinery – dynamin - has been shown to undergo sumoylation [46].

In this study, we show that SUMO2 or SUMO3 overexpression causes a strong inhibition of GHR degradation. The same phenomenon occurs if cells are depleted from the SUMO conjugase, Ubc9. In contrast to GluR6, the GHR itself does not seem to be modified

by SUMO. Our data indicate that degradation of GHR is inhibited, probably due to interference with sorting at the level of multi vesicular bodies (MVBs). Additionally, our preliminary data suggest that SUMO3 overexpression increases the nuclear localization of GHR.

Materials and Methods

Antibodies, reagents and plasmids
Mouse monoclonal antibody against Jak2 was purchased from Invitrogen (AHO1352), anti-SUMO2/3 from Abcam (ab81371), anti-KDEL from Merck (10C3), anti-GAPDH from Millipore (MAB374) and anti-actin from MP Biomedicals Inc (clone C4). Mouse monoclonal anti-HA tag 12CA5 antibody, used for immunoblotting, was from Babco (Richmond CA) and anti-HA antibody (HA.11 Covens), used for microscopy, was from Covance. Rabbit polyclonal antibody anti-EE1 was from BD Biosciences, San Diego, CA and anti-Ubc9 from Santa Cruz (sc-10759). Rabbit polyclonal anti-GHR B antibody used for western blot detections and anti-GHR T antibody used for immunoprecipitations were previously described [3, 47]. Rabbit monoclonal anti-histone H3 antibody used both for immunobloting and microscopy were from Cell Signaling (D1H2). The secondary antibodies Alexa Fluor 680 and IR 800-conjugated goat anti mouse and anti-rabbit IgGs were obtained from Molecular Probes. Alexa488-transferrin was from Molecular Probes and EGF-Alexa fluor 488 streptavidin from Invitrogen. Rodamine-EGF and rodamine-transferrin were from Molecular Probe. Fluorescently labeled secondary mouse and rabbit antibodies used for microscopy (Alexa Fluor 488, 568 and 647) were from Molecular Probe. ProteinA beads were purchased from RepliGen Corporation. Human GH was kindly provided by Eli Lilly & Co. Research Labs (Indianapolis, IN). Culture media, fetal calf serum (FCS), L-glutamine and antibiotics for tissue culture were purchased from Invitrogen. The pcDNA3 HA-SUMO1, SUMO2 and SUMO3 plasmids were kind gift from Frauke Melchior. pSG213 plasmid expressing Jak2 was described elsewhere [2].

Cell culture, transient transfections and gene silencing
All cell lines were cultured under standard conditions in DMEM containing 10% FCS, supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin. The GHR-expressing Hek293 cell line (Hek-wt GHR) was cultured in media supplemented with 0.6 mg/ml Geneticin (G418; Gibco). Hek293 cell line expressing both GHR and HA-SUMO3 under a doxycycline-controlled pcDNA4 vector, was grown in the same medium, supplemented with 300 μg/ml Zeocin (Invitrogen). GHR-expressing γ2A cells were cultured in media supplemented with 0.6 mg/ml Geneticin (G418; Gibco) and γ2A cell line expressing GHR and Jak2 described in [2] were grown in the same medium, supplemented with puromycin and hygromycine.U2OS stably expressing GHR were grown in media supplemented with 150 μg/ml hygromycine and U2OS expressing EGFR with 125 μg/ml zeocin. Transfections were performed with Fugene (Roche). Cells were plated at 40% confluency (106 cells in 6 cm dish format) 24 hours before the transfections, and then transfected with 2 μg of DNA and 6 μl of Fugene. Cells were silenced for 48 hours with Lipofectamine2000 according to standard conditions. siRNA used for Ubc9 silencing was purchased from Ambion (s14591).

Cell lysis and immunoprecipitations
Cells were washed once with PBS and harvested in denaturing lysis buffer (1 mM EDTA, 1% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide in PBS). The lysates were boiled for 6 min, sheared 5 times with a 25G needle, boiled again for 3 min, vortexed and clarified for 5 min at 16,100g. Supernatants were diluted to a final concentration of 0.5% SDS with Immunomix special: 2% TritonX-100, 1 mM EDTA, 1% BSA, 0.5% DOC, and inhibitors (1

mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide) in PBS. For GHR immunoprecipitations lysates were incubated with rabbit anti-GHR(T) antibody for 2 h before adding Protein A beads for 45 min in an end-over-end rotator at 4°C. Beads were washed two times with immunomix (1% Triton-X100, 0.1% SDS, 1 mM EDTA, 1% BSA, 0.5% DOC, and inhibitors in PBSa) and two times with 0.1 concentration of PBSa and boiled with sample loading buffer for 5 min.

Subcellular fractionations
The high salt nuclear fraction protocol was performed as follows. Briefly, Hek293 cells in a 10-cm dish were scraped into 1 ml of ice-cold PBS, and centrifugated for 5 min in 9,300g. Supernatant was discarded and cells were resuspended in 200 μl of buffer 1 (25 mM HEPES pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 1 mM DTT, and inhibitors) and lysed by adding 200 μl of buffer 2 (buffer 1 supplemented with 1% NP-40) and incubating for 15 min end-over-end at 4°C. Nuclei were pelleted at 500g for 5 min and washed once with buffer 3 (1:1 mixture of buffer 1 and 2). The cytoplasmic fraction was collected and immunoprecipitation was performed by adding 500 μl of buffer (2% Trition-X100, 1% SDS, 1 mM EDTA, 1% BSA, 0.5% DOC, and inhibitors in PBS), followed by incubation with the appropriate antibody as described above. To the nuclear fraction 500 μl of denaturing lysis buffer was added and procedure was performed as described in the Cell lysis and immunoprecipitations section.

Immune fluorescence microscopy
Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier's instructions (Amersham Biosciences). Transfected cells, grown on coverslips, were incubated with Cy3-GH (0.5 μg/ml), Alexa488-labeled transferrin (0.8 μg/ml), Alexa594-labbeled transferrin (25 μg/ml), Alexa488-labeled EGF or rhodamine-EGF (10 ng/ml). Cells were washed with PBS to remove unbound label and fixed for 1 h in 3% paraformaldehyde in phosphate buffer, pH 7.4. After fixation, the cells were permeabilized in 0.1% saponin for 1 h and the cover slips were incubated with anti-HA and fluorescently labeled secondary antibodies. For anti-histon H3 staining cells were fixed for 10 min with ice cold methanol. The cover slips were embedded in Prolong DAPI (company) and visualization was performed using a Zeiss LSM 510 meta system.

Results

Overexpression of SUMO2/3 causes accumulation of GHR
In order to determine whether GHR is sumoylated, GHR expressing Hek293 cells were stimulated with GH and anti-GHR imunoprecipitations were performed. No sumo signal on GHR was detectable neither with anti-SUMO2/3 (Fig. 1A) nor with anti-SUMO1 or SUMO2 (data not shown). We conclude that under the conditions we used no SUMO modification of the GHR occurred. Next, we analyzed the effect of exogenous SUMO3 on GHR degradation. As shown in Fig. 1B, increased levels of SUMO3 caused a 2-fold accumulation of the mature GHR without affecting the levels of precursor GHR.
Next, we addressed the question whether the effect was specific for SUMO3. GHR expressing U2OS cells were transfected with HA-SUMO1, HA-SUMO2 and HA-SUMO3 and the amounts of GHR were analyzed on Western Blot. The results show that both SUMO2 and SUMO3 had a strong effect on GHR degradation (Fig. 1C). As the expression level of SUMO1 was consistently low, the effect of SUMO1 was unclear. The increased signal in the band partly overlapping the precursor (lane S3) is due to intermediate degradation products.

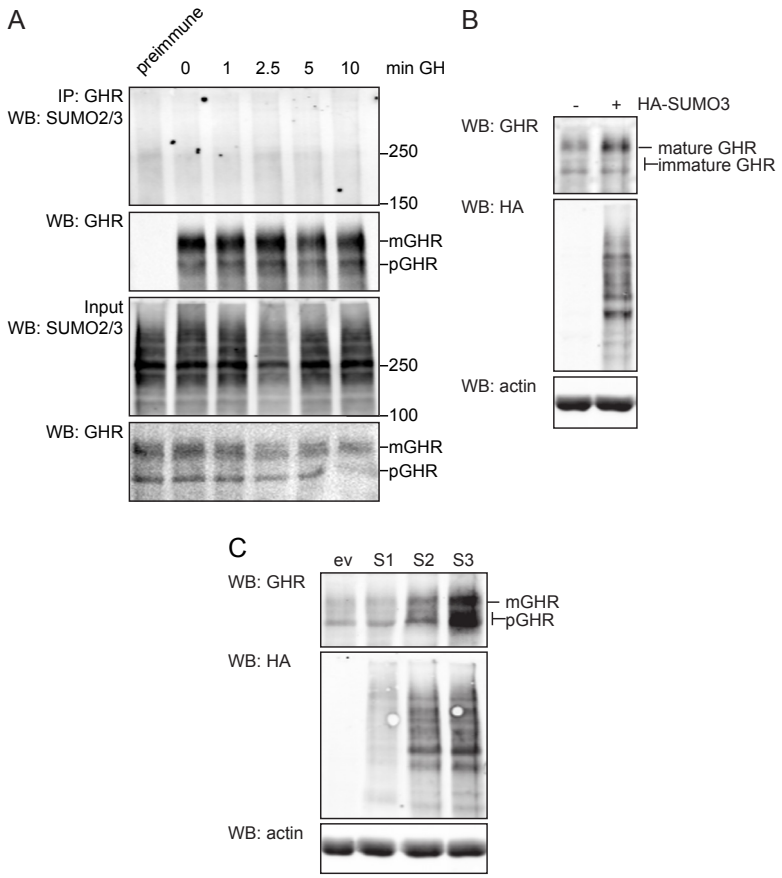

Figure 1. Excess of SUMO2/3 accumulates GHR. **A.** *Hek293 cells stably expressing GHR were stimulated with GH as indicated. Cells were lysed in denaturing conditions and GHR immunoprecipitations were performed. Sumoylation was assessed with anti-SUMO2/3 antibody.* **B.** *GHR-expressing Hek293 cells were transiently transfected with empty vector or HA-SUMO3. Amount of GHR was assessed with anti-GHR antibody* **C.** *U2OS cells stably expressing GHR were transiently transfected with empty vector (ev) HA-SUMO1 (S1), HA-SUMO2 (S2) or HA-SUMO3 (S3). Amount of GHR was analyzed with anti-GHR antibody.*

As sumoylation of the GHR does not seem to occur, and we recently identified Jak2, a major regulator of the GHR [2], as a SUMO3 substrate, we speculated that Jak2 might be implicated in the observed effect of SUMO3 overexpression on the GHR accumulation (Sedek and Strous, in preparation). The human fibrosarcoma cell line γ2A is an ideal tool for this because it lacks Jak2 [48]. We used GHR-expressing γ2A cells, transfected with empty vector, SUMO3 or with both SUMO3 and Jak2, and analyzed the amounts of GHR on western blot. As the transfection efficiency of γ2A cells generally amounts more than 50% and introduction of Jak2 did not change the effect of SUMO3 on GHR levels, we conclude that GHR accumulated independent of Jak2 (Fig. 2A). Thus, SUMO3 acts in the degradation of the GHR neither through sumoylation of the GHR nor via Jak2.
Previously, we identified Ubc9 as being involved in GHR degradation (Slotman, et al., in preparation). This supported our hypothesis that the sumoylation machinery is involved

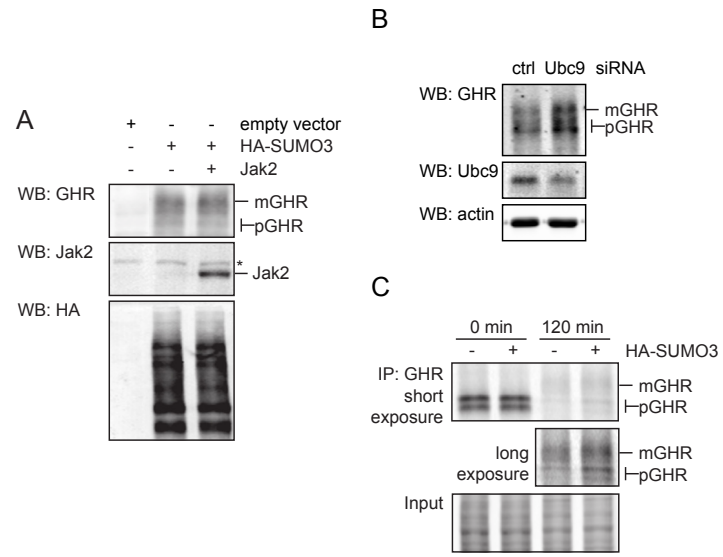

Figure 2. GHR accumulation is Jak2 independent. **A**. *γ2A cell line stably expressing GHR was transfected with empty vector or HA-SUMO3 or HA-SUMO3 and Jak2.* **B.** *Ubc9 silencing cause accumulation of GHR. Hek293 stably expressing GHR was silenced with anti-Ubc9 siRNA for 48 h. Amount of GHR was assessed with anti-GHR antibody. Silencing was controlled with anti-Ubc9 antibody and decreased 50%.* **C.** *GHR expressing U2OS cells were transiently transfected with HA-SUMO3. Cells were pulse labeled with radioactive [35S] methionine for 15 min and chased for 0 min or 120 min. Anti-GHR immunoprecipitations were performed and signal was determined with a STORM imaging system.*

in GHR trafficking. Therefore, we investigated the effect of Ubc9 gene silencing on the amount of GHR in steady state. Like SUMO2/3 overexpression, Ubc9 gene silencing induced an accumulation of mature GHR (Fig. 2B). The increased label at the position of immature GHR is due to degradation process or proteolytic cleavage of mature GHR (da Silva Almeida et al., in preparation). This result is in line with the effect of SUMO3 overexpression, supporting the hypothesis that sumoylation is required for sorting at the endosomes.
Since many transcription factors are SUMO substrates, we investigated whether synthesis of GHR is increased in excess of SUMO3. [35S] methionine was added to the cells for 15 min (pulse), GHR immunoprecipitations were performed and the amount of radioactive GHR was analyzed. While the synthesis of the receptor was not increased the degradation of the mature GHR was clearly inhibited, taking into account that the half-life of the GHR under steady state conditions is approximately 75 min (Fig. 2C, long exposure) [49]. This result is in favor of our hypothesis that exogenous SUMO3 inhibits GHR degradation.

GHR trafficking is affected by SUMO3 overexpression

To further analyze the mechanism of GHR accumulation, we prepared a Hek239 cell line that allowed varying expression levels of SUMO3 upon doxycycline (Supplementary Fig. 1A and B). Cells were treated with doxycycline, stimulated for 30 min with Cy3GH or rhodamine-transferrin and analyzed under the microscope. A clear increase in the amount of Cy3-GH was visible in the doxycycline-treated cells, compared to untreated cells (Fig. 3A). As the Cy3GH accumulated inside the cells with little label on the cell

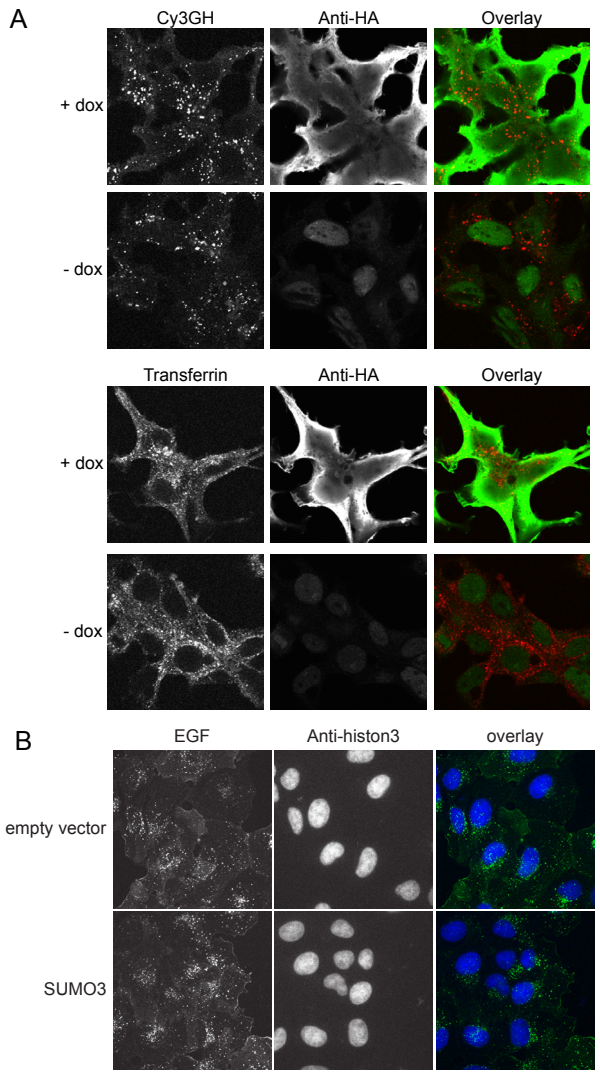

Figure 3. Trafficking of GHR is affected by excess of SUMO3. **A.** *Hek293 cell line stably expressing plasmid coding for HA-SUMO3 under doxycycline stimulating promoter was treated or not with doxycycline. Cells were grown on cover slips and incubated with cy3-labeled GH (Cy3GH – upper panel, red) or Rhodamine-labeled transferrin (Transferrin – lower panel, red) for 30min prior to fixation with formaldehyde. SUMO3 expression was assessed with primary anti-HA antibody and secondary Alexa488 conjugated antibody (green).* **B.** *EGFR expressing U2OS cells were transiently transfected with HA-SUMO3. Cells were grown on cover slips and incubated with Alexa488-labbeled EGF (green) for 2 h prior to fixation with methanol. Nucleus was stained with primary anti-histon H3 antibody and secondary Alexa647-conjugated antibody (blue).*

surface we conclude that SUMO3 overexpression does not affect the endocytosis of the GHR. Interestingly, neither the amount nor the distribution of transferrin was affected. Additionally, morphology of endosomes seems not to be altered. Only increased number of Cy3-GH containing vesicles can be observed in cells treated with doxycycline.

To further analyze the specificity of GHR accumulation in response to SUMO3 we determined both amount and localization of the EGF receptor (EGFR) as measured by fluorescent EGF. As illustrated in Fig. 3B, neither localization nor amount of EGFR was affected. This shows that accumulation of receptor upon SUMO3 overexpression is specific for GHR.

GHR is endocytosed via clathrin-coated pits and targeted to degradation in lysosomes via MVBs [50]. However, when βTrCP2 or Tsg101 are depleted the receptor is recycling back to the plasma membrane [4, 5]. We wanted to know whether SUMO3 overexpression is modifying GHR endocytosis by targeting it to the recycling route. That would result in

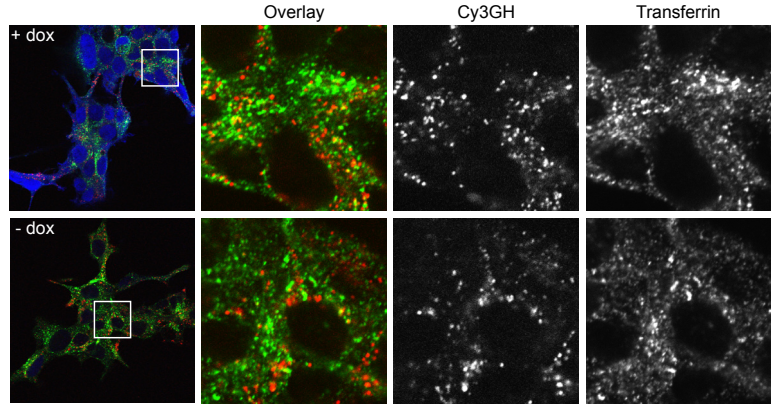

Figure 4. There is no increase in targeting GHR to the recycling route upon SUMO3 overexpression. *Hek293 cell line stably expressing plasmid coding for HA-SUMO3 under doxycycline stimulating promoter was treated or not with doxycycline. Cells were grown on cover slips and incubated with Cy3GH for 30 min. Media was exchanged and cells were incubated with Alexa488-labbeled transferrin (Transferrin) for another 30min. Cells were fixated with formaldehyde. SUMO3 expression was assessed with primary anti-HA antibody and secondary Alexa647-conjugated antibody. For clarity anti-HA staining was removed in magnifications. Red –Cy3GH, Green – Tf, Blue – Anti-HA.*

accumulation of GHR both at the cell surface and in endosomes. About 80% of transferrin receptors (TfR) normally reside within recycling endosomes and recycle continuously between the cell surface and endosomes [51]. Thus, we decided to analyze co-localization of GHR and TfR upon SUMO3 overexpression. Increased co-localization with TfR would point towards increased recycling of GHR as possible explanation for accumulation. GHR expressing Hek293 were stimulated with doxycycline to increase SUMO3 production. Next, cells were stimulated for 30 min with Cy3GH, washed and chased for another 30 min in media containing Tf. Results were analyzed under the microscope. As can be seen in Fig. 4 there is no increase in co-localization between GH (red) and Tf (green) upon SUMO3 overexpression.

Our results show that excess of sumo is affecting neither EGFR nor TfR amounts and localization. Upon SUMO3 overexpression endocytosis of GHR is not inhibited and there is no increase in targeting GHR to recycling route. Thus, the data indicate that excess sumo(ylation) specifically affects the GHR sorting machinery at endosomes, possibly at the level of MVBs resulting in GHR accumulation.

SUMO3 overexpression increases GHR in the nucleus

Since SUMO2/3 overexpression causes accumulation of GHR and sumoylation plays a role in nuclear translocation, we investigated whether exogenous SUMO might alter Cy3GH localization. Previously, it has been reported that GHR may localize to the nucleus (reviewed in [52]). This phenomenon has been well documented for EGF receptor [14, 16]. The data show that it takes 2 h for the EGFR to translocate to the nucleus upon EGF stimulation. Therefore, we stimulated EGFR-expressing U2OS cells with EGF for 30 min and chased for 90 min. Likewise, we stimulated GHR-expressing Hek293 cell with Cy3GH. Both cell lines were transiently transfected with SUMO3. As can be seen in Fig. 5A, nuclear localization of EGFR and GHR is similar. It has been suggested before that GHR might utilize the same route as EGFR on the way to nucleus.

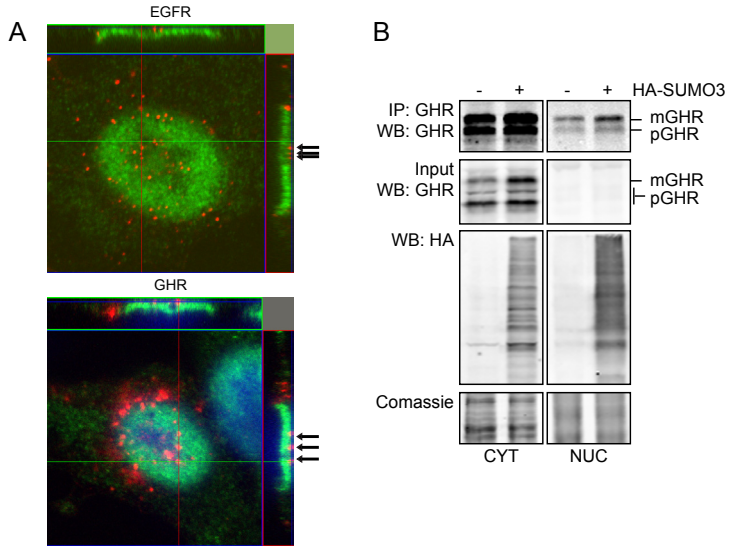

Figure 5. SUMO3 overexpression increase amount of nuclear GHR. **A.** *Cell lines were transiently transfected with SUMO3. To assess nuclear localization of EGFR, U2OS cells stably expressing EGFR were cultured on cover slips, stimulated with Rodamine-EGF (red) for 2 h and fixated with methanol. To access GHR nuclear localization, Hek293 stably expressing GHR were cultured on cover slips and stimulated for 2 h with Cy3GH (red) prior to fixation with methanol. In both cases nucleus was stained with primary anti-histon H3 antibody and secondary Alexa647-conjugated antibody (green). Z-stack pictures were made under confocal microscope.* **B.** *GHR expressing Hek293 were transiently transfected with HA-SUMO3. Cytoplasmic-nuclear fractionation was performed and anti-GHR immunoprecipitation were made in denaturing conditions.*

Next we asked whether SUMO3 overexpression alters GHR localization, targeting it to the nucleus. GHR expressing Hek293 were transiently transfected with HA-SUMO3 and cytosolic/nuclear fractionation was performed (Supplementary Fig. 1C). Anti-GHR immunoprecipitations were performed and samples were analyzed on Western Blot. As can be seen in Fig. 5B, there is a visible increase in GHR nuclear localization comparing to non transfected sample. This data suggest that upon SUMO3 overexpression there is an increased targeting of GHR to the nucleus which could partially explain accumulation of the receptor. However, our results need further testing and have to be treated as preliminary data.

Discussion

In this study we show that GHR, which is not subjected to sumoylation, accumulates upon overexpression of SUMO2/3. Additionally, we show that Ubc9 silencing gives the same phenotype. GHR accumulation is Jak2-independent and is specific for GHR. Metabolic labeling showed that exogenous SUMO3 causes a clear accumulation of the receptor without interfering with synthesis, indicating inhibition of degradation. Our results also show that endocytosis of GHR is not inhibited without an increase in receptor recycling back to the cell surface. Our preliminary study also shows that SUMO3 overexpression might direct part of the Cy3GH-linked GHR to the nucleus. Together, we provide evidence that excess of SUMO2/3 specifically inhibits degradation of the GHR

affecting neither EGFR and nor Tf transport. Very likely, excess of SUMO2/3 increases nuclear translocation, aspecifically, for both EGFR and GHR.

The only GHR-interacting protein known to be sumoylated is Jak2 tyrosine kinase (Sedek and Strous, in preparation). Although the SUMO3-induced accumulation of GHR was Jak2 independent (Fig. 2B), the results do not formally exclude that Jak2, sumoylated or not, may affect the homeostasis of the GHR.

Our data suggest that the mechanism of receptor accumulation is specific for GHR. This is supported by the observation that excess of SUMO3 does not seem to alter the morphology of the endosomal system. Amount and localization of both TfR and EGFR was not affected by SUMO3 overexpression. Different selection mechanisms of GHR, EGFR and TfR, both at the cell surface and in endosomes, may explain differences in phenotypes. Since GHR endocytosis is not inhibited, and there is no increase in targeting of the receptor to the recycling route, the data suggest that GHR is accumulating due to inhibition of degradation. In this scenario accumulation of GHR is caused by affected sorting at the level of MVBs that does not allow proper targeting to lysosomes.

Both upregulation and downregulation of sumoylation machinery caused GHR accumulation which indicates that the underlying mechanism may be similar. Accumulation of GHR after Ubc9 depletion suggests a possible scenario. Since GHR itself seems not to be sumoylated, a yet to be identified factor needs to be modified for the proper sorting of receptor at MVBs. Several candidate proteins might be involved in this event. Two E3 ubiquitin ligases, βTrCP2 and CHIP are required for GHR endocytosis but also for proper sorting at the level of MVBs [5] (Thesis Slotman). Additionally, two members of ESCRT machinery – Tsg101 and Hrs - have been implicated in GHR sorting to lysosomes [5]. Both βTrCP2 and Tsg101 depletion caused increased targeting of GHR to the recycling route. Depletion of Hrs caused a general trafficking defect that affected both the TfR and GHR route. Under these conditions, GHR could not be properly sorted to lysosomes. A third factor, the ubiquitin ligase, Triad1, has also been shown to be important for GHR sorting at MVBs (Hassik et al., submitted). Depletion of Triad1 affected both GHR and EGFR sorting. None of these proteins have been reported to be a substrate for sumoylation machinery. Since βTrCP2 seems to act specifically on GHR, it is a potential candidate for being a sumo substrate itself. However, preliminary data suggest that βTrCP2 is not sumoylated (data not shown). Until now, sumoylation have not been implicated in receptor trafficking at the level of endosomes. However, since the ubiquitylation pathway is to a large extent involved in sorting at the level of MVBs, it is conceivable to assume that the sumoylation pathway is equally important in a yet to be identified way. Our data imply that the ESCRT machinery proteins and other proteins acting at the level of MVBs are potential substrates for the sumoylation machinery. Therefore, our study shows the importance for the screening of sumoylation of proteins modulating protein trafficking.

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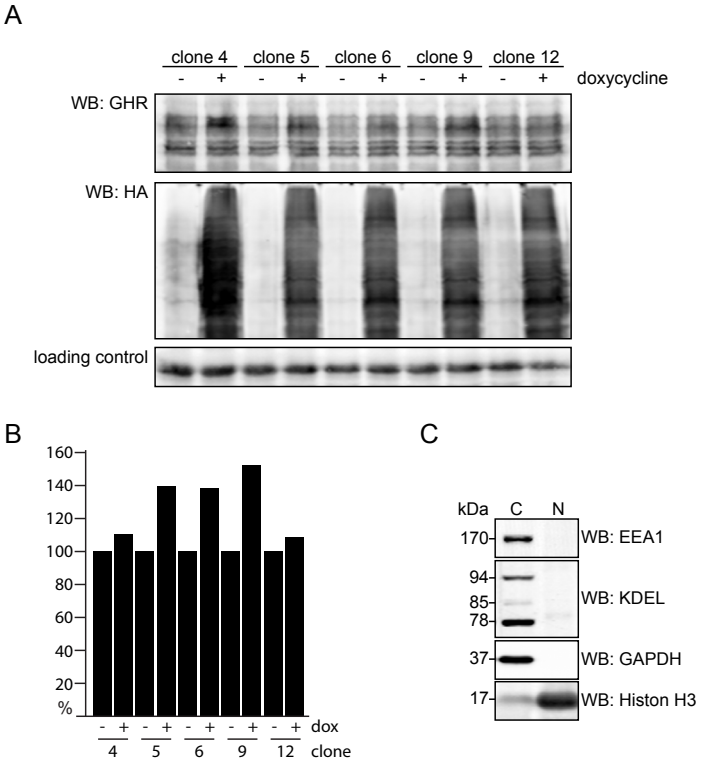
Supplementary data



Supplementary Fig. 1. **A.** *Hek293 cell line stably expressing GHR was transfected with plasmid coding for HA-SUMO3 under doxycycline stimulating promoter. Several clones were picked and tested. Clones were treated or not with doxycycline and amount of GHR and SUMO3 were accessed with anti-GHR and anti-HA antibodies.* **B.** *Graph representing relative amounts of GHR from part A. Clone 9 was picked and used in experiments.* **C.** *Several markers of fractionation purity were checked. Hek293 cells were fractionated and analyzed for the presence of markers of the early endosomes (EEA1), the ER (KDEL), the cytoplasm (GAPDH) and nuclei (histone H3).*

CHAPTER 6

Summarizing discussion

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Summarizing discussion

Since GHR is a class I cytokine receptor lacking enzymatic activity, it needs Jak2 kinase activity in order to perform signal transduction upon GH stimulation. GHR/Jak2 signaling regulates many important processes in the cell and its deregulation often leads to malignant transformation. Thus, tight control of their actions is crucial for cells. Jak2 is a binding partner for several other cytokine receptors except GHR. Because there are only 4 Jak2 family members that serve several dozens of cytokine receptors, understanding how their activities lead to specific downstream effect is crucial for future strategies to cure illnesses related to immunity and cancer. For Jak2 the focus is on myeloproliferative diseases and malignancies. In this thesis we analyzed how phosphorylation, sumoylation and ubiquitylation, together, orchestrate Jak2 localization and function. We took the GHR as a prototype cytokine receptor and studied the mechanism of its endocytosis as a key important factor in GH sensitivity regulation of cells.

In chapter 2 of the thesis we provide evidence that Jak2 is modified by SUMO2/3 up to high molecular weight on multiple lysines (Fig. 1). Additionally, we show that phosphorylated Jak2 is monosumoylated as depicted in the cartoon of Fig. 1, stage 1. It is thought that Jak2 adopts a close conformation, where both FERM and kinase domains are blocked, due to binding to the pseudokinase domain [1]. The first step in kinase activation is exposing the FERM domain that mediates receptor binding [2-6]. If we assume that monosumoylated Jak2, identified in Fig. 7C (chapter 2), is present in the protein complex that is isolated via a Strep-tagged GH in Fig. 4D (chapter 3, to be confirmed), we can conclude that monosumoylated Jak2 might be an intermediate in the Jak2 signalosome, present on the octameric GHR (Fig. 1-1). As such, monosumoylation might serve several functions, decoded by SUMO binding proteins via their SUMO interactive motifs. Currently, there is little known about these factors and their functions. It is conceivable that monosumoylation is required for Jak2 to unfold to be able to increase the binding to Box1 or that it is involved in the actual binding to the multimeric tails. It is also possible that it serves as a docking factor for signaling adaptors. As we identified Ubc9 as a positive factor in GHR degradation, the first possibility is most likely. Thus, we speculate that attachment of SUMO molecule to Jak2 may represent a general mechanism, where the modification changes its conformation allowing firm attachment to the receptor. In such a scenario, a drug inhibiting this modification would be a powerful tool in the treatment of Jak2 related diseases, especially the ones caused by kinase hyperactivity including myeloproliferative disorders.

As shown in chapter 2, polysumoylation of Jak2 can be induced both by GH stimulation and by certain stressors (Fig. 1-7). GH binding causes full activation of the kinase due to changes in Jak2 conformation itself [1, 7, 8]. We speculate that this open structure may serve as a substrate for the sumoylation machinery (Fig. 1-3). Since GH stimulation can also be considered a stressor, the mechanism of Jak2 polysumoylation may be similar in both cases. We hypothesize that stress causes Jak2 transition into a (partially) open conformation, which allows binding of SUMO E3. Probably, a small fraction of Jak2 is sumoylated in the cell without any additional stimuli. As sumoylation is often connected with phosphorylation, we might speculate one step further. In addition to being a tyrosine kinase that can phosphorylate itself on tyrosine residues, Jak2 is also constitutively phosphorylated on serine 523, which inhibits its kinase activation [9, 10]. It

has been proposed that this weak inhibition can be overcome by addition of ligand [11]. It is possible that Jak2 phosphorylation on serine 523 may stimulate polysumoylation, leading to low levels of sumoylated Jak2, present in the cell in the absence of external stimuli. Since polysumoylated Jak2 is not present on the receptor, we speculate that modification of the kinase with sumo chains serves as a mechanism that keeps Jak2 in an inactive state, non-responsive to cytokine stimulation. Thus, polysumoylation during cytokine signaling would serve as one of the mechanism to terminate the signaling cascade. Modification with polysumo chains in response to stresses would prevent uncontrolled activation of kinase in the situation, where cell first has to cope with the stressor.

A second important feature of our study is that polysumoylation facilitates Jak2 translocation to the nucleus (Fig. 1-6). Although we cannot fully exclude that sumoylation prevents Jak2 from being exported from the nucleus, a nuclear targeting function for the polysumo chains on Jak2 is most obvious. In such a scenario, specific stressors would be able to downregulate Jak2-related cytokine receptor signaling by decreasing the availability of Jak2 in the cytosol. Whether a comparable strategy also applies for the other three Jak family members remains to be investigated. Although we did not probe the reversibility of the system, nuclear segregation via polysumoylation offers a reversible mechanism to the cell to respond to specific stresses. As Jak2 is a stable protein it is not likely that stress-induced polysumoylation leads to proteasomal degradation.

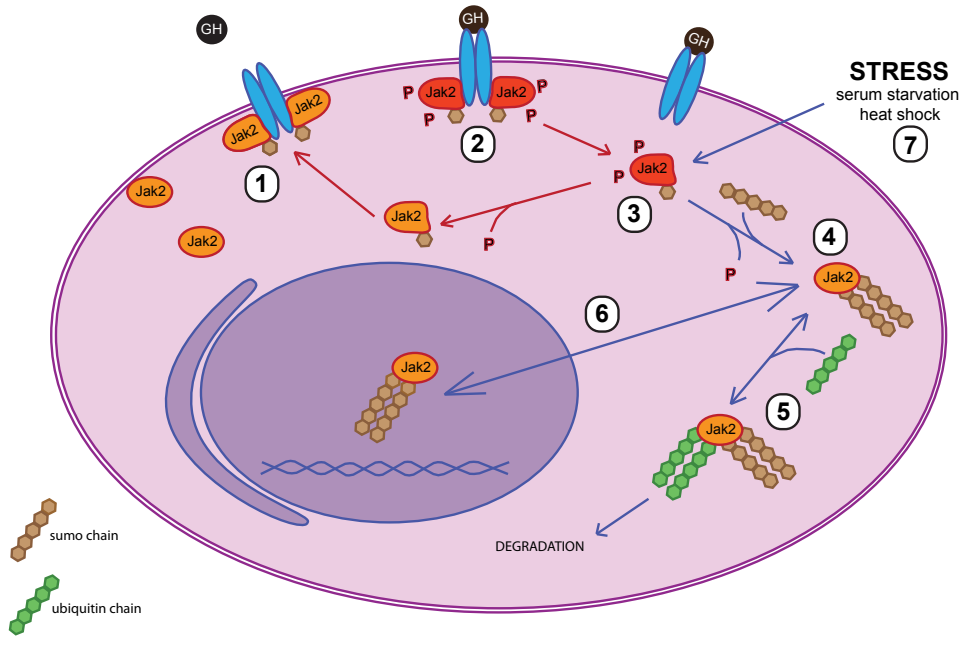

Figure 1. Schematic representation of a model of Jak2 regulation via sumoylation.
1. Jak2 in an open conformation due to monosumoylation binds the receptor. 2. Upon GH stimulation, monosumoylated Jak2 transphosphorylates itself and the GHR, and initiates the signaling cascade. 3. Monosumoylated phosphorylated Jak2 detaches from the receptor. Jak2 can become dephosphorylated and take part in another round of receptor activation. 4. Alternatively, sumo chains are added to the kinase (polysumoylation), which makes it signaling-defective. 5. Sumoylation stimulates polyubiquitylation of Jak2 which leads to its proteasome-dependent degradation. 6. Polysumoylation serves as a signal for nuclear translocation. 7. Independently of cytokine stimulation, multiple stresses stimulate Jak2 polysumoylation.

However, it remains to be investigated how the cells regulate the balance between nuclear translocation and degradation of Jak2 (Fig. 1-5).
Aberrant Jak2 signaling has been reported in many malignancies [12-18]. Dawson et al found that Jak2 translocates to the nucleus where it phosphorylates histon H3 on Y41 (H3Y41) which leads to increased expression of hematopoietic oncogene LMO2 [18]. They showed that inhibition of Jak2 activity in human leukemic cells decreases both the expression of the LMO2 and the phosphorylation of H3Y41. Additionally, it was reported that Jak2 V617F, bearing the most common mutation found in patients with myeloproliferative diseases that leads to constitutive activation, is found more often in the nucleus of hematopoietic cells [17]. The selective Jak2 inhibitor was able to chase the kinase back to cytoplasm and normalized LMO2 levels in vitro. The authors suggested that blocking Jak2 nuclear translocation could be a new treatment strategy for patients bearing V617F mutation. Those results indicate that nuclear translocation of the kinase may be mainly responsible for the symptoms of myeloproliferative diseases. This goes in line with our results showing increased nuclear localization of V617F mutant. However, our results indicate that nuclear translocation is connected with sumoylation rather than phosphorylation. Alternatively Jak2 activation increases sumoylation and nuclear translocation of mutant Jak2. Indeed, we show an increased sumoylation of V617F, which is likely due to constitutive phosphorylation. Thus, a drug that interferes with Jak2 polysumoylation would inhibit nuclear translocation and might be equally efficient as drugs that inhibit the kinase activity. Additionally, the kinase would still be able to perform its cytoplasmic actions that may be beneficial for the cells.
In chapter 3, we analyzed GHR complexes with a novel method, 2D BN/SDS-PAGE. GHR is synthesized in the ER, where the receptors dimerize (Fig. 2-0) [19]. However, our results indicate that immature receptor complexes can also exist as tetramers and higher molecular complexes. The smallest GHR complex present on the plasma membrane is a tetramer (Fig. 2-2). There are also many higher molecular weight GHR complexes present on the plasma membrane that probably represent an array of GHR in complexes with ancillary proteins, like Jak2, βTrCP2, CHIP, phosphatases, DUBs and others (Fig. 2-1). Additionally, we show that GH binding on ice, rather than activation, is sufficient to cause dimerization of the tetramer (Fig. 2-3,4). Both complexes seem to be endocytosis-competent (Fig. 2-6). This finding has consequences for our perception of the functionality of both the ligand binding and the intracellular domains. The experiments with proteinase K show that the specificity of the multimerization does not depend on the GH binding domain.
The effect of GH binding remains enigmatic. Previous binding studies with 125I-GH showed a stoichiometry of two GHR tails for one GH [20, 21]. In the experiments of chapter 3 there are two striking effects of GH: Firstly, its binding brings about the disappearance of the higher heterogeneous complexes that might happen on ice, and, secondly, the disappearance of the tetrameric complex that requires incubation at 37ºC. The first effect (GH binding on ice) probably represents the detachment of ancillary proteins from the receptor tails. As it is unlikely that membrane fluidity at 0ºC allows GH binding to induce conformational changes of the cytosolic tail, it is feasible that the ancillary proteins at the receptor tails detach from the membrane due to energy shortage (low ATP levels). This is in line with our previous observation that rapid GHR deubiquitylation occurs, if cells are kept in ice. The second observation is fascinating.

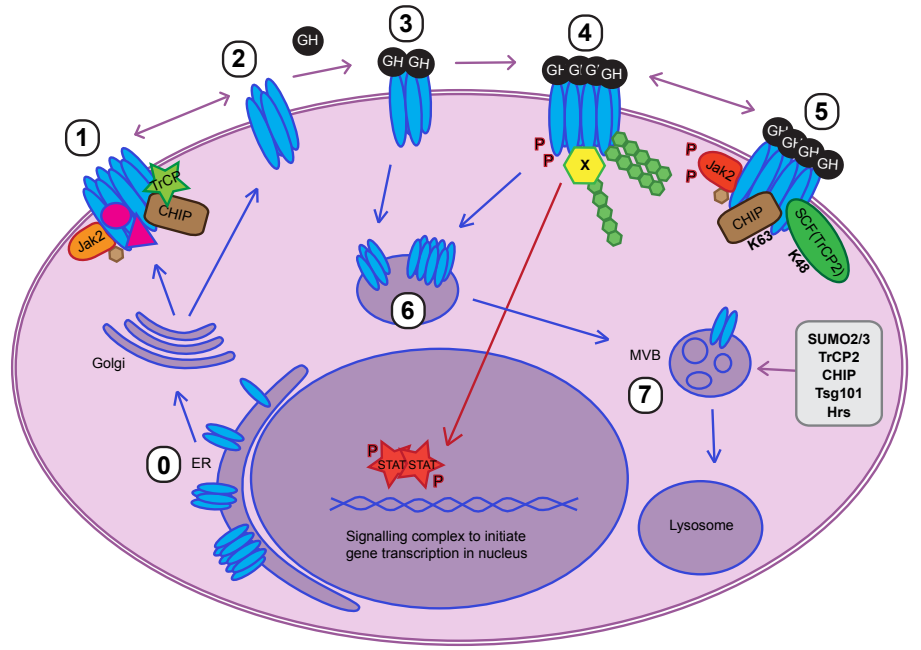

Figure 2. Model of GHR trafficking.
0. GHR is synthesized in the ER where receptor dimers, tetramers and octamers are formed. 1. Tetramers and higher molecular weight GHR complexes on plasma membrane have attached ancillary proteins. 2. The smallest GHR complex on the plasma membrane is a tetramer. 3. Upon GH stimulation GHR tetramers dimerize to octamers. 4. Phosphorylated receptors trigger signaling cascade via STAT5/MAPK. Receptors within octamers are phosphorylated and ubiquitylated. Presumably yet to be identified factor X has to be ubiquitylated and degraded in proteasome. 5. Binding of enzymes to GHR octamer is transient. Jak2 catalyzes GHR phosphorylation, SCFTrCP2 makes K48 and CHIP K63-linked ubiquitin chains. 6. Both GHR tetramers and octamers are endocytosis-competent. 7. Receptors are targeted to MVB and subsequently degraded in the lysosomes. Several factors are involved in GHR selection at the level of MVBs.

It shows that GH binding induces a complex that potentially can bind 4 GH molecules (Fig. 2-4). As it is a time consuming process, it is likely that there exists cooperation between the intra- and extracellular domains. It is currently unknown whether initiation of signaling is required for the dimerization. An obvious approach would be to repeat the experiment with Strep-tagged GH antagonist that has only one binding site. The stoichiometry of complex formation with the (ubiquitylating, phosphorylating and sumoylating) enzymes is another interesting problem. It is clear that they are present in the steady state. This is anticipated from the fact that complexes containing GHR with a mutated UbE binding motif are considerably smaller than the ones with a wild type GHR (chapter 3, Fig. 1).
What would be the physiological reason for GHR multimerization? Each GHR molecule contains one Jak2 binding site - Box1, one βTrCP2 binding site - Box2 (UbE motif) and one CHIP binding site [22] (Slotman et al., submitted). Additionally, βTrCP2 acts in a SCF complex together with Skp1, Cul1, Rbx1, Nedd8 and an E2 [23]. Together, they create a big complex that is thought to be subject to dimerization [24-26]. Therefore, accommodation of all enzymes on GHR molecule may remain a challenge. Dimerization could serve as a mechanism to increase the binding surface on receptor molecules. GHR octamer and tetramer could easily accommodate all the factors, Jak2, SCFβTrCP2 and CHIP at the

same time (Fig. 2-5). We show that binding of enzymes to GHR complexes is transient. It is also possible that receptors bind one enzyme at a time. Only receptors present in octamers are subjected to phosphorylation and ubiquitylation, which suggests that only the octameric GHR complex can serve as a bona-fide signaling platform (Fig. 2-4). The interesting question is whether different GHR molecules within octamer are subject to phosphorylation and different to ubiquitylation. Our results indicate that ubiquitylated GHRs are not phosphorylated. Further studies are needed to fully elucidate this matter.
The analysis of GHR complexes on 2D BN/SDS-PAGE after overexpression of K63R and K48R mutant ubiquitin reveals that both chains are involved in GHR endocytosis and indicate that the receptor can be modified by both types of ubiquitin chains. The function of receptor ubiquitylation has not been elucidated yet. 399K-less GHR mutant lacking all lysine residues is normally endocytosed [20]. However, GHR internalization depends on a functional ubiquitin-proteasome system [27]. It has been shown that the GHR is ubiquitylated by K48-linked chains [28]. This is in line with the finding that βTrCP2, necessary for GHR endocytosis, is responsible for making K48 chains and is able to directly ubiquitylate the receptor [29] (da Silva Almeida, manuscript in preparation). A possible scenario implies that an unknown factor (X) has to be ubiquitylated in order for receptor to be endocytosed (Fig. 2-4). In this thesis we have analyzed GHR complexes of K399K-less mutant GHR. Even though this mutant is not ubiquitylated, we could observe a weak ubiquitylation signal in receptor complexes. Whether the ubiquitylation signal comes from such a factor (X) remains to be determined. In our group we identified CHIP as a second E3 ubiquitin ligase, necessary for GHR endocytosis (Slotman et al., submitted). This ligase creates K63-linked polyubiquitin chains and is able to ubiquitylate GHR in vitro (Thesis Nespital). However, how those two types of ubiquitylations together orchestrate GHR endocytosis remains to be determined.
Our study, showing how sumoylation regulate Jak2 function, contributes to the development of new drugs both against GH/GHR related malignancies and Jak2 driven disorders. Our experience with optimizing 2D BN/SDS-PAGE method to study GHR complexes may be used in the future to elucidate the mechanisms of other membrane receptor-driven signaling pathways.

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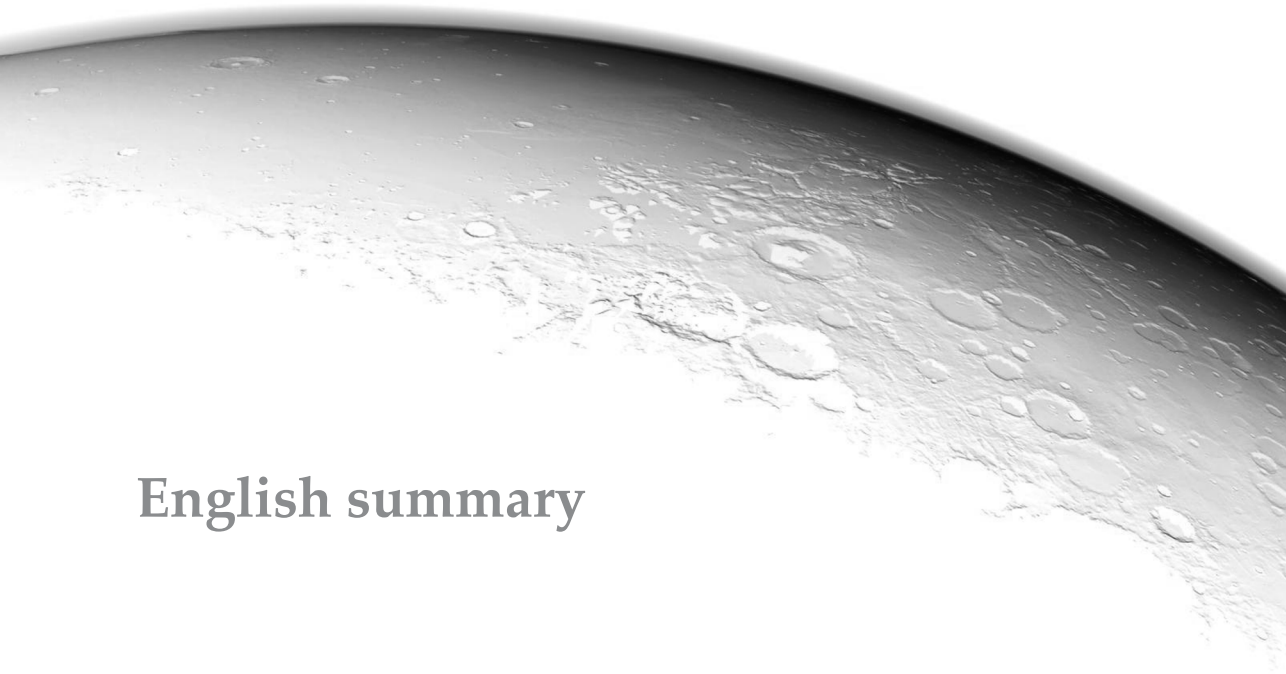


English summary

English summary

Growth hormone (GH) is the main regulator of human growth. The substance is secreted by an anterior lobe of a pituitary gland. Insufficient amount of GH secreted in the childhood leads to dwarfism and excess causes a condition characterized by excessive growth called acromegaly. Apart from that GH regulates other important processes in mammals like carbohydrate and lipid metabolism, muscle growth, fertility, immunity, cardiac development, ageing and others.

In order for the cells to be able to respond to GH in the blood they need a growth hormone receptor (GHR) on their surface. GHR is a protein that protrudes through the cell membrane. Almost all cells of the body produce GHR. Circulating GH binds to the part of GHR that is localized outside the cells. This binding causes the part of GHR that is inside the cell to change shape what induces signaling cascade. This change in shape activates Janus kinase 2 (Jak2) which phosphorylates GHR, Jak2 itself and certain members of STAT family of transcription factors (STAT5). Next, STATs form dimers and translocate to the nucleus where they initiate transcription of specific proteins. Those proteins are necessary for GH-dependent actions. With sufficient nutrition, GH together with IGF-1 promotes the preservation of muscle proteins. During fasting the burning of the fat is stimulated. As a result, the protein degradation is prevented allowing the body to stay strong. Extensive GH stimulation leads to insulin resistance (diabetes mellitus) and cancer. The intensity of GH signaling depends on amount of GHR present at the surface of the cells. Our research shows that during constant synthesis the number of receptors is regulated by its degradation. Receptors on the surface are continuously taken inside the cell in special vesicles in a process called endocytosis. Next, GHRs are transported via endosomes to lysosomes for ultimate destruction. The endocytosis process of GHR is controlled by two posttranslational modifications: ubiquitylation by ubiquitin ligase complex $SCF^{\beta TrCP2}$ and CHIP, and phosphorylation by Jak2.

In this thesis I focused on posttranslational modifications of proteins that regulate the sensitivity of cells to GH. These types of modifications regulate many important features of proteins. Attachment of relatively small phosphor or acetyl group can lead to protein activation or deactivation, respectively. However, also attachment of whole proteins is common in cellular systems. Attachment of a protein called ubiquitin very often serves as degradation signal and attachment of a protein called SUMO (family of ubiquitin) can stimulate change in protein localization but also activity. In this thesis I studied how those modifications, alone and together, orchestrate the function of GHR and Jak2.

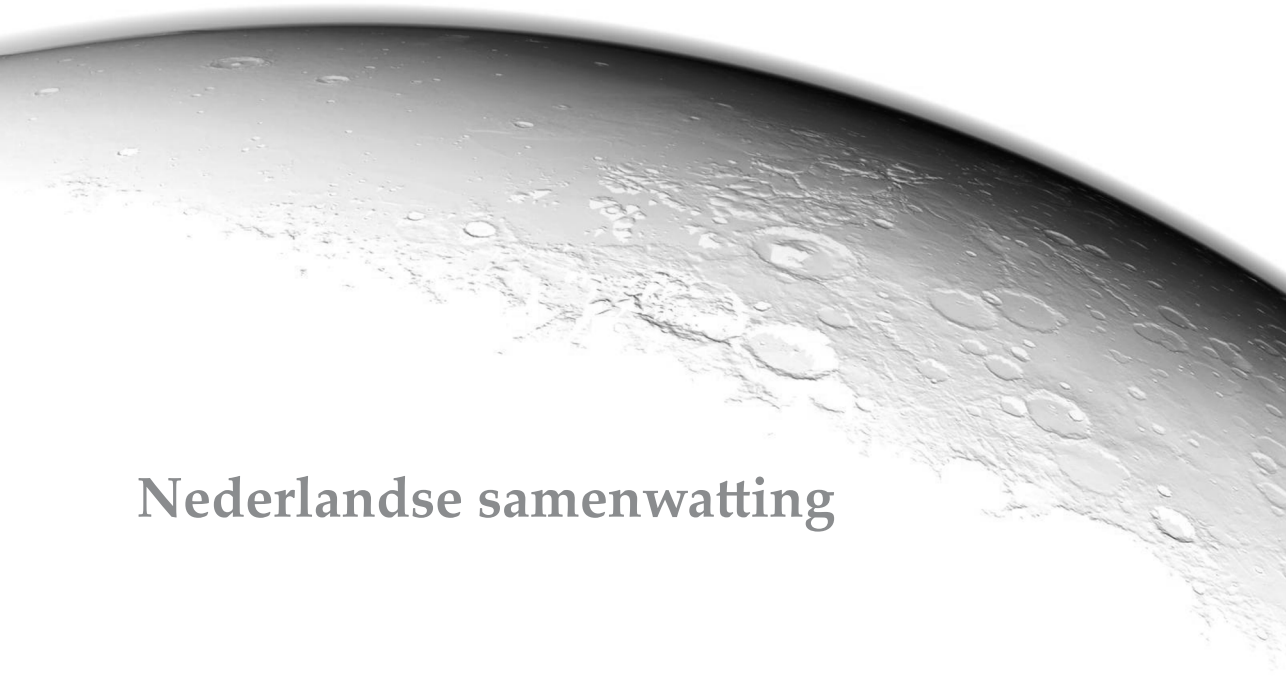
Jak2 is mainly found in the cytoplasm of the cells where it associates with proteins (receptors) located at the cell surface. However, small fraction of kinase can translocate to the nucleus and activate gene transcription which in turn may lead to cancer transformation. Mutations in Jak2 causing its constitutive activity lead to blood disorders (myeloproliferative diseases) like polycythemia vera (PV), essential thrombocytosis (ET) and idiopathic myelofibrosis. It has been proposed that nuclear fraction of constitutively active V617F Jak2 is especially malignant. Therefore interfering with nuclear translocation would be beneficial for patients. In the thesis for the first time I show that Jak2 is SUMOylated. High molecular weight sumoylation regulates Jak2 shuttling between nucleus and cytoplasm. Thus, a drug inhibiting kinase sumoylation

and nuclear translocation would provide new therapeutic strategy for patients suffering from myeloproliferative neoplasms. Additionally our study showed that Jak2 is monoSUMOylated and we hypothesize that this modification regulates kinase binding to the receptor. Since Jak2 in the absence of GH stabilize the GHR, and in presence of GH promotes its degradation, sumoylation can be a crucial mechanism regulating GHR signaling. Our results indicate a previously unknown role of SUMO as Jak2 regulator which needs to be further investigated.

In chapter 5 I show that sumoylation is also involved in GHR degradation. Excess of SUMO causes GHR accumulation on the plasma membrane and inside the cell. However neither synthesis nor endocytosis rate are affected. This phenomenon is independent of Jak2 but it cannot be excluded that presence of kinase is contributing to this effect. My results suggest that the mechanism of receptor accumulation is specific for GHR and not for other signaling receptors. I provide evidence that GHR segregation in endosomes is affected, which leads to delayed degradation in lysosomes. The data show that ESCRT proteins that take part in proper segregation of proteins in endosomes can be potential substrates of sumoylation machinery.

Many cancer and AIDS patients suffer from uncontrolled muscle wasting condition called cachexia due to insufficient amount of GHRs at the plasma membrane of their cells. This in turn causes insensitivity of the cells to GH leading to high amount of circulating hormone in the blood. Opposite situation (high amount of GHRs at the cell surface and/or high production of GH by pituitary gland or other cells in the body) results in cancer. Therefore it is important to understand how the availability of the receptor for GH signaling is regulated. This important knowledge can be obtained by studying posttranslational modifications of the proteins working together in large protein complexes that regulate GH sensitivity. In the thesis I optimized the existing technique “blue native electrophoresis” (BN-PAGE) to analyze GHR complexes at early stages of receptor endocytosis. In this method Coomassie Brilliant Blue is used that binds nonspecifically to protein complexes giving them negative charge without causing denaturation. Thus the complexes can be separated according to their masses. Combining BN-PAGE with second dimension SDS-PAGE allows analysis of proteins within the complexes with mass spectrometry or western blotting. Cells were stimulated with GH containing Strep-tag that allowed me to pull down the receptor complexes on beads and elution without disruption. I identified two main GHR complexes at the plasma membrane whose sizes correspond to tetramers and octamers, respectively. Upon GH binding the tetramer dimerized to an octameric complex. Only receptors within the octameric complex were subjected to posttranslational modifications: ubiquitylation and phosphorylation. Additionally, both Jak2 and a component of ubiquitin ligase complex, βTrCP2, formed transient high molecular weight complexes presumably with octameric GHRs. Together, my data obtained with BN-PAGE are an important confirmation of earlier results obtained by colleagues from Prof. Strous group. Apart from that they offer a new and fascinating insight into the mechanism underlying the biosynthesis and degradation of GHR. Since in BN-PAGE stoichiometry of different proteins within the protein complexes is preserved, it provides better insight into the functioning of signaling and membrane transport systems than simple pull down techniques. The results contribute to the understanding of the mechanism of GHR endocytosis and in the future may help to design new treatments for patients.

The results of this thesis represent a significant progress in the understanding of how posttranslational modifications regulate GHR and Jak2 function. My findings can be applied to design new therapeutic strategies for patients with myeloproliferative diseases and GHR/Jak2 related malignancies.



Nederlandse samenwatting

Nederlandse samenvatting

Groeihormoon (GH) is nodig voor de lengtegroei van het menselijk lichaam. Het wordt gemaakt in de voorkwab van de hypofyse. Onvoldoende productie van het hormoon gedurende de jeugd leidt tot dwerggroei, terwijl een overmatige hoeveelheid GH acromegalie veroorzaakt. Behalve groei is GH ook betrokken bij andere belangrijke processen zoals koolhydraat- en vetstofwisseling, spiergroei, vruchtbaarheid, afweer, de ontwikkeling van het hart en verouderingsprocessen.

Om te kunnen reageren op GH in het bloed hebben cellen de groeihormoonreceptor (GHR) nodig aan hun oppervlak. GHR is een eiwit dat door het celmembraan heen steekt. Vrijwel alle cellen van het lichaam maken GHR. Circulerend GH bindt aan het gedeelte van de GHR, dat zich aan de buitenkant van de cellen bevindt. Deze binding aan de buitenkant zorgt ervoor dat het GHR-gedeelte, dat binnenin zit, van vorm verandert, waardoor er een signaal ontstaat in de cel. Deze vormverandering activeert Janus kinase 2 (Jak2), waardoor de GHR, Jak2 zelf, en bepaalde leden van de STAT-familie van transcriptiefactoren (STAT5) gefosforyleerd worden. De STATs vormen daardoor dimeren, verhuizen naar de celkern en activeren de transcriptie van specifieke eiwitfactoren. Deze factoren zijn noodzakelijk voor de GH-afhankelijke acties. Bij voldoende voeding bevordert GH, samen met IGF-1, het behoud van spiereiwitten. Bij vasten wordt de verbranding van vet wordt bevorderd. Het gevolg is dat eiwitafbraak wordt voorkomen, waardoor het lichaam krachtig blijft en vet wordt verbrand. Teveel GH signalering zorgt voor insuline-ongevoeligheid (diabetes mellitus) en kanker. De intensiteit van de GH signalering hangt in belangrijke mate af van het aantal GHRs dat zich op een cel bevindt. Uit ons onderzoek blijkt dat, bij gelijkblijvende synthese, dit aantal wordt geregeld door middel van afbraak van de GHR. Receptoren aan het celoppervlak worden voortdurend naar binnen gehaald door middel van afknoppende blaasjes middels een process dat endocytosis wordt genoemd. Vervolgens worden de GHRs via endosomen naar lysosomen vervoerd en afgebroken. Het endocytose-proces van de GHR wordt meticuleus geregeld door twee posttranslationele modificaties: ubiquitylering middels de ubiquitineligases $SCF^{\beta TrCP2}$ en CHIP, en fosforylering middels Jak2.

In de hier beschreven onderzoeken heb ik me bezig gehouden met de posttranslationele modificaties van eiwitten die de gevoeligheid van cellen voor GH regelen. Dit soort modificaties regelen talrijke belangrijke functies van eiwitten. Aanhechting van een relatief kleine fosfosfaat- of acetylgroep kan het ene enzym activeren en het andere de-activeren. Ook aanhechting van hele eiwitten komt veelvuldig voor in cellulaire regelsystemen. Zo kan ubiquitylering o.a. werken als een afbraaksignaal terwijl de aanhechting van SUMO (een familielid van ubiquitine) kan leiden tot locatieverandering van een eiwit, maar ook tot functieverandering. In dit proefschrift heb ik bestudeerd hoe deze modificaties, alleen of samen, de functies van GHR en Jak2 regelen.

Jak2 komt vooral voor in het cytoplasma van cellen waar het zich kan binden aan eiwitten (receptoren) die zich aan het celoppervlak bevinden. Een klein gedeelte van de Jak2 moleculen kan naar de kern verhuizen en gentranscriptie activeren, waardoor kanker kan ontstaan. Mutaties in Jak2, die de kinase activiteit continue aanzetten, leiden tot

bloedziektes (myeloproliferatieve neoplasmen) zoals polycythemia vera (PV), essentiele thrombocythemia (ET) en idiopathische myelofibrosis. Vooral de nucleaire fractie van de V617F Jak2 mutatie wordt als bijzonder maligne beschouwd. Het tegengaan van de Jak2-verplaatsing naar de celkern zou wellicht al helpen bij deze patiënten. In dit proefschrift laat ik voor het eerst zien dat Jak2 wordt geSUMOyleerd. Hoogmoleculaire sumoylering blijkt de migratie van Jak2 tussen kern en cytoplasma te regelen. Een drug, die in staat is Jak2 sumoylering te remmen, zou wellicht de translocatie naar de kern kunnen voorkomen en daarmee kunnen dienen als een therapeuticum voor patiënten, die lijden aan myeloproliferatieve neoplasmen. Uit onze studie met SUMO bleek ook dat Jak2 kan worden "gemonoSUMOyleerd". Dit leidt tot de hypothese dat monoSUMOylering the binding van Jak2 aan de receptor regelt. Omdat Jak2, in afwezigheid van GH, de GHR stabiliseert en in aanwezigheid van GH zijn afbraak stimuleert, kan SUMOylering een cruciaal regelmechanisme vormen. Ons resultaat met mono-geSUMOyleerde Jak2 wijst op een nog onbekende rol van SUMO als Jak2-regulator, die nog verder moet worden uitgezocht.

In hoofdstuk 5 laat ik zien dat SUMOylering ook betrokken is bij de afbraak van de GHR. Overmaat SUMO veroorzaakte accumulatie van GHR aan het celoppervlak en in de cel. Noch synthese- noch endocytosesnelheid waren echter veranderd. Dit fenomeen was Jak2-omafhankelijk. Het kon echter niet worden uitgesloten dat de aanwezigheid van Jak2 in de cel aan dit effect bijdroeg. Mijn resultaten suggereren dat het mechanisme van receptor-accumulatie alleen voor de GHR geldt en niet voor andere sinalerende receptoren. Ik laat ook zien dat hoge cellulaire concentraties van SUMO de GHR segregatie in de endosomen verstoort de met een vertraagde afbraak in lysosomen als gevolg. De experimenten tonen aan dat ESCRT eiwitten, die het segregatieproces in endosomen bewerkstelligen, mogelijk zelf geSUMOyleerd kunnen worden.

Veel kanker- en AIDS patiënten lijden aan ongecontrolleerde spierafbraak, ook wel cachexia genoemd. Dit gaat gepaard met lage hoeveelheden GHR aan het oppervlak van cellen, waardoor GH-ongevoeligheid ontstaat, hetgeen weer leidt tot hoge GH spiegels. De tegenovergestelde situatie (veel GHRs aan het celoppervlak en/of hoge GH-productie in de hypofyse of andere cellen in het lichaam) kan leiden tot celtransformatie en kanker. Daarom is het van groot belang te begrijpen hoe de gevoeligheid van cellen voor GH wordt geregeld. Belangrijke kennis hiervoor kan worden verkregen door de studie van de posttranslationele modificaties, die eiwitten ondergaan, die samenwerken in de grote eiwitcomplexen, die de GH gevoeligheid regelen. In dit proefschrift heb ik de bestaande techniek "blue native electrophoresis" (BN-PAGE) geoptimaliseerd en aangepast voor de analyse van GHR bevattende eiwitcomplexen op het moment van endocytose. Bij deze methode worden eiwitcomplexen verzadigd met Coomassie Brilliant Blue, waardoor ze intact blijven en van een negatief geladen worden voorzien. De complexen kunnen dan gescheiden worden op grond van hun massa. Door in de tweede dimensie SDS-PAGE toe te passen kan dan de samenstelling van de complexen worden bestudeeerd middels massa spectrometrie of western blotting. Cellen werden daarvoor gestimuleerd met GH, dat voorzien was van een Strep-tag, die het mogelijk maakte om de GHR-bevattendende eiwitcomplexen door middel van affiniteitschromatografie te isoleren met behoud van hun integriteit. Ik heb twee GHR-complexen geïsoleerd vanaf het celmembraan, die qua grootte overeenkwamen met tetramere end octamere GHR complexen. GH-binding bleek, zelfs bij 0°C, in staat het tetramere complex om te zetten in

de octamere vorm. Alleen receptors in het octamere complex werden posttranslationeel gemodificeerd middels ubiquitylering en fosforylering. Ook Jak2 en de ubiquitine-ligase factor, βTrCP2, vormden hoog-moleculaire complexen, hoogstwaarschijnlijk alleen met octameer GHR. Mijn resultaten, verkregen met BN-PAGE, vormen een belangrijke bevestiging van vroegere resultaten, verkregen door medewerkers van de groep van Prof. Strous. Bovendien bieden ze nieuw en facinerend inzicht in de mechanismen die ten grondslag liggen aan de biosynthese- en afbraakmechanismen van de GHR. Doordat de BN-PAGE methode de stoichiometrie van de verschillende eiwtten in eiwitcomplexen intact laat biedt ze duidelijk meer inzicht in het functioneren van signalering- en membraantransport-systemen dan eenvoudige pull-down technieken. De resultaten dragen bij aan ons inzicht in de mechanismen van GHR-endocytose en kunnen in de toekomst van belang zijn bij het ontwikkelen van nieuwe behandelmethodes in de kliniek.

De resultaten, beschreven in dit proefschrift, rapporteren een significante vooruitgang in ons begrip van posttranslationele de modificaties die GHR- en Jak2-functies regelen. Mijn resultaten zullen ongetwijfeld worden toegepast bij de ontwikkeling van nieuwe therapeutische strategieën voor patiënten met myeloproliferatieve ziekten en andere GHR/Jak2 gerelateerde maligniteiten.

Podsumowanie po polsku

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Hormon wzrostu (growth hormone – GH) jest głównym regulatorem wzrostu u ludzi. Substancja jest wydzielana przez przedni płat przysadki mózgowej. Niewystarczająca produkcja GH prowadzi do karłowatości natomiast nadmar powoduje schorzenie charakteryzujące się nadmiernym wzrostem o nazwie akromegalia. Oprócz tego hormon reguluje wiele innych ważnych procesów u ssaków takich jak metabolizm cukrów i tłuszczy, wzrost mięśni, płodność, odporność, rozwój serca, starzenie się i inne.

Aby komórki mogły zareagować na GH znajdujący się we krwi, potrzebują receptora hormonu wzrostu (growth hormone receptor – GHR) na swojej powierzchni. GHR jest białkiem które wystaje przez błonę komórkową. Prawie wszystkie komórki ciała produkują GHR. Hormon krążący we krwi przyłącza się do części receptora znajdującego się na zewnątrz komórki. Przyłączenie hormonu powoduje, że cześć GHR która znajduje się w środku komórki zmienia kształt co inicjuje kaskadę sygnałową. Zmiana kształtu powoduje aktywację kinazy Janus 2 (Jak2), która fosforyluje GHR, siebie oraz niektóre białka z rodziny czynników transkrypcyjnych STAT (STAT5). Następnie, białka STAT tworzą dimery i przemieszczają się do jądra komórkowego gdzie inicjują transkrypcję białek niezbędnych do działań zależnych od GH. Przy odpowiedniej ilości pożywienia GH wraz z IGF-1 sprzyja ochronie białek mięśni. Z drugiej strony post stymuluje spalanie tłuszczów. Zapobiega to degradacji białek i pozwala organizmowi zachować siłę. Nadmierna stymulacja hormonem wzrostu prowadzi do insulinooporności (cukrzyca) oraz nowotworów. Intensywność sygnalingu zapoczątkowanego przez GH zależy od ilości GHR na powierzchni komórek. Nasze badania wskazują że przy stałej syntezie, ilość receptorów jest regulowana przez ich degradację. Receptory z powierzchni komórki są stale pobierane do wnętrza komórki w specjalnych pęcherzykach w procesie zwanym endocytozą. Następnie receptory są transportowane poprzez system endosomalny do lizosomów gdzie zostaną zniszczone. Proces endocytozy jest kontrolowany przez dwie modyfikacje posttranslacyjne: ubikwitylację wykonywaną przez kompleks ligazy ubikwitynowo-białkowej $SCF^{\beta TrCP2}$ oraz CHIP i fosforylację wykonywaną przez Jak2.

W tej pracy przedstawione są wyniki badań nad modyfikacjami posttranslacyjnymi białek które regulują wrażliwość komórek na GH. Modyfikacje posttranslacyjne regulują wiele ważnych cech u białek. Przyłączenie stosunkowo małej grupy fosforanowej lub acetylowej może prowadzić do odpowiednio aktywacji lub deaktywacji białka. Przyłączanie całych białek jest również rozpowszechnionym procesem w komórkach. Przyłączenie białka o nazwie ubikwityna bardzo często stymuluje degradację białka natomiast przyłączenie białka o nazwie SUMO (pochodzącego z tej samej rodziny co ubikwityna) może stymulować zmianę lokalizacji jak również aktywności białka. W tej pracy badałam jak te modyfikacje, odzielnie oraz razem, regulują funkcjonowanie GHR oraz Jak2.

Jak2 znajduje się głownie w cytoplazmie komórkowej gdzie przyłącza się do białek receptorowych znajdujących się na powierzchni komórki. Jednakże niewielka ilość kinazy może przemieszczać się do jądra gdzie aktywuje transkrypcję genów co może prowadzić do tranformacji nowotworowej. Mutacje powodujące konstytutywną aktywność kinazy prowadzą do chorób krwi zwanych chorobami mieloproliferacyjnymi takich jak czerwienica prawdziwa, nadplytkowosc samoistna oraz mielofibroza. Wyniki

badań pokazują że jądrowa frakcją konstytutywnie aktywnej kinazy V617F jest najbardziej chorobotwórcza. Dlatego też zablokowanie translokacji do jądra byłoby korzystne dla pacjentów. W tej pracy pokazałam po raz pierwszy że Jak2 jest SUMOylowany. Polisumoylacja reguluje transport kinazy między jądrem a cytoplazmą. Dlatego też lekarstwo blokujące sumoylację, i tym samym transport kinazy do jądra, byłoby nową strategią leczenia dla pacjentów cierpiących z powodu chorób mieloproliferacyjnych. Oprócz tego nasze badania pokazały że Jak2 jest również monosumoylowany co może odpowiadać za przyłączanie kinazy do receptora. Ponieważ Jak2 w przpadku braku GH stabilizuje GHR, natomiast w obecności GH stymuluje degradację receptora, sumoylacja może być ważnym mechanizmem regulującym sygnaling GHR. Nasze wyniki wskazują na uprzednio nieznaną rolę białka SUMO w regulacji kinazy Jak2

W rozdziale 5 pokazałam że sumoylacja jest również zaangażowana w degradację GHR. Nadmiar SUMO powoduje akumulację GHR zarówno na powierzchni jak i wewnątrz komórki. Jednakże ani synteza ani endocytoza receptora nie są zaburzone. Efekt akumulacji występuje niezależnie od obecności Jak2, jednakże nie można wykluczyć że kinaza przyczynia się do tego efektu. Moje wyniki sugerują, że mechanizm regulujący akumulację receptora jest specyficzny dla GHR ale nie dla innych receptorów. Oprócz tego, segregacja GHR w endosomach jest zaburzona co prowadzi do opóźnienia degradacji w lizosomach. Wyniki te wskazują że białka ESCRT, które biorą udział w prawidłowej segregacji białek w endosomach są potencjalnymi substatami SUMO.

Wielu pacjentów chorych na raka lub AIDS cierpi z powodu niekontrolowanej utraty mięśni. Stan ten nazywa się kacheksją i jest spowodowany niewystarczająca ilością GHR na powierzchni komórek co prowadzi do braku wrażliwości komórek na GH i wysokich ilości GH we krwi. Odwrotna sytuacja (wysoka ilość GHR na powierzchni komórek oraz/lub wysoka produkcja GH w przysadce lub innej cześci ciała) prowadzi do nowotworów. Dlatego też ważne jest zrozumienie w jaki sposób regulowana jest dostępność receptora dla sygnalingu GH. Tą ważną wiedzę można uzyskać badając modyfikacje posttranslacyjne białek działających razem w dużych kompleksach białkowych regulujących wrażliwość komórek na GH. W tej pracy zaadaptowałam niebieską elektroforezę w warunkach natywnych (blue native elektrophoresis – BN-PAGE) do analizy kompleksów białkowych receptora hormonu wzrostu na wczesnych etapach endocytozy. W tej metodzie używa się substancji o nazwie Coomassie Briljant Blue która łączy się niespecyficznie z białkami nadając im negatywny ładunek ale nie powodując ich denaturacji. Dzięki temu kompleksy białkowe mogą zostać rozdzielone w zależności od masy. Połączenie elektroforezy natywnej z dwukierunkową elektroforezą w obecności SDS pozwala dodatkowo na analizę białek znajdujących się wewnątrz kompleksów białkowych przy pomocy spektrometrii mas lub metody western błot. Komórki były stymulowane hormonem wzrostu który zawierał Strep-tag pozwalający na izolację kompleksów na kuleczkach opłaszczonych białkiem łączącym się z tym tagiem a następnie elucję w natywnych warunkach. Analiza wyników pokazała że głownie dwa kompleksy GHR znajdują się na powierzchni komórki wielkością opowiadające tetramerom i octamerom. Tylko receptory znajdujące się w oktamerze są zarówno fosforylowane jak i ubikwitynowane. Oprócz tego pod wpływem hormonu wzrostu mniejszy kompleks przechodzi w większy co sugeruje dimeryzację tetrameru. Zarówno kinaza Jak2 jak i komponent kompleksu ligazy ubikwitynowo-białkowej βTrCP2 znajdują

się jedynie przejściowo w kompleksach receptora o wysokiej masie. Podsumowując, moje wyniki uzyskane przy pomocy BN-PAGE są ważnym potwierdzeniem wcześniejszych wyników uzyskanych przez innych członków grupy Prof. Strousa. Oprócz tego oferują nowy i fascynujący wgląd w mechanizm lezący u podłoża syntezy i degradacji GHR. Ponieważ w metodzie BN-PAGE, stechiometria rożnych białek jest zachowana, dostarcza to lepszego wglądu w funkcjonowanie systemów sygnalingu i transportu membranowego niż prosta metoda izolacji na kuleczkach. Moje wyniki przyczyniają się do zrozumienia mechanizmu endoctozy GHR i w przyszłości mogą pomóc w opracowaniu nowych metod leczenia dla pacjentów.

Wyniki tej pracy stanowią znaczny postęp w zrozumieniu, jak modyfikacje posttranslacyjne regulują funkconowanie GHR i Jak2. Moje wyniki mogą być zastosowane do opracowania nowych strategii terapeutycznych dla pacjentów cierpiących w powodu chorób mieloproliferacyjnych oraz nowotworów spowodowanych rozregulowaniem sygnalingu na linii GHR/Jak2.

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Curriculum Vitae

Magdalena Sedek was born in Warsaw, Poland, on 10th April 1981. In 2000 she completed Tadeusz Reytan high school in Warsaw. In the same year in September she started her studies in Psychology at the Faculty of Psychology at Warsaw University. In 2001 in September she started her parallel studies in Biology at Faculty of Biology at Warsaw University. In 2002 she was awarded Socrates Erasmus scholarship to study one year at Middlesex University in London, Great Britain. In January 2007 she defended a master thesis at Faculty of Psychology with a specialization in Clinical Neuropsychology. From 2003 till 2007 she was performing an internship at Department of Genetics at Polish Academy of Science under the supervision of Prof. Teresa Zoladek. In June 2007 she defended her master thesis at Faculty of Biology with a specialization in Biotechnology. In July 2007 she moved to the Netherlands to start her PhD studies under the supervision of Prof. Ger Strous within the Marie Curie Network program "UbiRegulators".

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