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# DIPLOMARBEIT

# The role of Stat1 serine phosphorylation in Stat1 transcriptional activity

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# Table of contents

# Abstract

Interferons (IFNs) regulate immune responses upon viral and bacterial infections by triggering the JAK/Stat signaling pathway. In these signaling responses, signal transducers and activators of transcription (Stats) become tyrosine phosphorylated, translocate into the nucleus, bind to DNA and activate transcription of individual target genes. Besides the mandatory tyrosine phosphorylation of Stat proteins, a second phosphorylation at a serine residue, located in a conserved consensus sequence inside their transactivation domain, was discovered. It was shown that for Stat1 this serine phosphorylation is required for full fledged IFN-γ dependent innate immunity in mice. The results from the work done in our lab revealed that Stat1 has to be tyrosine phosphorylated and assembled into transcriptional complexes to become S727 phosphorylated. However, the effect of this modulating serine phosphorylation on the expression of individual Stat1 targets genes and the kinase responsible for this modification have not been characterized yet.

In the first project of my diploma thesis time course studies in mouse embryonic fibroblasts (MEFs) revealed that individual Stat1 target genes vary in their dependence on Stat1 S727 phosphorylation for full transcriptional activation in interferon gamma responses. Furthermore, chromatin immunoprecipitation assays in MEFs showed that Stat1 binding to its target promoters precedes the appearance of serine phosphorylated Stat1. This is being consistent with publications stating that Stat1 gets serine phosphorylated once it is bound to chromatin. Future studies will have to clarify how the promoters of individual Stat1 target genes differ and how these differences affect the dependence on Stat serine phosphorylation for full transcriptional activation.

The second objective of this thesis was to identify the kinase mediating Stat1 S727 phosphorylation and to clarify whether other Stat members than Stat1 are also serine phosphorylated by the same subset of enzymes. Inhibitor studies in human and murine cells using Flavopiridol, a cyclin-dependent kinase (CDK) inhibitor with increased specificity for nuclear CDKs, revealed that Stat1 and Stat3 serine phosphorylation was

abolished by Flavopiridol in interferon gamma and IL-6 responses, respectively. However, the precedent tyrosine phosphorylation was not affected. Due to the highly conserved serine phosphorylation site inside the transactivation domain across all the Stat members, these findings implicate that more Stat proteins than Stat1 and Stat3 are phosphorylated by the same subset of kinases.

# Kurzfassung

Interferone regulieren Immunantworten nach viralen und bakteriellen Infektionen indem sie die sogenannten JAK/Stat Signaltransduktionswege aktivieren. Im Laufe dieser Signalübertragung werden signal transducers and activators of transcription (Stats) Tyrosin-phosphoryliert, sie translozieren in den Nukleus, binden dort an spezielle DNA Sequenzen und aktivieren schließlich die Transkription spezieller Zielgenen. Neben der zwingend erforderlichen Tyrosin-Phosphorylierung der Stat-Proteine wurde eine zweite Phosphorylierungsstelle, ein hoch konserviertes Serin innerhalb der Transaktivierungsdomäne der Stats, identifiziert. Für Stat1 konnte gezeigt werden, dass diese Serin-Phosphorylierung für eine maximale Aktivierung der Interferon gamma abhängigen Immunantwort in Mäusen notwendig ist. Ergebnisse aus unserem Labor zeigten, dass Stat1 Tyrosin-phosphoryliert und in einem nuklären Transkriptionskomplex integriert sein muss, um an dem besagten Serin phosphoryliert werden zu können. Die genauen Auswirkungen der modulierenden Serin-Phosphorylierung auf die Expression individueller Stat1 Zielgene als auch die Kinase, die diese Phosphorylierung vermittelt, sind nicht bekannt.

Im ersten Teil meiner Diplomarbeit zeigten Kinetikstudien, welche an embryonalen Mausfibroblasten (MEFs) durchgeführt wurden, dass die Abhängigkeit der Aktivierung individueller Stat1 Zielgene von der Stat1 Serin727-Phosphorylierung in Interferon Antworten unterschiedlicher Natur ist. Interessanterweise konnte in Chromatinimmunopräzipitationen gezeigt werden, dass das Erscheinen von Stat1 an den Promotoren der untersuchten Zielgene dem Aufkommen von phoshoserin-Stat1 zeitlich vorhergeht. Dies ist im Einklang mit Studien die postulieren, dass Stat1 nur Serin-phosphoryliert werden kann wenn es an Chromatin gebunden vorliegt. Zukünftige Arbeiten werden das Ziel haben, die Unterschiede in den Promotern einzelner Zielgene zu analysieren um herauszufinden, was diese Differenzen für die Serinabhängigkeit der Expression dieser Gene aussagen.

Der zweite Teil meiner Arbeit beschäftigte sich mit der Identifizierung der Kinase, welche die Stat1 Serin-Phosphorylierung vermittelt, und der Fragen, ob andere

Mitglieder der Stat-Familie von den gleichen Kinasen wie Stat1 Serin-phosphoryliert werden. Inhibitorstudien in humanen Zellen und Mauszellen konnten zeigen, dass Flavopiridol, ein Cyclin-abhängiger Kinase (CDK) Inhibitor der eine erhöhte Wirksamkeit gegen nukleäre CDKs aufweist, die Serin-Phosphorylierung von Stat1 und Stat3 in Gamma-Interferon und IL-6 Antworten verhindern konnte, ohne die vorangehende Tyrosin-Phosphorylierung zu beeinflussen. Aufgrund der hohen Konservierung des phosphorylierbaren Serins innerhalb der einzelnen Mitglieder der Stat-Familie implizieren die erhaltenen Resultate, dass mehrere Stat-Proteine von derselben Gruppe an Kinasen Serin-phosphoryliert werden.

# 1 Introduction

## 1.1 Interferons

### 1.1.1 Interferons and interferon-like cytokines

Interferons were first discovered by Isaac Lindemann in 1957 as substance that allowed protection against viral pathogens (1). Interferons (IFNs) themselves represent proteins with antiviral activities that are secreted from cells under a variety of different conditions (2).

The interferon family comprises type I, type II and type III IFNs, which all belong to the family of interferons because of their shared function in defense against bacterial and viral pathogens. There is however no common molecular structure among all the interferon members. IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-δ, IFN-ε, IFN-τ as well as other reported IFN like cytokines such as limitin belong to the type I interferons, that all bind to a common membrane receptor, consisting of IFNAR-1 (interferon alpha receptor 1) and INFAR2c (3). Since the production of type I IFNs is a major mechanism of the innate immune system against viral infections, type I interferons are heavily expressed after viral encounter. Among others stimuli, the recognition of pathogens by toll-like receptors plays a major role in IFN expression (4). Type II interferons consist of only one known member, IFN- γ. While type I interferons can be produced by almost every mammalian cell type, type II interferons are mainly induced and secreted in T cells and natural killer (NK) cells. The recently classified type III interferons (IFN-λ1, IFN-λ2 and IFN-λ3) signal via a distinct receptor, consisting of IL10R2 and IFNLR1 (5). IFN-λ1, IFN-λ2 and IFN-λ3, also called IL29, IL28A and IL28B respectively, have been reported to share functional properties with type I IFNs by activating similar intracellular signaling cascades, which lead to similar biological activities, including an antiviral state (6).

### 1.1.2 Role and effects in the immune system

Interferons are defined by their ability to fight off viral challenges. On one hand, IFN stimulation enables cells to eliminate viral pathogens in infected cells and on the other hand, interferons induce an “antiviral state” in uninfected cells, protecting them from future infection. IFNs induce a pro-inflammatory state by regulating genes involved in inflammation, apoptosis, cell cycle and cell differentiation (7).

From the approximately 300 genes induced by type I interferon signaling, relatively few have any defined function in establishing the antiviral state. Most of the induced genes encode pattern-recognition receptors (PRRs) and transcription factors that form an amplification loop, leading to an enhanced interferon production. The dsRNA-activated protein kinase (PKR), which is constantly expressed at low levels in all mammalian cells, is up-regulated upon type I and type III IFN exposure (8). The impact of PKR on proliferation and its antiviral properties can be attributed to its ability to phosphorylate the elongation initiation factor 2α (eIF2α). By chemically modifying eIF2α, PKR inhibits the translation of cellular as well as viral mRNA. Activation of 2’5’ oligoadenylate synthase (OAS) leads to the polymerization of ATP into oligomers of adenosine by 2’, 5’-linked phosphodiester bonds. These unique 2’, 5’-oligomers then specifically activate RNaseL, which subsequently mediates RNA degradation (9). RNA degraded by RNaseL can be recognized by other cytoplasmic PRRs, such as RIGI and MDA5, leading to the expression of type I interferon genes (10). Type I and type III interferons also induce the expression of several GTP-hydrolysing proteins. Among those, the Mx proteins have a defined antiviral role (11). Mx proteins can trap essential viral components by their influence on vesicle trafficking, stopping viral replication at early stages (12). One of the most strongly induced IFN-stimulated genes (ISGs) is the ISG15 gene. ISG15 has been characterized as a 15 kDa ubiquitin homologue. Many proteins playing a role in the type I interferon response, including Jak1, Stat1 and PKR, have been reported to undergo ISGylation (13). Independent of its intracellular function, ISG15 has been shown to act as a cytokine after being secreted in large amounts (14). Several families of guanosine triphosphatases (GTPases), including 47-

kD immunity-related GTPases (p47 IRGs), 65- to 73-kD guanylate-binding proteins (p65Gbps), and large inducible GTPases (Vligs/Gvins), were found to be of the most abundantly induced proteins in interferon gamma responses (15). Recent studies demonstrated that at least Gbp1, Gbp6, Gbp7 and Gbp10 showed protective functions against bacterial pathogens, while Gbp1, Gbp2 and Gbp5 displayed weak antiviral properties (16,17). The RNA editing protein adenosine deaminase acting on RNA 1 (ADAR1) that catalyzes the deamination of adenosine to inosine, was shown to be upregulated in type I interferon responses (18). However, the outcome of RNA alterations by ADAR1 can lead to either enhanced or reduced virus persistence, depending on the virus (19). Furthermore members of the IFITM protein family were identified as viral restriction factors that are induced by interferons (20). Apart from their role in the defense against viral infections, IFNs also participate in the clearance of non-viral pathogens and in tumor growth restriction (21,22). The activity of cytotoxic T-cells as well as macrophage activation and Th1 T cell-differentiation is partly IFN dependent (7).

However, IFNs have also been shown to induce anti-inflammatory genes such as the RNA binding protein tristetraprolin (TTP) or the matrix metalloproteases (MMPs) (23,24). TTP mediates the decay of pro-inflammatory messenger RNAs, while MMPs limit tissue destruction caused by inflammation.

### 1.1.3 Induction of IFNs during infection

Toll-like receptors are membrane-spanning proteins that play a crucial role in recognizing pathogen-associated molecular patterns (PAMPs), such as viral DNA or bacterial lipopolysaccharide (LPS) (25). The recognition of PAMPs by pattern recognition receptors (PRRs) leads to the activation of various pro-inflammatory transcription factors and IFN regulatory factors (IRFs), resulting in the expression of pro-inflammatory cytokines and type I interferons (26). Among the toll-like receptors, the endosome located TLR3, TLR7, TLR8 and TLR9 are of outstanding importance in antiviral immunity. TLR7 and TLR8 recognize guanosine- or uridine rich ss-RNA, while

TLR3 senses ds-RNA. TLR9 specifically detects unmethylated RNA (27). TLR3, TLR7, TLR8 and TLR9 signaling is mediated by the recruitment of different adaptor proteins such as MyD88, TRIF, TRAM and IPS-1. These adaptors subsequently lead to the activation of downstream kinases like TBK-1 and members of the IKK family. The activated IFN regulatory factors (IRF1, IRF3 and IRF7) together with the nuclear factor kappa B (NF-κB) and the activating protein 1 (AP-1) potently induce type I IFNs (28).

TLR 4, which is not associated with viral recognition, can also drive IFN production by signaling via TRAM/TRIF. After binding bacterial lipopolysaccharide (LPS), signal transduction downstream of TLR4 can result in the production of pro-inflammatory cytokines like TNF by signaling via TIRAP/MyD88, or in type I IFN production by using the adaptors TRAM/TRIF (29).

Another family of IFN-inducing pattern-recognition receptors is the family of retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs). Two members of this family, the cytosolic RNA helicases RIG-I and MDA5, facilitate recognition of foreign RNA (30). To efficiently eradicate viral challenges, a positive feedback loop between the type I interferon regulated expression of RIG-I and MDA5 and the RLR induced IFN production is formed. Another RLR family member called LGP2 has recently been identified. Since LGP2 lacks the caspase-recruitment and the activation domains (CARDs), which play a crucial role in RIG-I and MDA5 signaling, it has been postulated that LGP2 acts as a negative regulator by competing with RIG-I and MDA5 (31).

The ds-DNA sensor protein ZBP1, which is also known as a DNA-dependent activator of IFN-regulatory factor (DAI), has been reported to induce IFN production after binding to bacterial or viral ds-DNA (32).

## 1.2 Interferon signaling

### 1.2.1 JAK/Stat signaling pathway

Interferons and interferon like cytokines conventionally signal through the JAK/Stat pathway. The JAK/Stat signaling pathway is triggered by the binding of a cytokine to its transmembrane cell surface receptor, resulting in receptor dimerization (33). Subsequently, permanently receptor associated kinases known as Janus Kinases (JAKs) are brought to close proximity to each other and thereby get activated. Specific tyrosine residues on the receptors and on the Janus Kinases are then phosphorylated by the activated JAKs. A family of latent cytoplasmic transcription factors known as Stats (Signal Transducers and Activators of Transcription) recognizes and binds the tyrosine phosphorylated receptors via their SH2 domain (34). Stats themselves are then phosphorylated by Janus kinases at a conserved tyrosine residue, leading to a conformational change of Stat dimers by the reciprocal interaction between the phosphorylated tyrosine residue of one Stat molecule with the SH2 domain of the other monomer and vice versa. Thereby, Stat dimers are able to leave the receptor and translocate into the nucleus, where they activate gene transcription by binding to specific DNA sequences. The mammalian JAK family consists of four members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), each containing a conserved kinase domain and second catalytically inactive pseudo-kinase domain (34). Seven mammalian Stats, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 were identified. All Stat proteins show a high homology in their SRC homology 2 (SH2) domain and the DNA binding domain (35,36). Except Stat2 and Stat6, all Stat members can be additionally phosphorylated at a serine residue, located in a conserved consensus sequence inside their transactivation domain. Recent data indicate that Stat serine phosphorylation is required for their full transcriptional activity (37–39).

### 1.2.2 Signal transducers and activators of transcription (Stats)

Signal transducers and activators of transcription (Stats) are transcription factors that directly transduce the signal, generated at the cell surface, into the nucleus. A special feature of Stat mediated signaling compared to other signaling cascades, such as the Map Kinase Pathways, is, that no other signaling molecule other than the Stat protein itself is required. Stats display a dual function by acting as signal transducers and as transcription factors in one molecule. Stat proteins are activated by various stimuli, including cytokines and some growth factors. Thus, signal transducers and activators of transcription control essential biological processes such as immune responses, cell growth, cell differentiation and apoptosis.

In human and mouse genomes, seven members of the Stat family have been identified so far. Stat homologues were also identified in non mammalian species like Xenopus laevis, Caenorhabditis elegans and Drosophila Melanogaster (40). Mammalian Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6, which are all encoded by different gene loci, are 750–850 amino acids long and share six functionally conserved domains (41) (Figure 1). The N-terminal domain has been shown to play an important role in the dimerization of unphosphorylated Stat monomers (42,43). Furthermore the interaction of Stat proteins with other co-activators, such as the CREB binding protein (CBP)/p300, is facilitated by the N-terminal domain (15). In addition this domain is essential for the inactivation of Stat dimers, which involves tyrosine dephosphorylation and nuclear export (44). The adjacent coiled-coil domain consists of four α-helices and is known to mediate associations with other proteins, such as the Stat1/IRF9 or the Stat3/cJUN interaction (45,46). The Stat3 coiled-coil domain has been reported to be important in the recruitment and binding of Stat3 to the interleukin 6 (IL-6) receptor (47). The highly conserved DNA-binding domain (DBD) is needed for the binding of Stat proteins to specific DNA sequences. Small differences in the amino acid sequences of this domain across the Stat members may determine the different DNA binding specificities of each Stat to individual target genes (48). Stat1 has been shown to be predominantly dimeric prior to activation in an anti-parallel dimer conformation (49). In this anti-parallel dimer orientation, the dimerization involves reciprocal interactions between the N-

terminal domain of one monomer and the coiled-coil domain of the other (50). The α-helical linker domain (LD) links the DNA-binding domain and the SH2 domain. The LD has been shown to be crucial for a stable association between Stat1 and chromatin after IFN-γ stimulation (51). Recent data indicates a role of the linker domain in the constitutive nucleocytoplasmic shuttling of unphosphorylated Stat molecules in resting cells (52). The most conserved domain among all Stat proteins is the Src-Homology 2 (SH2) domain. This domain is needed for the interaction of Stats with phosphorylated tyrosine residues and is therefore important for receptor association and a conformational change in Stat dimers upon activation (53). The C-terminal transactivation domain (TAD) is the least conserved domain among all Stat members (54). However, the highly conserved C-terminally located tyrosine activation motif, which is phosphorylated by Janus kinases, can interact with the SH2 domain of other Stat molecules, driving Stat dimerization (41). Additionally, all members of the Stat family except Stat2 and Stat6 contain a serine residue inside their TAD, located in a conserved consensus site, PMSP (Stat1, Stat3 and Stat4) or PSP (Stat5) (Figure 2). This serine phosphorylation site further modulates Stat activity and is required for their full transcriptional activity Stat proteins (37,38). The transactivation domain was shown to interact with several proteins, including minichromosome maintenance complex component 5 (MCM5), breast cancer 1 susceptibility protein (BRCA1) and CBP/p300 (55,56).

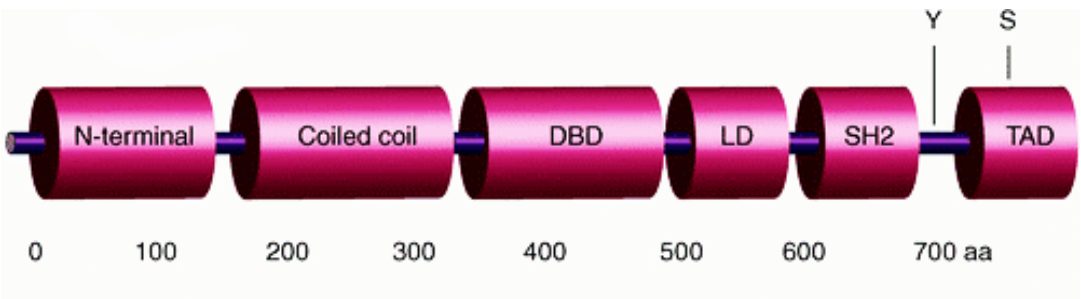



**Figure 1| Structural domains of signal transducers and activators of transcription (Stats).** Stats contain six conserved domains: NTD: N-terminal domain; CCD: coil–coiled domain; DBD: DNA binding domain; LD: linker domain; SH2: Src-Homology 2 domain; TAD: transactivation domain; two phosphorylation residues were identified: an activating tyrosine (Y) and modulating serine (S) phosphorylation (modified from (57))

Stat proteins exist either as full-length proteins or as c-terminally truncated isoforms, known as α- and β-isoforms, respectively. These alternatively spliced β-isoforms were so far identified for Stat1, Stat3 and Stat5 (58,59). Although Stat β-isoforms can be tyrosine phosphorylated, translocated into the nucleus and used to bind specific promoter sequences, their resulting transcriptional activation of target genes is diminished compared to full length α-isoforms. It has been suggested that Stat1β may negatively regulate Stat1α by binding to DNA as a nonfunctional transcription factor or by sequestering the functional STAT1α isoform. Recent studies have however revealed a mechanism by which Stat1β triggers cell death through a distinct, p53 independent pathway (60).

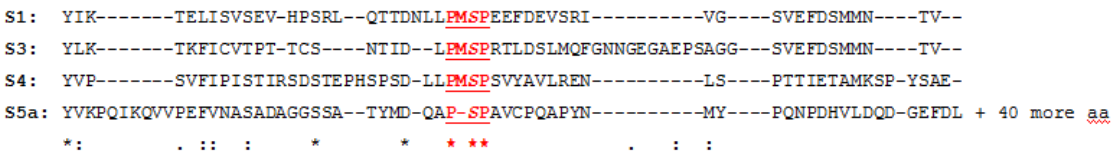



**Figure 2 | Multiple sequence alignment of murine signal transducers and activators of transcription (Stats).** Stat1, Stat3 , Stat4 and Stat5 contain a serine residue inside their TAD, located in a conserved consensus site, PMSP (Stat1-S727, Stat3-S727 and Stat4-S721) or PSP (Stat5-S725); conserved motif highlighted in red.

### 1.2.3 Post-translational modifications of Stats and their effects

The intensity to which Stats are activated after a given signal partly depends on various post-translational modifications altering Stat activities. All Stat proteins can be tyrosine phosphorylated at a highly conserved tyrosine activation motif around the position 700 by receptor associated Janus kinases (54). The phosphorylated tyrosine residue then drives a conformational change in Stat dimers. Stat1 has been shown to be predominantly dimeric prior to activation and that the dimer is mediated by N-terminal domain interactions (49). After being tyrosine phosphorylated at residue Y701, Stat1 dimers change from a previous anti-parallel conformation to a parallel state (61). In the parallel orientation, the formerly hidden nuclear localization signals (NLS), which

were described to be located in the N-terminus and in the DNA binding domain, become accessible, resulting in an importin mediated translocation of Stat1 into the nucleus (62,63) (Figure 3). In addition, all members of the Stat family except Stat2 and Stat6 contain a conserved, phosphorylatable serine residue inside their TAD (64). Stat1 S727 phosphorylation was shown to be required for full transcriptional activity of Stat1 after IFN-γ stimulation (37). JAK/Stat independent signaling pathways, such as the MAP kinase pathways, were shown to cause Stat1 and Stat3 serine phosphorylation without the need of Stat proteins to be previously tyrosine phosphorylated (65). The mechanism underlying the tyrosine-independent serine phosphorylation is autonomous from the interferon induced serine phosphorylation of Stats.

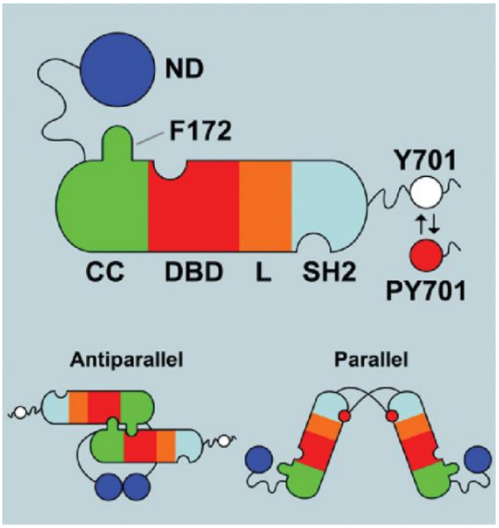



**Figure 3** | **Schematic features of the anti-parallel and parallel conformation of Stat1 homodimers**: ND: N-terminal domain; CC: coil–coiled domain; DBD: DNA binding domain; L: linker domain; SH2: Src-Homology 2 domain; Y701 is phosphorylated (red dot) when the molecule is activated (modified from (61))

The proteasome-mediated protein degradation via lysine ubiquitination plays an important regulatory role in JAK/Stat pathways. The degradation of both Stat1 and Stat4 has been shown to be promoted by the nuclear E3 ubiquitin ligase SLIM (66). ISG15, an ubiquitin-like protein that is highly induced by interferons, can also be

conjugated to Stat proteins (67). The biological consequences of ISGylation of Stat proteins are however not well understood and it is believed that the modification modulates responses to interferons, depending on the stimulus and cell type. Furthermore, SUMOylation of the c-terminally located lysine 703 of Stat1 has been reported (68). Methylation of Stat1 on arginine 31 by the methylase PRMT1 after IFNα/β treatment has been described (69). The modification was suggested to have an impact on Stat-induced transcription by preventing the binding of PIAS (negative regulator protein inhibitors of activated Stat) to Stat1. Recent data however claims the absence of arginine methylation of Stat1 and Stat3 (70). Stat1 acetylation was reported to regulate tyrosine dephosphorylation in response to type I interferons and to interfere with NF-κB-dependent transcription (71,72). The issue of acetylation/dephosphorylation switch is however controversial since a report exits that excludes Stat1 acetylation (73).

#### 1.2.3.1 Stat1 and Stat3 serine phosphorylation

All members of the Stat family except Stat2 and Stat6 contain a conserved phosphorylatable serine residue inside their TAD. For Stat1, Stat3 and Stat4 this serine is located inside a conserved PMSP sequence, while Stat5 is modified at a PLSP motif. Full activation of transcription by Stat1 and Stat3 has been shown to be dependent on tyrosine and serine phosphorylation (37). However, not all IFN-γ target genes require Stat1 S727 phosphorylation to be maximally transcribed (74).

Stat1 modification at S727 can be induced by IFN-γ stimulation or by stress induced p38 MAP kinase signaling. After IFN-γ stimulation, Stat1 dimers only become serine phosphorylated if a preceding phosphorylation at tyrosine 701 took place. Furthermore it was reported that tyrosine phosphorylated Stat1 dimers have to translocate into the nucleus and bind to chromatin to become modified at S727 (75). Stat1 Serine phosphorylation linked to p38MAPK activity is independent of the canonical JAK/Stat pathway and of SH2 domain based protein interactions (76). Even though MAPK pathways may be activated by IFN-α signaling, IFN induced serine

phosphorylation of Stat1 is not connected to classical MAPK pathways (74,77). Previous studies indicate that stress-induced activation of the p38 MAPK pathway increases the expression of the IFN-γ induced Stat1 target gene IRF1, compared to gamma-interferon stimulation alone. The enhanced expression of IFN-γ induced genes by p38 activity was shown to be independent of increased Stat1 serine phosphorylation by the p38 pathway (78). The role of Stat1 molecules phosphorylated on S727 but not on Y701 is still elusive and needs further clarification.

The biological roles of Stat1 and Stat3 differ drastically. While Stat1 mainly drives the expression of genes responsible for pro-inflammatory and anti-proliferative responses, Stat3 activation is connected to proliferation and anti-inflammatory cytokine production (79). Therefore it was suggested that Stat3 serine phosphorylation might be regulated by different cytokines and signaling pathways than Stat1, although both Stats contain a highly conserved phosphorylatable PMSP motif in their transactivation domain. Upon Interleukin (IL)-6 stimulation Stat3 becomes phosphorylated on a single tyrosine residue (Y705), resulting in Stat3 dimerization, nuclear translocation and in the binding of Stat3 to its target genes (80). Recent publications have shown that in response to Interleukin (IL)-6, IFN-γ and epidermal growth factor (EGF), Stat3 molecules become additionally phosphorylated on a single Serine (S727) residue (81). Studies have revealed that growth factor receptors signal to Stat3 through the ERK pathway (82). Activating Raf and its downstream effectors was reported to induce Stat3 S727 phosphorylation without the need of a previous tyrosine phosphorylation (83). Similar to Stat1, Stat3 S727 phosphorylation has been shown to enhance Stat3 transcriptional activity (37). Recent data revealed a role of Stat3 in metabolic function in mitochondria, where it supports Ras-dependent oncogenic transformation independent of its nuclear role as transcription factor (84).

### 1.2.4 Nuclear export and dephosphorylation of Stats

The Stat1 activation-inactivation cycle depends on the ability of Stat dimers to shuttle between nucleus and cytoplasm. Studies have revealed that, upon IFN-γ stimulation, the nuclear localization of Stat1 dimers is only transient and that Stat1 molecules rapidly recycle into the cytoplasm (85). Publications suggest that Stat1 is tyrosine dephosphorylated inside the nucleus, resulting in the inactivation of Stat1 dimers and the subsequent nuclear export (86). A recently proposed model claims that after DNA disassociation, structural changes of tyrosine-phosphorylated, parallel-arranged Stat1 dimers to anti-parallel dimers are crucial for Stat1 to become tyrosine dephosphorylated. After tyrosine-phosphorylated Stat1 dimers disengage from DNA, the N-terminal domains dimerize, causing the disconnection of the reciprocal phosphor-tyrosine/SH2 interactions between the monomers. Thereafter, a reciprocal association of the DNA-binding and the coiled: coil domains is favored. The resulting anti-parallel Stat1 dimer is thought to be easier accessible for tyrosine phosphatases (44) (Figure 4).

The tyrosine-phosphatases T-cell protein tyrosine phosphatase (TC-PTP) and SHP2 were proposed to dephosphorylate Stat1 on Y701 (87,88). However, the nature of the phosphatase and the precious mechanism regulating Stat tyrosine dephosphorylation have not been totally clarified yet.

Recent data indicates that the nuclear export of Stat proteins is mediated by the binding of chromosome region maintenance 1 protein (CRM1) to nuclear export signals within Stats. CRM1 was shown to recognize specific hydrophobic amino acids that are situated within the DNA binding domain of Stat1. The proper location of the CRM1 binding site within the DNA binding domain of Stat1 leads to the assumption that Stat1 dimers have to dissociate from DNA to allow CRM1/Stat1 interactions (89).

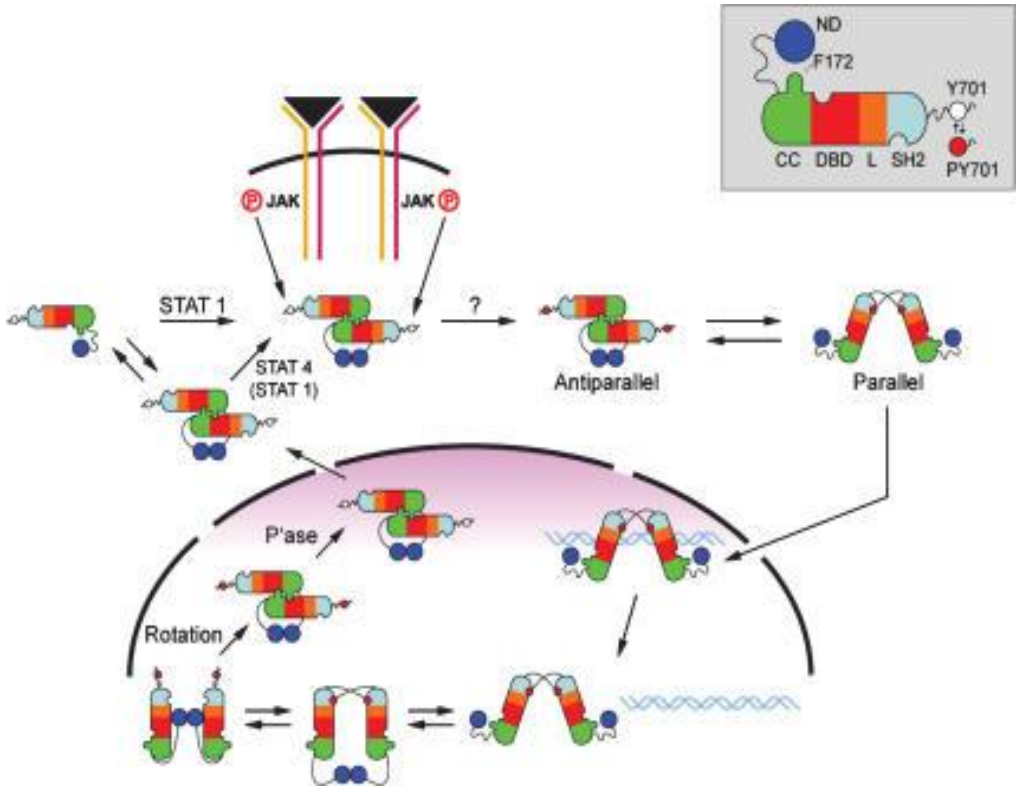



**Figure 4 | Model for Stat1 dephosphorylation**. After DNA disassociation, Stat1 dimers undergo spatial reorientations from parallel-arranged dimers to an anti-parallel form. The structural change then allows easy access to a phosphatase, resulting in Stat1 Y701 dephosphorylation. (modified from (44))

### 1.2.5 Regulation of the JAK/Stat signaling in the immune system

Since an overreaction of Stat signaling in immune responses can lead to potentially dangerous outcomes, a transient activation of Stat proteins has to be guaranteed. Recent studies have revealed various mechanisms controlling JAK/Stat signaling (90) (Figure 5).

Suppressors of cytokine signaling (SOCS) are rapidly induced by various cytokines and are known to inhibit JAK/Stat signaling by forming a typical negative feedback-loop (91). SOCS proteins can interact directly with tyrosine phosphorylated JAKs via their SH2 domain, resulting in the inhibition of JAK activity (92). CIS, a member of the SOCS family, has shown to prevent Stat activation by competing with Stat dimers for binding sites on cytokine receptors (93). Also a role of SOCS in targeting components of the

JAK/Stat signaling pathway for degradation via the ubiquitin-proteasome pathway has been proposed (94). Several protein tyrosine phosphatases (PTPs) have been identified to regulate JAK/Stat signaling. PTP1B and TCPTP have demonstrated to dephosphorylate tyrosine phosphorylated JAK2/TYK2 and JAK1/JAK3, respectively (95,96). An increased level of tyrosine-phosphorylated JAK1 after IFN-γ stimulation was reported in SHP2 deficient fibroblasts (97). Protein inhibitors of activated Stat (PIAS) were reported to inhibit Stat signaling in a cytokine dependent manner, as PIAS proteins fail to interact with Stats in unstimulated cells (98). PIAS1 and PIAS3 inhibit the DNA-binding properties of activated Stat1 and Stat3, respectively (99,100). PIASX and PIASY were shown to negatively regulate Stat4 and Stat1 dependent gene expression without impeding Stat dimers from chromatin binding (101). Moreover, PIAS proteins were implicated to play a role in Stat1 SUMOylation (102). The in vivo role of SUMOylated Stat1 has however not yet been clarified (103).

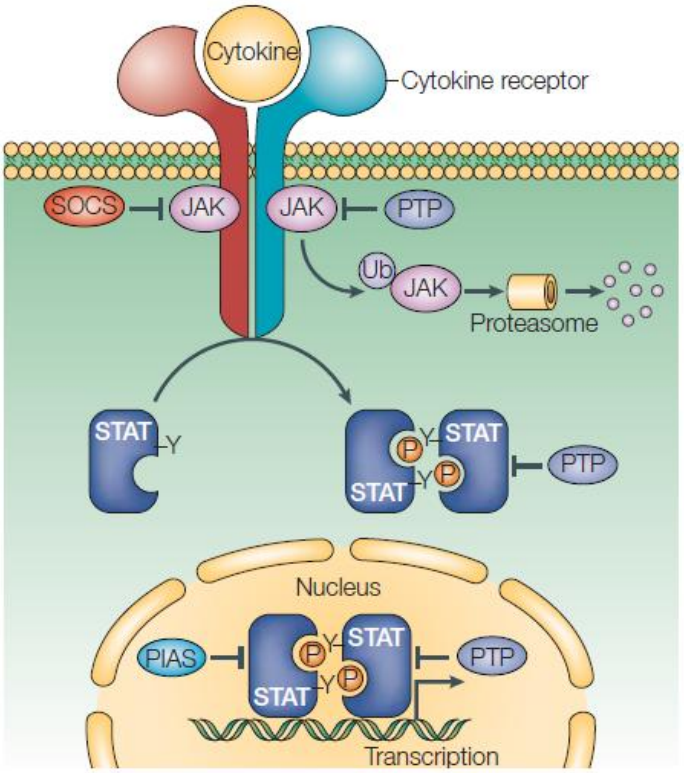



**Figure 5 | Negative regulators of the JAK/Stat signaling pathway**. The JAK-Stat signaling can be negatively regulated amongst others by suppressors of cytokine signaling (SOCS) proteins, protein tyrosine phosphatases (PTPs) and protein inhibitors of activated Stat (PIAS) ( modified from(90))

### 1.2.6 Type I and type II IFN signaling

All type I interferons bind to a common receptor consisting of two subunits called IFNAR-1 (interferon alpha receptor 1) and INFAR2c (104). The receptor associated Janus kinases JAK1 and Tyk2 then predominantly activate Stat1/Stat2 heterodimers and, to a smaller extent, Stat1/Stat1 homodimers. However, Stat3, Stat4 and Stat5 can also be involved in type I interferon responses in various cell types under diverse conditions (105,106). Tyrosine-phosphorylated Stat1/Stat2 heterodimers as well as activated Stat1/Stat1 homodimers then leave the promoter and translocate into the nucleus. Prior to DNA binding, Stat1/Stat2 heterodimers associate with the interferon regulatory factor 9 (IRF9) to form the transcriptionally active ISGF3 (interferon stimulated gene factor-3) complex (107). ISGF3 complexes bind specific promoter consensus sequences ($AGTTTN_3TTTC$) known as interferon stimulated response elements (ISREs) (108). Sat1/Stat1 homodimers, which are also activated to a smaller extent upon IFN-α stimulation, recognize different DNA sequences known as gamma activating sites (GAS) (109) (Figure 3). The Stat1/Stat2 heterodimer driven expression of genes containing GAS-like sequences known as pIREs (palindromic IFN-response elements) without the help of IRF9 has been reported (107).

The only known type II interferon, IFN-γ, binds to a receptor consisting of IFNGR1 and IFNGR2 chains (110). After cytokine binding and receptor oligomerization, the receptor associated kinases JAK1 and JAK2 become activated, resulting in the phosphorylation of IFNGR1 at tyrosine 440 by JAK1, which has been previously activated by JAK2 (111,112). The tyrosine phosphorylated IFNGR1 molecule then serves as a docking site for Stat1 homodimers, recognizing the modified receptor via their SH2 domain. After receptor binding, Stat1 becomes phosphorylated at tyrosine 701, causing a conformational change in Stat1 homodimers and the subsequent translocation into the nucleus (7). IFN-γ activated Stat1 dimers specifically recognize promoters containing GAS elements with the consensus sequence $TTCN_{2-4}GAA$ (113) (Figure 6). Recent reports suggest that members of the Stat family other than Stat1, like Stat3, can also be activated in response to type II IFNs (114).

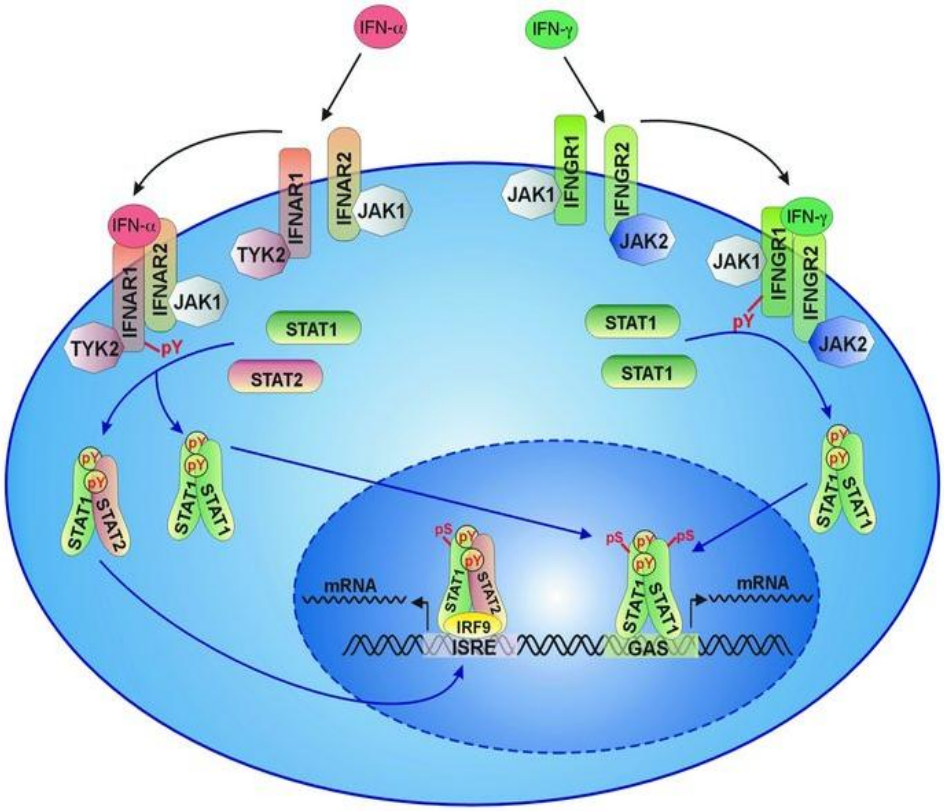



**Figure 6| JAK/Stat signaling transduction in response to type I and type II interferons. (modified from (115))**

### 1.2.7 Interferon target genes

Upon interferon stimulation, latent cytoplasmic transcription factors, called Stats, become activated and subsequently translocate into the nucleus where they provoke the expression of IFN-stimulated genes (ISGs). By this signal transduction pathway, type I, type II and type III IFNs induce partially overlapping sets of genes. The similarities and differences in the expression pattern of different interferons are partly determined by the use of overlapping transcription factor complexes (116).

By first analyzing IFN-α responses, stretches of DNA that were crucial for type I interferon signaling were identified. The so called interferon-stimulated response elements (ISREs) display a conserved consensus sequence of AGTTTN$_3$TTTC (117). After

type I interferon stimulation, a multimeric complex called ISGF3, consisting of a Stat1/Stat2 heterodimer associated with IRF9, which is able to specifically recognize ISRE sites, was found (46). IRF9 was characterized as a 48 kDa protein, which is predominately located in the cytoplasm of untreated cells (118).

The study of the guanylate-binding protein (GBP) gene, that is rapidly induced after IFN-α as well as IFN-γ stimulation, lead to the discovery of the IFN-γ activation site (GAS) (119,120). GAS elements with the consensus sequence $TTCN_{2-4}GAA$ were shown to be crucial in IFN-γ responses by being recognized by Stat1 homodimers (113). Although it has been reported that IFN-γ primarily activates Stat1 homodimers, other IFN-γ induced Stat complexes, namely Stat1/Stat2 heterodimers, Stat3 homodimers and Stat1/Stat3 heterodimers are formed (121,122). Evidence exists that complexes of Stat1 homodimers associated with IRF9 are able to bind ISRE sites, offering an explanation for the overlapping expression of ISRE containing genes in type I and type II IFN responses (123).

The family of interferon regulatory transcription factors (IRFs) currently consists of nine members (IRF1-9). IRFs were shown to play important roles in immune responses, apoptosis and cell cycle regulation (124). Previous studies have revealed that IRF1, IRF2, IRF3, IRF5, IRF7 and IRF9 are induced by IFN-α/β, while IFN-γ stimulation only drives the expression of IRF1, IRF8 and IRF9 (125). Some IRFs have been indicated to function as regulators of ISG expression in response to different types of IFNs. IRF1 was shown to bind to a subset of ISRE containing genes and thereby regulating their activation (126). Furthermore, IRF3, IRF4, IRF7 and IRF8 have been reported to modulate ISG expression (127). The transcription of genes, containing both an ISRE and a GAS site by cooperation of Stat1 and IRF1 in response to IFN-γ, has been described (128).

## 1.3 Nuclear CDKs and their role in transcriptional regulation

The transcriptional regulation of genes is one of the most important steps in controlling gene expression. The initiation of transcription is a tightly controlled mechanism, which is partly regulated by the formation of the Pre-initiation Complex (PIC) (129). The PIC is assembled from general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), RNA polymerase II (RNA pol II) and the mediator complex. Two cyclin-dependent kinases, CDK7 and CDK9, have demonstrated to be involved in transcriptional regulation (130). The CDK7/cyclin H complex phosphorylates the carboxyl-terminal domain (CTD) of RNA pol II at serine 5 inside the YSPTSPS heptarepeat and thereby promotes initiation of transcription (131). The transcription elongation factor b (P-TEFb), consisting of CDK9 and cyclin T, was reported to play a role in efficient transcriptional elongation by phosphorylating the CTD of RNA pol II at serine 2 (131).

After RNA pol II has escaped the promoter and started transcription, some components of the PIC remained promoter bound, forming the so called scaffold complex (scaffold-PIC) (132). The scaffold-PIC, which serves as a reinitiation intermediate for the assembly of new transcription complexes, consists of most PIC components, including mediator, TFIID, TFIIA, TFIIH and TFIIE. By this mechanism of rapid reinitiation, a fast and high gene induction is warranted.

### 1.3.1 CDK8 subcomplex controls mediator function

The mammalian mediator complex exists in two forms, the so called core mediator complex and a complex of the mediator, reversibly associated with the CDK8 subcomplex (CDK8-Mediator complex). The CDK8 subcomplex consists of CDK8, cyclin C, Med12, and Med13. The core mediator complex and the CDK8-Mediator complex were shown to be highly similar in subunit composition, only differing in the presence of Med26 and the CDK8 subcomplex, respectively (133). Publications have demonstrated that the core mediator complex alone displays coactivatory functions,

while the CDK8-Mediator complex inhibits transcriptional reinitiation (134). The core mediator complex was reported to form a stable binary complex with RNA pol II and to stimulate TFIIH-mediated phosphorylation of the C-terminal domain of polymerase II, resulting in promoter escape (135,136).

In yeast, CDK8 kinase activity was shown to negatively regulate transcription by phosphorylating the CTD of RNA pol II, causing a disassociation of mediator and pol II (137). Furthermore, a mechanism by which CDK8 prevents initiation by phosphorylating cyclin H has been described (138). Elmlund et. al were able to demonstrate that the binding of the CDK8-submodule to the Mediator alone, without the need of CDK8 kinase activity, was sufficient to sterically block RNA pol II/Mediator interactions (139). Recent data suggests that the reversible formation of the CDK8-Mediator complex functions as a switch that regulates transcriptional initiation and reinitiation events (134) (Figure 7).

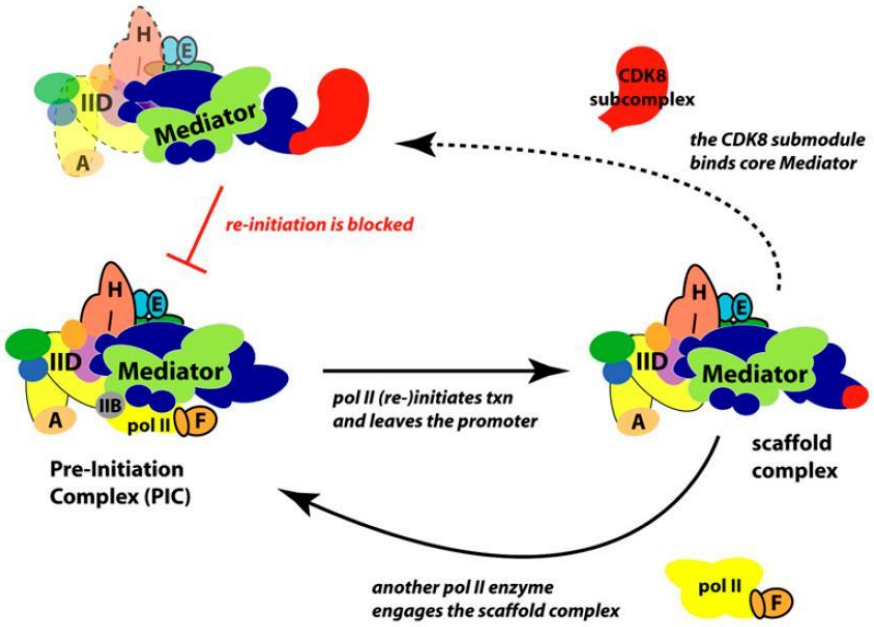



**Figure 1 | Transcriptional regulation by the CDK8 subcomplex**. Following PIC formation and initiation, the escaped pol II leaves behind a scaffold complex at the promoter. The association of the CDK8 subcomplex to the Mediator leads to the formation of the CDK8-Mediator complex, which blocks re-initiation. The scaffold-PIC without the CDK8 subcomplex is however able to rapidly reinitiate new rounds of transcriptional initiation. (modified from 134)

### 1.3.2 Nuclear CDKs couple activation and turnover of transcription factors

Latent cytoplasmic transcription factors such as Stat, Smad, Notch and NF-κB are signaling molecules that reside in an inactive form in the cytoplasm but rapidly translocate into the nucleus upon activation. Recently published data suggests models in which nuclear Cyclin-dependent kinases (CDKs) engaged in transcription facilitate the coordination of transcriptional activation and subsequent turnover of latent cytoplasmic transcription factors (140,141).

After members of the transforming growth factor β (TGF-β) family bind to their serine/threonine kinase receptor complexes, transcription factors known as Smads become phosphorylated at their C-terminus. This phosphorylation event allows Smad proteins to translocate into the nucleus, where they cooperate with sequence-specific transcription factors to drive the expression of specific target genes (142). Recent studies have revealed that receptor-activated Smads are phosphorylated inside the nucleus at an interdomain linker region by CDK8 and CDK9 (140). These phosphorylations were shown to enhance Smad transcriptional activity. At the same time, specific ubiquitin ligases are able to recognize the phosphorylated linker, resulting in the ubiquitination of Smad and the subsequent proteasome-mediated turnover of the activated transcription factor (140).

A similar phenomenon was observed in Notch signaling. Upon ligand binding, the Notch transmembrane receptor undergoes proteolysis, followed by the release of the Notch receptor intracellular domain (ICD) (143). Inside the nucleus, ICD interacts with the DNA binding protein CBF1 and the coactivator MAM to induce the expression of Notch responsive genes (144). Recent data indicates that CDK8 phosphorylates the Notch ICD inside the nucleus, targeting the transcription factor for degradation via the ubiquitin-proteasome pathway (141).

# 2 Aims

## 2.1 Project 1: The role of Stat1 S727 phosphorylation in Stat1 transcriptional activity

The IFN-γ induced phosphorylation of Stat1 at its transactivation domain has been shown to be required for full-fledged interferon gamma dependent innate immunity. In this context, the dependence of Stat1 on serine 727 phosphorylation, for maximal transcriptional activity of Stat1, has been proposed. The first objective of this thesis was to study the role of Stat1 S727 phosphorylation on the activation of individual Stat1 target genes in interferon responses.

## 2.2 Project 2: Studies on the kinase and kinetics of Stat serine phosphorylation

All members of the Stat family except Stat2 and Stat6 have been reported to contain a serine residue inside their transactivation domain, located in a conserved consensus site, PMSP (Stat1, Stat3 and Stat4) or PLSP (Stat5). Tyrosine phosphorylated Stat1 has to translocate into the nucleus and to bind chromatin to become S727 phosphorylated in interferon responses. So far, the kinase as well as the precious kinetics of this process have not been identified. Recent data obtained in our lab suggests that the nuclear cyclin-dependent kinases, CDK7, CDK8 or CDK9, might play a role in the serine phosphorylation of Stat proteins. Therefore, the second part of my thesis was to indentify the nature of the kinase as well as the kinetics underlying Stat1 S727 phosphorylation in interferon responses. Furthermore, the question whether other Stat members than Stat1 are phosphorylated by the same subset of kinases at this conserved serine residue, was addressed.

# 3 Results

## 3.1 Project 1: The role of Stat1 S727 phosphorylation in Stat1 transcriptional activity

### 3.1.1 Characterization of Stat1 target genes upon IFN-γ stimulation

Stat1 activation in response to IFN-γ has been reported to result in the phosphorylation of Stat1 at tyrosine 701 and at serine 727 (65). Data have shown that maximal activation of transcription by Stat1 requires the activating tyrosine phosphorylation as well as the modulating serine phosphorylation (37). Publications however indicate that not all genes activated by Stat1 depend on Stat1 S727 phosphorylation to become maximally transcribed (74). Studies using Stat1-deficient U3A cells reconstituted with Stat1-S727A showed that Stat1 molecules that could not be serine phosphorylated were impaired in establishing an antiviral state upon IFN stimulation. The transcription of IRF1, a well known Stat1 target gene in IFN-γ responses, was described to be reduced by around 80% in U3A cells expressing the S727A mutant Stat1 protein (37).

This study addressed the question whether the lack of Stat1 S727 phosphorylation generally reduces the transcription of all Stat1 dependent genes after IFN-γ stimulation or whether it is a gene specific phenomenon. Apart from IRF1, the expression of two other well known Stat1 target genes in IFN-γ responses, namely GBP2 and TAP1, was assessed. The promoter of IRF1 was described to contain one gamma activation site (GAS) downstream of the transcriptional start site, whereas an interferon stimulated response element (ISRE) as well as a GAS element is present in the TAP1 promoter region (145,146). The GBP2 promoter was characterized to contain a proximal GAS site as well as distally located GAS and ISRE elements (147) (Figure8) .

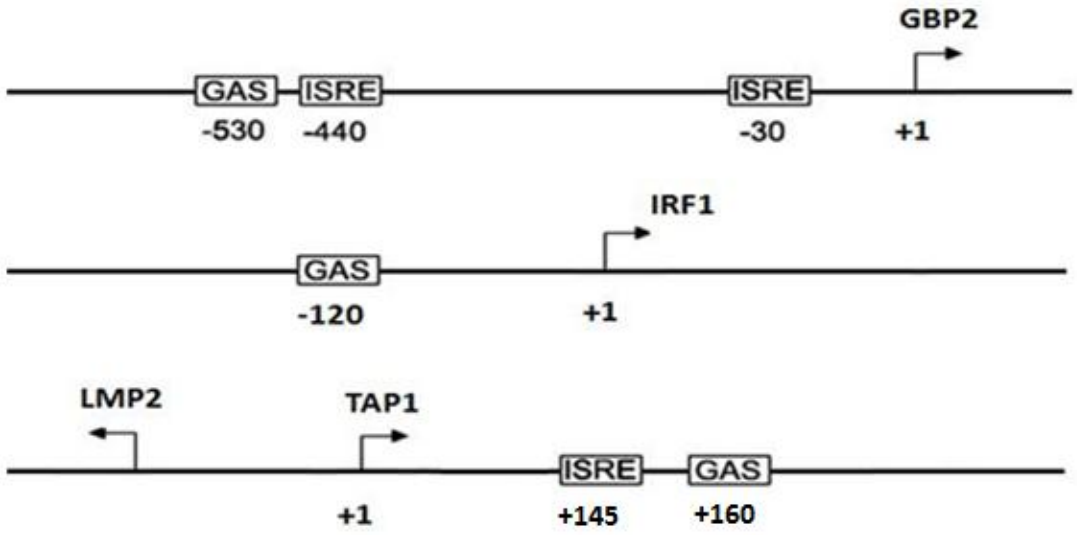



**Figure 2 | Schematic representation of the IRF1, GBP2 and TAP1 promoter structure.** GAS: gamma activating site, ISRE: interferon-stimulated response element

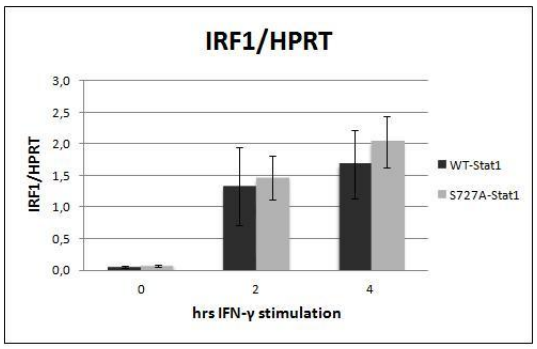
### 3.1.2 mRNA levels and inducibility of Stat1 target genes IRF1, TAP1 and GBP2 in IFN-γ stimulated MEFs I

To test whether Stat1 S727 phosphorylation in IFN-γ responses effects individual Stat1 target genes differently, interferon induced GBP2, IRF1 and TAP1 mRNA levels of immortalized (3T3) mouse embryonic fibroblasts (MEFs) derived from mice expressing either wild type Stat1 (Stat1-wt) or S727A-Stat1, were compared. Cells were treated with IFN-γ (5 ng/ml) for 0, 120 and 240 min and thereafter RNA was isolated. mRNA levels were then quantified by qRT-PCR analysis. For the normalization of RNA levels, qRT-PCR analysis was also carried out for the internal housekeeping gene HPRT.

This experiment revealed that if normalized to HPRT, IRF1 mRNA levels were slightly increased at all observed time points in Stat1-S727A 3T3 MEFs compared to Stat1-wt 3T3 MEFs. In both cell lines full activation of the IRF1 gene was reached after 2 hours of IFN stimulation and stayed at that level for at least 4 hours. The basal as well as the stimulated IRF1 mRNA levels normalized to HPRT were increased by about 20% in

Stat1-S727A MEFs compared to Stat1-wt MEFs. The inducibility between the mutant and the wild-type transcription factor displayed no significant difference (Figure 9).
The mRNA levels of GBP2 in unstimulated cells differed massively between Stat-wt MEFs and Stat1-S727A fibroblasts if normalized to HPRT. The basal expression of GBP2 in immortalized mouse embryonic fibroblasts expressing the S727A mutant protein was reduced by around 80% compared to wild-type cells. However, this difference in mRNA levels decreased along time. Two and four hours after IFN-γ stimulation, 3T3 S727A-MEFs displayed 37% and 46% of the mRNA levels observed in Stat1 wild-type cells, respectively. The fact, that GBP2 mRNA levels were increased in wild-type Stat1 MEFs compared to fibroblasts expressing the mutant protein, was however not reflected in GBP2 inducibility. While after two hours of IFN stimulation the inducibility between Stat1-wt MEFs and S727A-Stat1 MEFs was nearly alike, the GBP2 gene was drastically higher induced in fibroblasts expressing the mutant transcription factor after four hours of stimulation. The increase in relative mRNA levels of S727A-Stat1 MEFs compared to Stat1-wt MEFs along time, offers an explanation for this high inducibility in S727A-Stat1 fibroblasts at late time points (Figure 4).

The expression pattern seen for the TAP1 gene was similar to the one observed for GBP2. At basal levels, the S727A mutant cell line was heavily diminished in the ability to express TAP1 mRNA. Only 16% of the TAP1 mRNA level measured in unstimulated wild-type Stat1 MEFs was observed in S727A-Stat1 fibroblasts. As seen for GBP2, the differences in TAP1 mRNA levels between these two cell lines waned along stimulation time. Two and four hours after IFN-γ stimulation, mouse embryonic fibroblasts, expressing the mutant protein, displayed 25% and 43% of the mRNA levels assessed in Stat1-wt cells, respectively. Normalization to unstimulated samples revealed that TAP1 induction was higher in S727A-Stat1 fibroblasts than in wild-type Stat1 MEFs (
Figure **5**).

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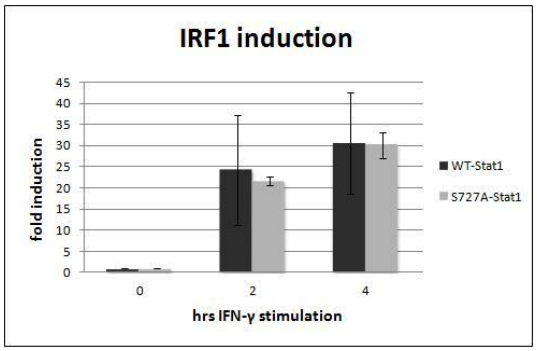
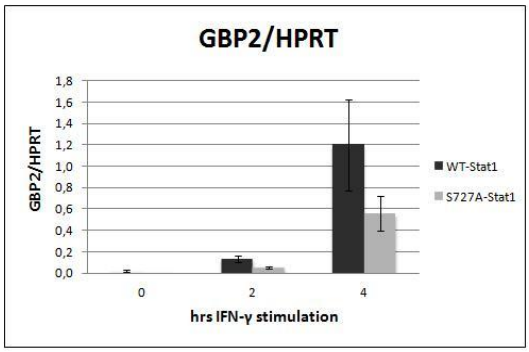



B

**Figure 3 |IRF1 mRNA levels and inducibility do not depend on Stat1 S727 phosphorylation in IFN-γ responses.** 3T3 MEFs expressing either wild type Stat1 (WT-Stat1) or S727A-Stat1 were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor IRF1 mRNA levels and inductions. S727A-Stat1 cells showed slightly higher IRF1 mRNA levels under resting and induced conditions if normalized to HPRT (A); however, normalization to unstimulated samples revealed no difference in IRF1 inductions (B). Results shown are derived from three independent experiments.

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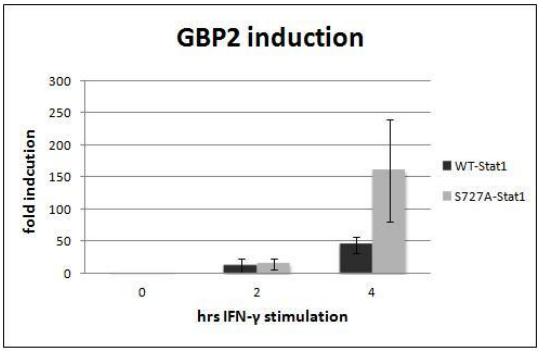
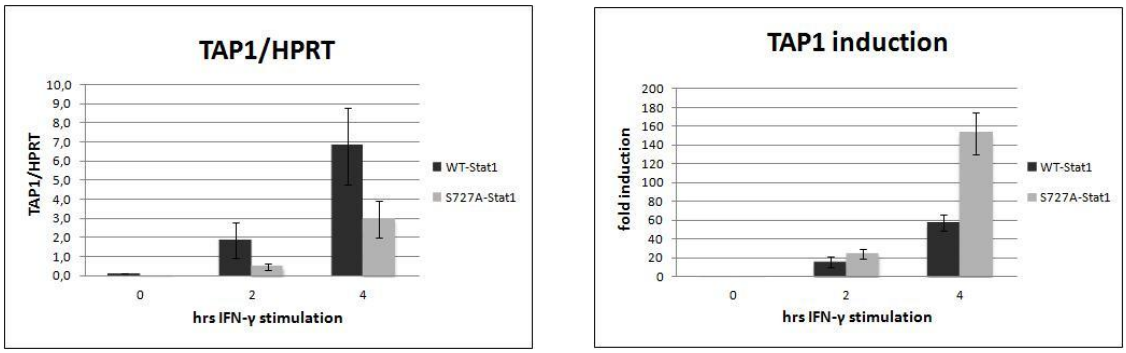



B

**Figure 4 | GBP2 mRNA levels and inducibility depend on Stat1 S727 phosphorylation in IFN-γ responses.** 3T3 MEFs expressing either wild type Stat1 (WT-Stat1) or S727A-Stat1 were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor GBP2 mRNA levels and inductions. S727A-Stat1 cells showed significantly lower GBP2 mRNA levels under resting and induced conditions if normalized to HPRT (A); however, normalization to unstimulated samples showed that GBP2 inducibility was higher in S727A-Stat1 fibroblasts (B). Results shown are derived from three independent experiments.




A B

**Figure 5 | TAP1 mRNA levels and inducibility depend on Stat1 S727 phosphorylation in IFN-γ responses.** 3T3 MEFs expressing either wild type Stat1 (WT-Stat1) or S727A-Stat1 were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor TAP1 mRNA levels and inductions. S727A-Stat1 cells showed significantly lower TAP1 mRNA levels under resting and induced conditions if normalized to HPRT (A); however, normalization to unstimulated samples showed that TAP1 inducibility was higher in S727A-Stat1 fibroblasts (B). Results shown are derived from three independent experiments.

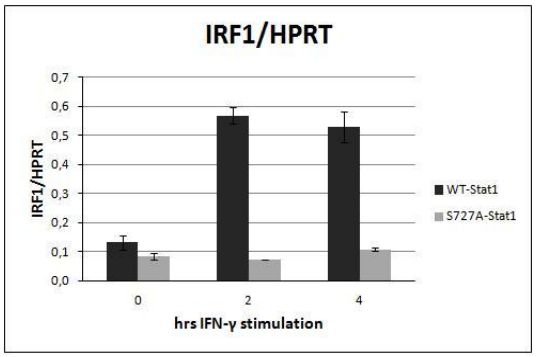
### 3.1.3 mRNA levels and inducibility of Stat1 target genes IRF1, TAP1 and GBP2 in IFN-γ stimulated MEFs II

To test whether the results obtained in immortalized (3T3) mouse embryonic fibroblasts (MEFs), which derived from mice expressing either wild type Stat1 (Stat1-wt) or S727A-Stat1, were also true in other cell lines, a similar experiment to the one described in chapter 3.1.2 was performed using different cell lines. Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 or the Stat1-S727A mutant were stimulated with IFN-γ for 0, 120 and 240 min. mRNA levels and gene inductions were assessed by qRT-PCR. For the normalization of RNA levels, qRT-PCR analysis was also carried out for the internal housekeeping gene HPRT.

This experiment revealed that if normalized to HPRT, IRF1 mRNA levels were drastically decreased at all induced time points in cells expressing the Stat1-S727A mutant compared to wild-type Stat1 fibroblasts. There was no activation of the IRF1 gene in

the Stat1 S727A mutant cell line. In fibroblasts expressing wild-type Stat1, the full activation of the IRF1 gene was reached after two hours of IFN stimulation and it lasted at that level for at least two more hours (Figure 6).

As seen for the IRF1 gene, GBP2 and TAP1 mRNA levels in Stat1-deficient 3T3 fibroblasts reconstituted with the Stat1-S727A mutant showed no increase at all after two and four hours of IFN stimulation. Cells expressing wild-type Stat1 displayed a similar expression pattern as observed in chapter 3.1.2. The mRNA levels of GBP2 and TAP1, if normalized to HPRT, showed an increase after two hours of interferon stimulation and reached their maximum after four hours of cytokine treatment (Figure 7 and Figure 8).

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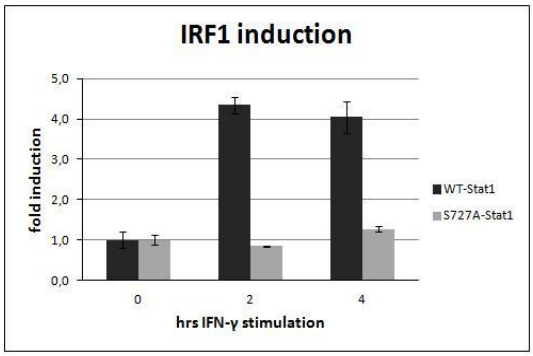
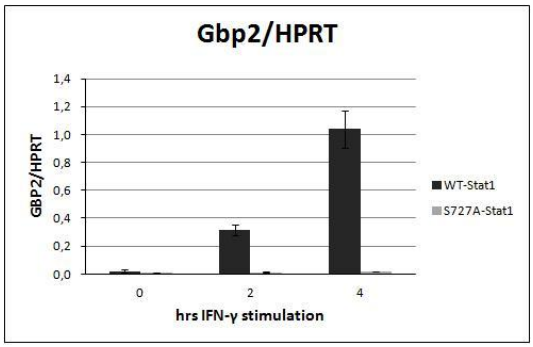



B

**Figure 6 | IRF1 mRNA induction abolished in Stat1-/- fibroblasts reconstituted with S727A-Stat1 in IFN-γ responses.** Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 or the Stat1-S727A mutant were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor IRF1 mRNA levels and inductions. S727A-Stat1 cells showed drastically decreased IRF1 mRNA levels under resting and induced conditions if normalized to HPRT (A); normalization to unstimulated samples revealed no IRF1 inductions in S727A-Stat1 fibroblasts. Results shown are derived two independent experiments (B).

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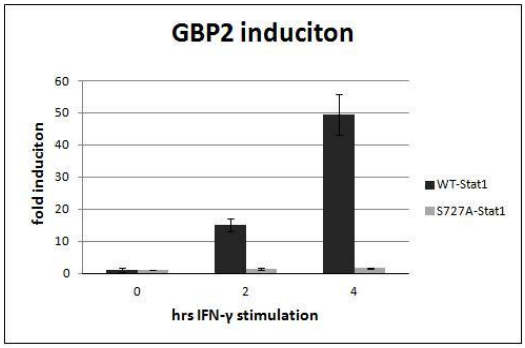
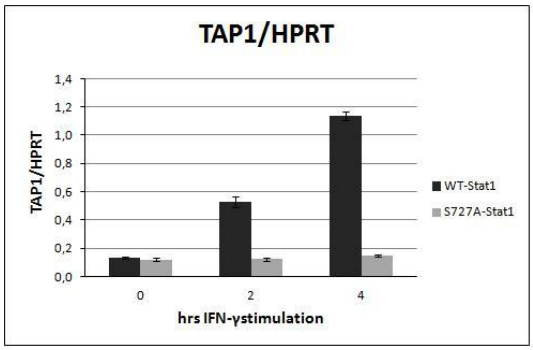



B

**Figure 7 | GBP2 mRNA induction abolished in Stat1-/- fibroblasts reconstituted with S727A-Stat1 in IFN-γ responses.** Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 or the Stat1-S727A mutant were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor GBP2 mRNA levels and inductions. S727A-Stat1 cells showed drastically decreased GBP2 mRNA levels under resting and induced conditions if normalized to HPRT (A); normalization to unstimulated samples revealed no GBP2 inductions in S727A-Stat1 fibroblasts. Results shown are derived two independent experiments (B).

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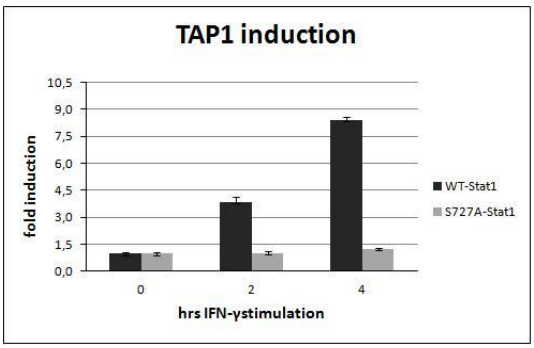



B

**Figure 8 | TAP1 mRNA induction abolished in Stat1-/- fibroblasts reconstituted with S727A-Stat1 in IFN-γ responses.** Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 or the Stat1-S727A mutant were stimulated with IFN-γ for the indicated time points. qRT-PCR was used to monitor TAP1 mRNA levels and inductions. S727A-Stat1 cells showed drastically decreased TAP1 mRNA levels under resting and induced conditions if normalized to HPRT (A); normalization to unstimulated samples revealed no TAP1 inductions in S727A-Stat1 fibroblasts. Results shown are derived two independent experiments (B).

## 3.2 Project 2: Studies on the kinase and kinetics of Stat serine phosphorylation

According to publications, Stat1 serine727 phosphorylation is crucial for full-fledged interferon gamma dependent innate immunity (38). The nature of the kinase responsible for Stat1 S727 phosphorylation has so far not been clarified. Data indicates that other Stat family members, namely Stat3, Stat4, Stat5a and Stat5b, can also be serine phosphorylated. Recent publications demonstrated that Stat1 and Stat3 have to be tyrosine-phosphorylated, translocated into the nucleus and bound to DNA to become S727 phosphorylated in interferon and IL-6 responses, respectively (75,148). All this data combined led to the hypothesis, that the nuclear CDKs engaged in transcription (CDK7, CDK8 and CDK9) might play a role in this phosphorylation event. These nuclear cyclin-dependent kinases seemed good candidates, because they are chromatin-bound proline-directed kinases that should be able to phosphorylate the conserved PMSP motif located in the Stat transactivation domain. Furthermore, CDK8 and CDK9 were reported to phosphorylated SMAD and Notch, two other latent cytoplasmic transcription factors (140,141).

### 3.2.1 Nuclear CDK inhibitor prevents Stat1 S727 phosphorylation in MEFs and human HepG2 cells

The group of Pavel Kovarik was able to show in murine bone marrow-derived macrophages (BMDMs), that IFN-induced Stat1 S727 phosphorylation as well as Stat3 S727 phosphorylation was inhibited by the chemical compound Flavopiridol (data not shown). Flavopiridol, a flavonoid derived from an Indian plant, was reported to potently inhibit CDK7, CDK8 and CDK9 (149). To reproduce the findings that CDK7, CDK8 or CDK9 were responsible for Stat1 S727 phosphorylation, further inhibitor studies were performed.

#### 3.2.1.1 Flavopiridol inhibits Stat1 S727 phosphorylation in IFN-γ stimulated MEFs

Immortalized mouse embryonic fibroblast were pretreated with the inhibitor Flavopiridol for 30 min and subsequently stimulated with IFN-γ or Anisomycin for 40 min. While the stimulation with Anisomycin causes only p38 MAPK-induced Stat1 S727 phosphorylation, IFNs induce phosphorylation of both Y701 and S727. p38 MAPK-induced S727 phosphorylation is mechanistically independent of interferon induced phosphorylation (78).

Western Blot analysis of whole cell extracts using specific antibodies against phosphorylated Stat1 S727 revealed that IFN-γ induced phosphorylation of Stat1 at Y701 and S727. Pretreatment with Flavopiridol prevented Stat1 to become serine phosphorylated in interferon responses. Stat1 Y701 phosphorylation, which is performed by receptor associated Janus kinases, was not affected by Flavopiridol after interferon stimulation. However, stimulation with the drug Anisomycin resulted in a S727 phosphorylation of Stat1 that was not inhibited by Flavopiridol. No Stat1 Y701 phosphorylation was detectable after Anisomycin treatment. In unstimulated cells the pretreatment with Flavopiridol further reduced the basal levels of pS727-Stat1. Untreated cells displayed basal Stat1 S727 or Y701 phosphorylation (Figure 15).

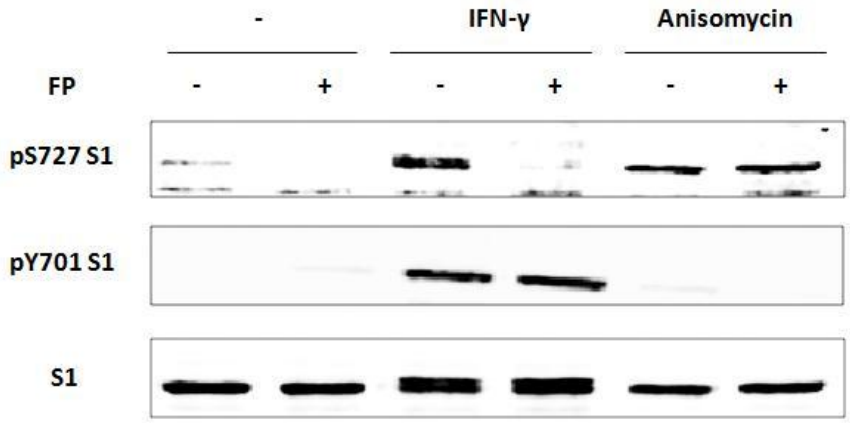



**Figure 9 | Stat1 S727 phosphorylation is inhibited by Flavopiridol in IFN-γ responses in MEFs.** Stat1-WT MEFs were pretreated with the nuclear CDK inhibitor Flavopiridol (FP, 500 nM) for 30 min and subsequently stimulated with IFN-γ (5 ng/ml) or Anisomycin (100 ng/ml) for 40 min.. Phosphorylation of Stat1 S727 (pS727 S1) was examined by Western blot analysis of whole-cell extracts with a pS727-Stat1 antibody. The membrane was reprobed with antibodies to phosphorylated Y701-Stat1 (pY701-S1) and Stat1-Ct (S1; loading control).

### 3.2.1.2 Flavopiridol inhibits Stat1 S727 phosphorylation in IFN-γ and IL-6 stimulated HepG2 cells

To test whether the results obtained in chapter 3.2.1.1 were also applicable in human cells, human liver hepatocellular carcinoma cells (HepG2) cells were pretreated with the inhibitor Flavopiridol for 30 min and subsequently stimulated with IFN-γ or Interleukin-6 (IL-6) for 40 min. IFN-γ as well as IL-6 are known to induce a JAK/Stat dependent serine 727 phosphorylation of Stat1. Western Blot analysis of whole cell extracts, using specific antibodies against phosphorylated Stat1 S727 and pY701-Stat1, revealed that IFN-γ as well as IL-6 induced the phosphorylation of Stat1 at Y701 and S727. The nuclear CDK inhibitor Flavopiridol was able to abolish Stat1 S727 phosphorylation in IL-6 and IFN-γ responses. The phosphorylation of Stat1 at tyrosine 701 was not affected by Flavopiridol in IL-6 or interferon signaling. Untreated cells displayed no Stat1 S727 or Y701 phosphorylation (Figure 10).

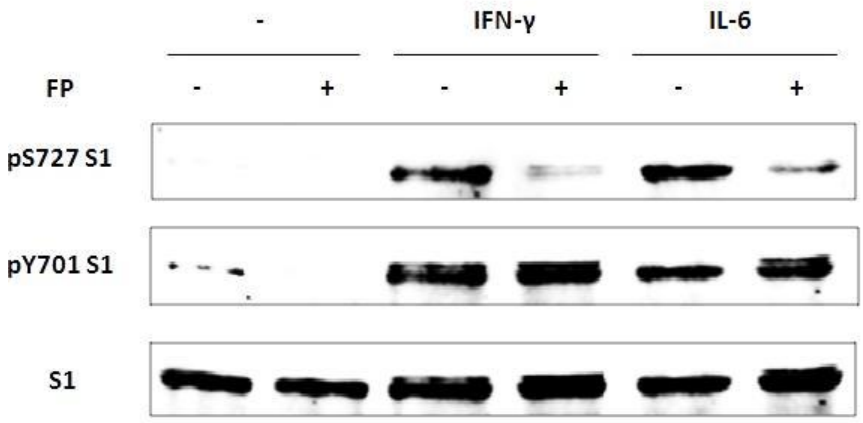



**Figure 10 | Stat1 S727 phosphorylation is inhibited by Flavopiridol in IFN-γ and IL-6 responses in HepG2 cells.** HepG2 cells were pretreated with the nuclear CDK inhibitor Flavopiridol (FP, 500 nM) for 30 min and subsequently stimulated with IFN-γ (10 ng/ml) or IL-6 (10 ng/ml) for 40 min. Phosphorylation of Stat1 S727 (pS727 S1) was examined by Western blot analysis of whole-cell extracts with a pS727-Stat1 antibody. The membrane was reprobed with antibodies to phosphorylated Y701-Stat1 (pY701-S1) and Stat1-Ct (S1; loading control).

#### 3.2.1.2 Nuclear CDK inhibitor prevents Stat3 S727 phosphorylation in HepG2 cells

To investigate whether the same kinase responsible for Stat1 S727 phosphorylation also mediates Stat3 S727 phosphorylation, HepG2 cells were stimulated with IL-6 for 40 min after being pretreated with Flavopiridol and/or UO126 for 30 min. Previous studies revealed that growth factor receptors signal to Stat3 through the ERK pathway, resulting in S727-phosphorylated Stat3 without the need of a preceding tyrosine phosphorylation (83). The use of a second inhibitor called UO126 that specifically inhibits MEK1/2 was therefore required. HepG2 cells stimulated with IL-6 without the addition of any inhibitors displayed Stat3 Y705 as well as S727 phosphorylation. The addition of either Flavopiridol or UO126 alone was not sufficient to inhibit Stat3 S727 phosphorylation. The Y705 phosphorylation was not affected by the inhibitors. The dual inhibition of the nuclear CDKs by Flavopiridol and MEK1/2 by UO126 abolished Stat3 S727 phosphorylation, but did not affect the tyrosine 701 phosphorylation in IL-6

responses. Untreated cells displayed basal Stat3 S727 or Y705 phosphorylation (Figure 11).

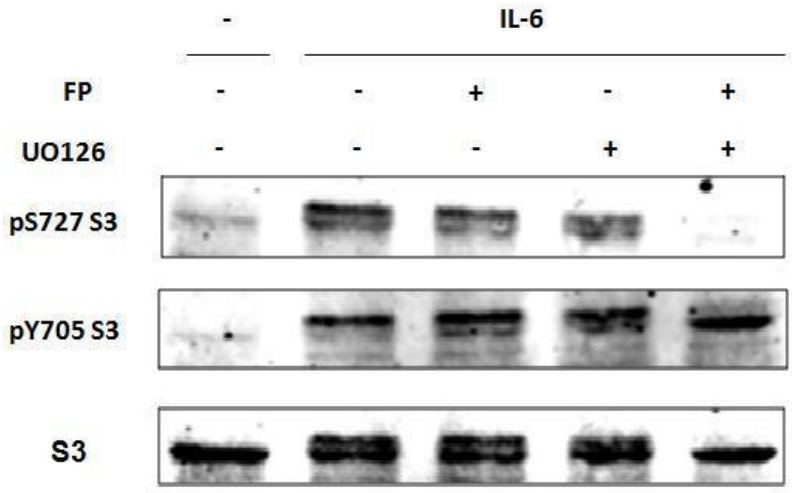
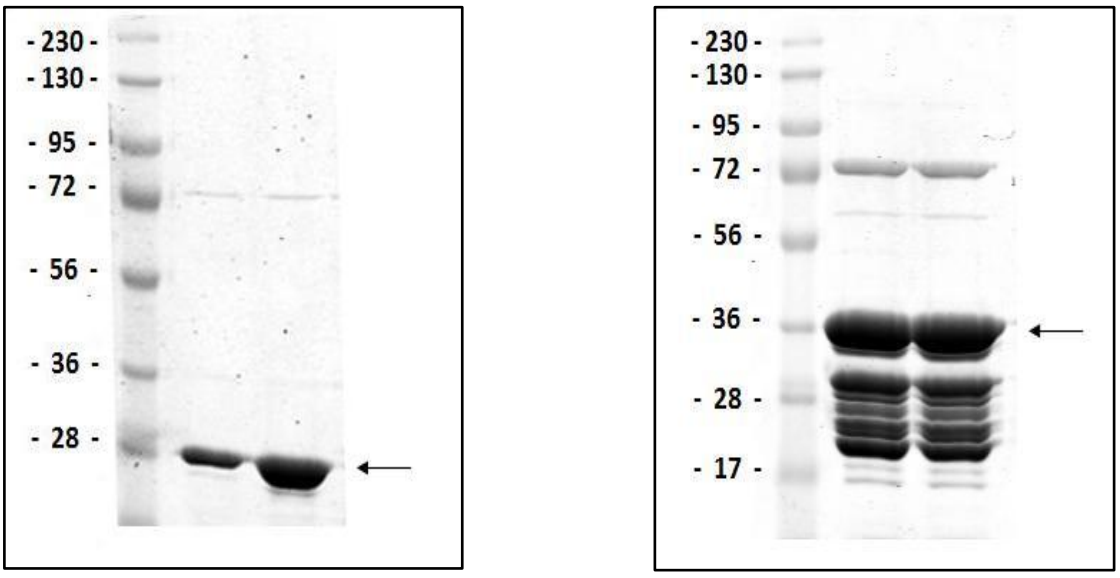



**Figure 11 | Stat3 S727 phosphorylation is inhibited by Flavopiridol in IL-6 responses in HepG2 cells.** HepG2 cells were pretreated with the nuclear CDK inhibitor Flavopiridol (FP, 500 nM) and/or the MEK1/2 Inhibitor UO126 (10 µM) for 30 min and subsequently stimulated with IL-6 (10 ng/ml) for 40 min. Phosphorylation of Stat3 S727 (pS727 S3) was examined by Western blot analysis of whole-cell extracts with a pS727-Stat3 antibody. The membrane was reprobed with antibodies to phosphorylated Y705-Stat3 (pY701 S3) and Stat3-Ct (S3; loading control).

### 3.2.2 GST-tagged protein expression and purification of Stat1 peptides

To identify which of the nuclear CDKs engaged in transcription (CDK7, CDK8 and CDK9) is able to phosphorylate Stat1 at serine 727 in vitro, Stat1 polypeptides fused to GST at their N-terminus were expressed and purified in order to perform in vitro kinase assays. One polypeptide contained the last 39 amino acids (short construct) of the C-terminus, while the other was 119 amino acids long (long construct). Both the long and the short construct were purified for the Stat1 wild-type protein and the S727A mutant (Figure 12). Kinase assays with CDK7, CDK8 and CDK9 were then performed by my colleague Joanna (data not shown.)




A B

**Figure 12| Coomassie blue staining of purified GST-tagged Stat1 polypeptides.** GST-tagged Stat1 polypeptides were expressed in BL21 competent E-coli and subsequently purified using Glutathione Sepharose 4B beads. A) Last 39 amino acids of Stat1 C-terminus of wild-type Stat1 and S727A mutant; B) Last 119 amino acids of Stat1 C-terminus of wild-type Stat1 and S727A mutant

### 3.2.3 Inducible shRNA-mediated silencing of CDK8 in Stat1-S727A MEFs

RNAi-mediated gene silencing of CDK7, CDK8 and CDK9 in Stat1 wild-type mouse embryonic fibroblast revealed that CDK8, and most likely not CDK7 or CDK9 is the kinase responsible for Stat1 S727 phosphorylation (Joanna Bancerek, data not shown). To further investigate the role of CDK8 in Stat1 transcriptional regulation, Stat1-deficient 3T3 fibroblasts reconstituted with the Stat1-S727A mutant protein, were stably transfected with a TRIPZ Lentiviral shRNAmir vector targeting specifically CDK8 (OPEN BIOSYSTEMS). The pTRIPZ lentiviral vector is engineered to be Tet-On. In the presence of doxycycline a single transcript consisting of TurboRFP and shRNAmir is induced. The expression of TurboRFP allows an easy visual observation of the shRNAmir expression under the fluorescence microscope (Figure 14).

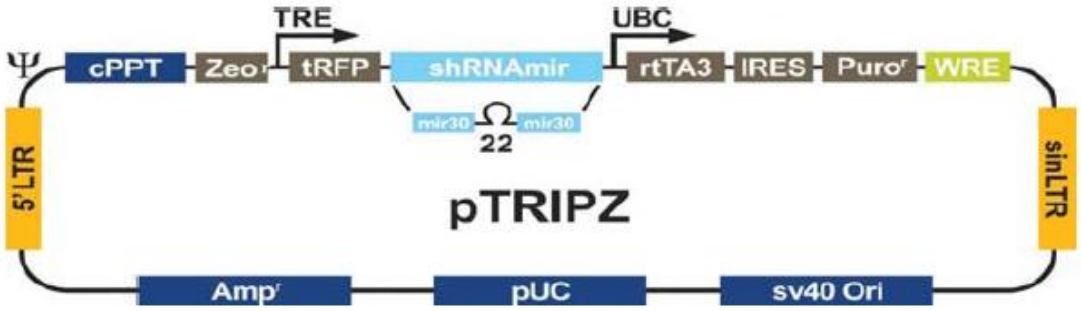



**Figure 13| Features of the pTRIPZ lentiviral vector**. IRES-Puro: mammalian selectable marker; turboRFP: marker to track shRNAmir expression; TRE: tetracycline responsive RNA Polymerase II promoter; rTA3: reverse tetracycline trasactivator; cPPT: cetnral Polypurine tract; WRE: enhances stabilty and translation of transcripts (http://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/)

Expression Arrest short hairpin RNA constructs are expressed as micro-RNA-30 (miR30) transcripts that are then further processed by Drosha and Dicer. The hairpin stem was engineered of 22nt of dsRNA and a 19nt loop from human miR30. The addition of a miR30 loop and a 125nt flanking sequence of miR30 were shown to increase the processing by Drosha and Dicer (Figure 14). The resulting siRNA then specifically targets the mRNA of interest for degradation.

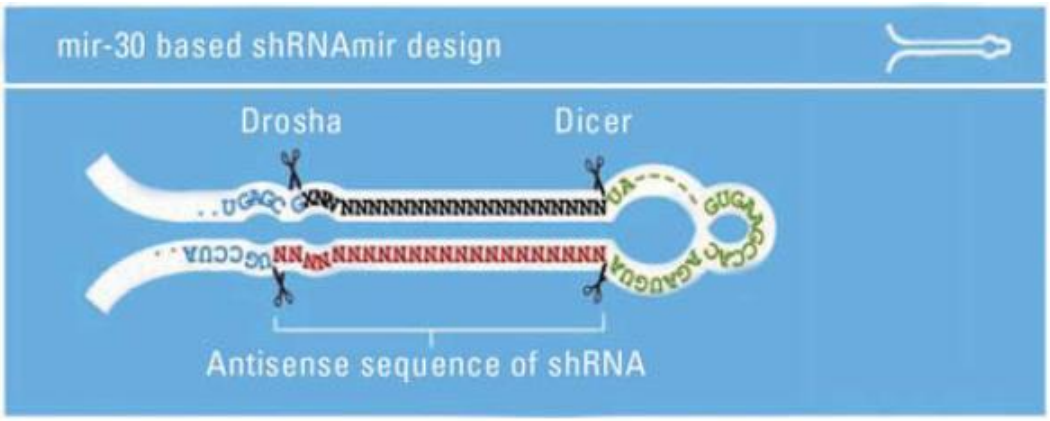



**Figure 14 | Expression Arrest shRNA are expressed as miR30 primary transcripts** (http://lentivirus.imb.uq.edu.au/downloads/pdfs/TRIPZ_Technical_Manual.pdf)

Stat1-deficient 3T3 fibroblasts reconstituted with the Stat1-S727A mutant protein were transfected with three different pTRIPZ Lentiviral shRNAmir vectors, targeting different parts of the CDK8 mRNA (V2THS_112909, V2THS_112910 and V2THS_112911, provided by OPEN BIOSYSTEMS), using the transfection reagent TurboFect (Fermentas). After transfection, cells were split into puromycin selection conditions to select for stable transfectants. Two weeks after selection, bulk cultures were tested for stable vector integration by doxycycline induced turboRFP expression using fluorescence microscopy. The addition of doxycycline resulted in the expression of turboRFP in the bulk cultures transfected with either V2THS_112909, V2THS_112910 or V2THS_112911. In the absence of doxycycline no turboRFP signal was detectable (Figure 15). However, Western Blot analysis and immune fluorescence microscopy of the bulk cultures revealed the loss of Stat1-S727A expression, rendering cells useless for our purposes (data not shown).

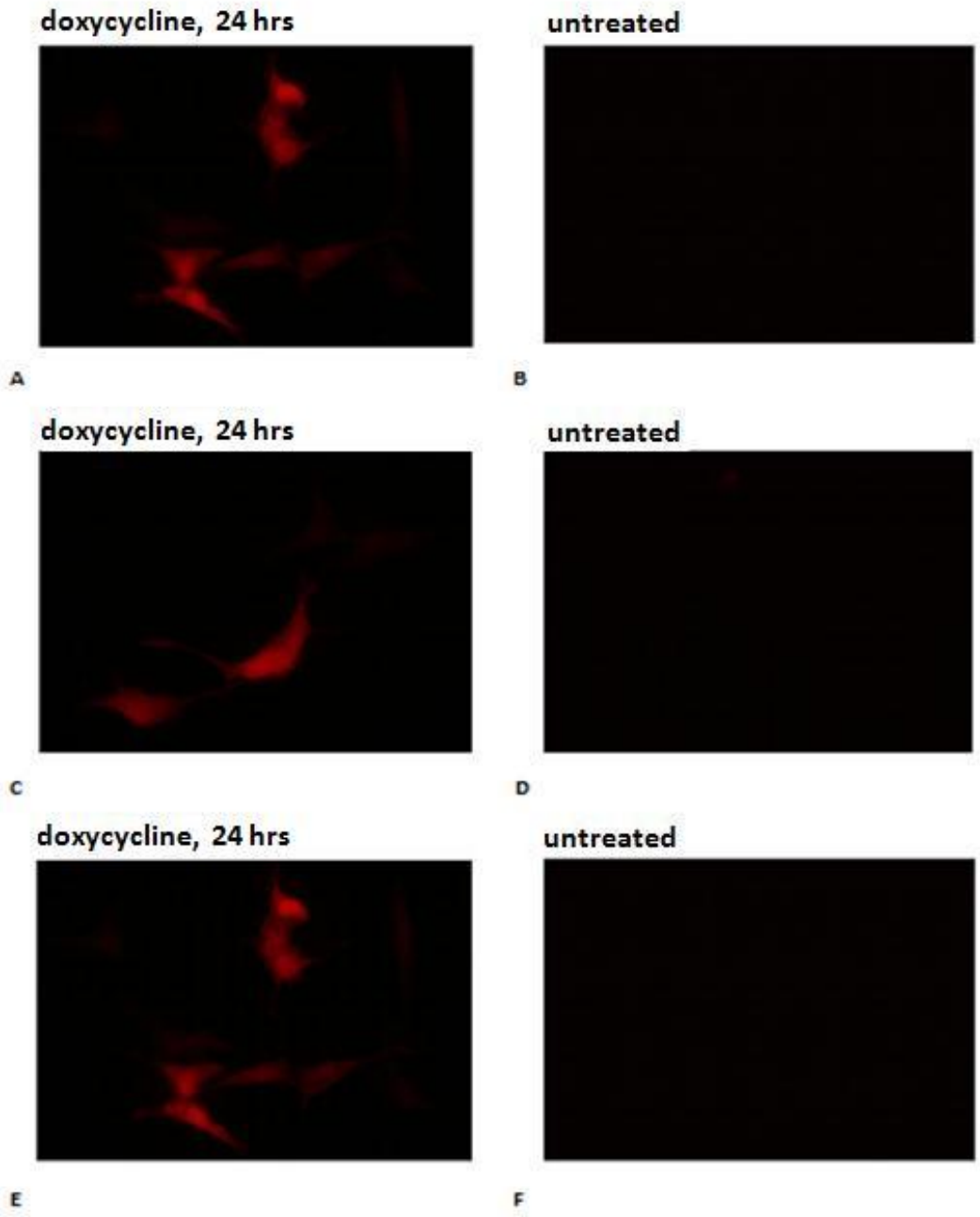



**Figure 15| TurboRFP expression from stably integrated doxycycline inducible pTRIPZ lentiviral vectors in Stat1-S727A MEFs.** Stat1-deficient 3T3 fibroblasts reconstituted with the Stat1-S727A mutant protein were stably transfected with three different pTRIPZ Lentiviral shRNAmir vectors, V2THS_112909 (A, B), V2THS_112910 (C, D) and V2THS_112911 (E, F), targeting different parts of the CDK8 mRNA. To asses stable integration, either no (B, D, F) or 1 µg/ml doxycycline (A, C, E) was added to batch culture aliquots for 24 hours. TurboRFP expression was visualized by fluorescence microscopy.

### 3.2.3 Chromatin Immunoprecipitation (ChIP) of pS727-Stat1 and Stat1 in MEFs after IFN-γ stimulation

Recent studies revealed that some kinases function as chromatin bound enzymes to modulate the activity of various transcription factors (150). Sadzak et.al demonstrated that Stat1 has to be integrated into chromatin associated transcriptional complexes to become S727-phosphorylated in IFN gamma responses (75). However, the kinetics underlying Stat1 S727 phosphorylation have not been clarified yet. Chromatin Immunoprecipitation (ChIP), which allows the observation of protein/DNA interactions in a cell at a distinct time point, is a powerful tool to address this question.

Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 were stimulated for 0, 5, 10, 20, 30 and 60 min with IFN-γ and subsequently pS727-Stat1 and Stat1-C were selectively immunoprecipitated. The recruitment of Stat1 molecules to the GAS sites located in the promoters of three well known Stat1 target genes in interferon responses, GBP2, TAP1 and IRF1, were then analyzed by PCR and qRT-PCR.

The immunoprecipitation of Stat1-C revealed that Stat1 bound to the promoters of TAP1 and LMP2 already 5 minutes after IFN-γ stimulation and reached its maximum recruitment after 10 min of interferon stimulation. The transcription factor stayed on the observed promoters for at least 1 hour under constant interferon stimulation. Serine 727 phosphorylated Stat1 was first slightly detectable on these promoters after 5 to 10 min of cytokine stimulation. Serine phosphorylated Stat1 molecules reached their maximum appearance on the promoters of TAP1 and GBP2 after 20 to 30 min of stimulation and stayed on the promoters of these two genes for at least 60 min under constant IFN-γ signaling. The IgG control (TTP preimmune serum) displayed no unspecific binding of either Stat1 or pS727-Stat1 to IgG antibodies (Figure 16).

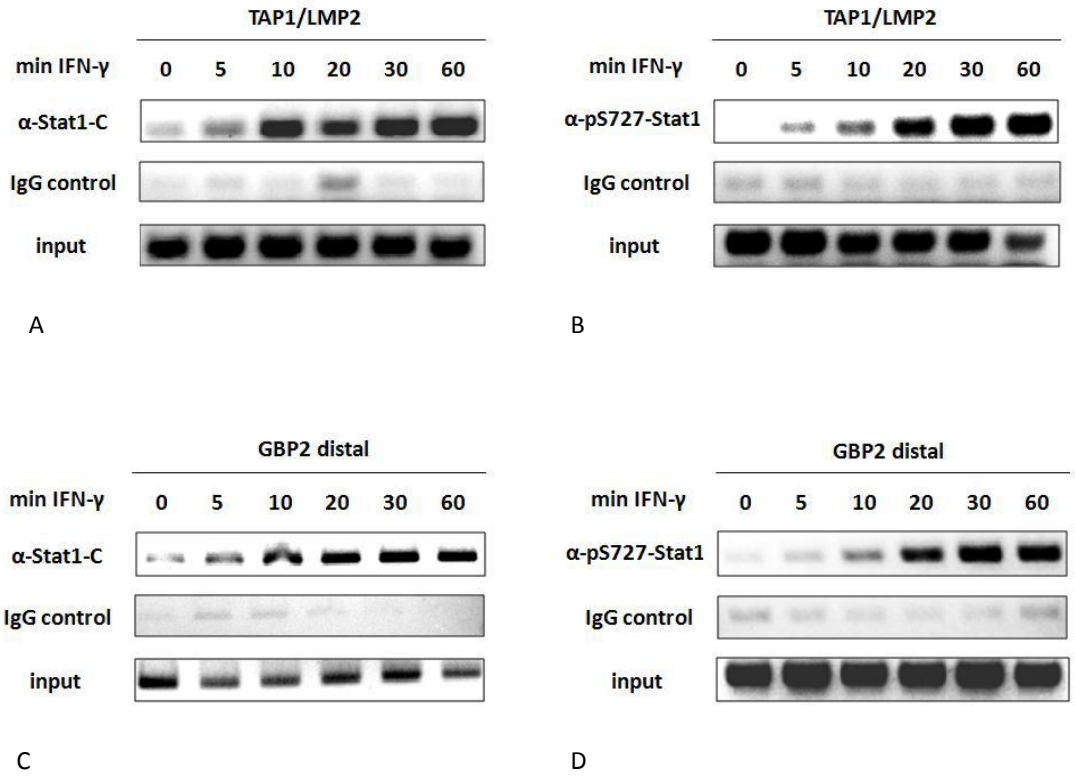
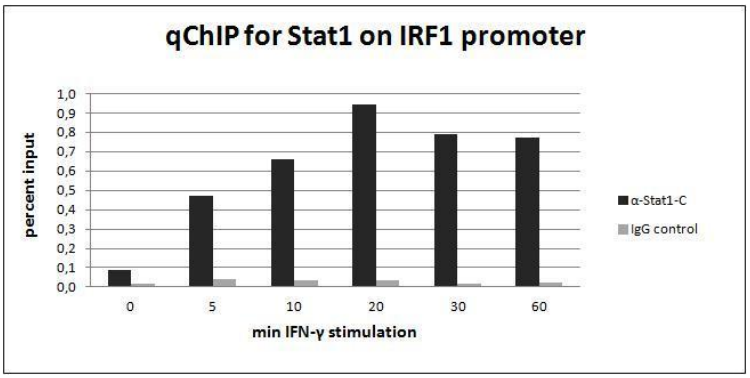
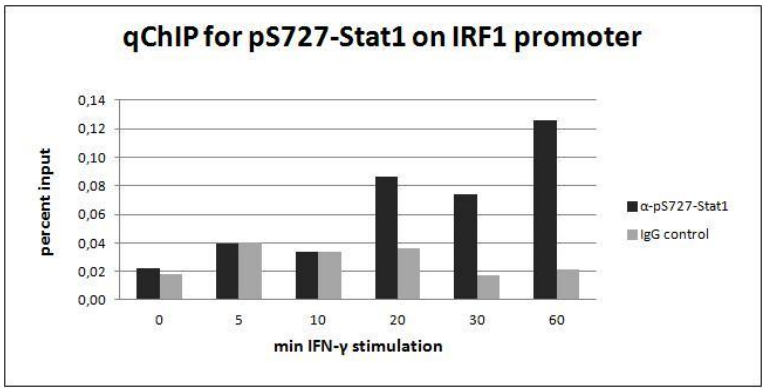



**Figure 16| Appearance of Stat1 precedes the appearance of pS727-Stat1 on the TAP1/LMP2 and GBP2 promoter in IFN responses.** Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 were stimulated with IFN-γ for the indicated time points and subsequently Stat1 (A, C) and pS727-Stat1 (B, D) were selectively immunoprecipitated. The recruitment of Stat1 and pSS727-Stat1 to the promoters was monitored by PCR.

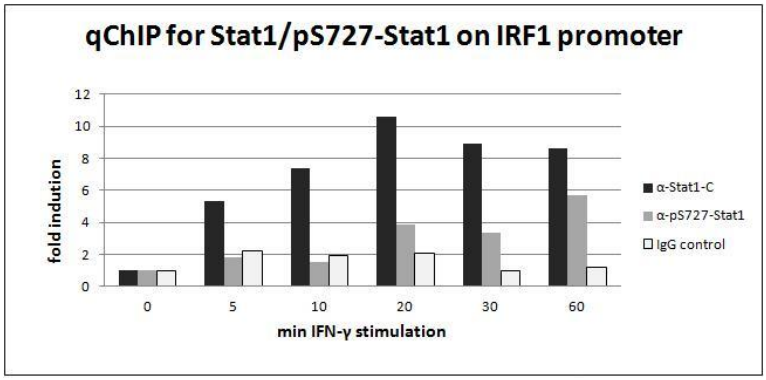
Similar data as seen for the GBP2 and TAP1 promoters were obtained for the IRF1 gene. The analysis of the IRF1 promoter revealed that Stat1 was first recruited to the IRF1 GAS site after 5 min of IFN-γ stimulation and reached its maximum after 10 to 20 min of constant cytokine signaling. Stat1 stayed on the IRF1 promoter for at least 60 min. The level of S727 phosphorylated Stat1 increased after 20 min of IFN stimulation and was detectable on the IRF1 promoter for at least 60 min under these conditions. The IgG control (TTP preimmune serum) displayed no unspecific binding of either Stat1 or pS727-Stat1 to IgG antibodies (Figure 17).




A




B




C

**Figure 17| Appearance of Stat1 precedes the appearance of pS727-Stat1 on the IRF1 promoter in IFN** responses. Stat1-deficient 3T3 fibroblasts reconstituted with wild-type Stat1 were stimulated with IFN-γ for the indicated time points and subsequently Stat1 (A, C) and pS727-Stat1 (B, C) were immunoprecipitated. The recruitment of Stat1 and pSS727-Stat1 to the promoter was monitored by qRT-PCR.

# 4 Discussion

## 4.1 Role of Stat1 S727 phosphorylation on Stat1 transcriptional activity

Interferons are one of the main cytokines coordinating responses of the immune system during infections. While a too weak IFN response may not be able to properly eradicate invading pathogens, a too strong response can potentially harm the host (151). One level of regulation was described as an additional modulating phosphorylation of Stat1 at S727, next to its mandatory tyrosine phosphorylation in interferon gamma responses. This chemical modulation of the Stat1 transactivation domain was shown to be needed for full-fledged IFN-γ dependent innate immunity in mice (38).

For the analysis of the role of Stat1 S727 phosphorylation in IFN-γ responses, mouse embryonic fibroblasts, expressing either the wild-type or a S727A mutant Stat protein, were stimulated with IFN-γ and the expression levels of Stat1 target genes were monitored. Three well known Stat1 target genes in interferon responses, namely IRF1, GBP2 and TAP1, were analyzed. Interestingly, the comparison of immortalized (3T3) mouse embryonic fibroblasts (MEFs) derived from mice, expressing either wild-type Stat1 or the S727A mutant, showed no impairment of the mutant transcription factor in its ability to induce the expression of IRF1 after two and four hours of IFN-γ stimulation. If normalized to the internal housekeeping gene HPRT, Stat1-S727A MEFs displayed even slightly higher IRF1 mRNA levels in the unstimulated as well as in the induced state compared to Stat1-wt MEFs. This was however not true for the two other monitored genes GBP2 and TAP1. Stat1-S727A MEFs were heavily impaired in the expression of GBP2 and TAP1 in interferon gamma responses, at basal as well as at induced conditions, if compared to HPRT. The observation, that unstimulated Stat1-S727A MEFs show a drastically decreased expression of GBP2 and TAP1, indicates that the modulating serine phosphorylation may play a major role in the constitutive basal

IFN/Stat1 signaling in resting cells. However, the importance of the S727 phosphorylation seems to wane over time, if stimulated with high amounts of interferon gamma. GBP2 and TAP1 mRNA levels of the mutant cells increasingly resembled more the levels of wild-type Stat1 fibroblasts after two and four hours of constant interferon signaling. This fact also explains the higher inducibility of Stat1-S727A cells compared to Stat1 wild-type fibroblasts after longer periods of interferon stimulation.

The results obtained from of Stat1-defcient 3T3 fibroblasts reconstituted with wild-type Stat1 or the Stat1-S727A mutant differed from the ones described above. No interferon mediated inductions of IRF1, GBP2 or TAP1 were detectable in cells expressing Stat1-S727A. Later studies revealed that Stat1-defcient 3T3 fibroblasts, stably reconstituted with the Stat1-S727A mutant, had the tendency to lose the expression of Stat1. If the striking impairment in IRF1, GBP2 and TAP1 expression in these cells is due to a loss of Stat1 is not totally clear. Stat1 deficient fibroblasts stably reconstituted with wild-type Stat1 displayed a similar expression pattern after interferon gamma stimulation as MEFs derived from mice expressing the wild-type transcription factor.

Together these data indicate that individual Stat1 target genes depend differently on Stat1 S727 phosphorylation in interferon gamma responses. While the lack of Stat1 S727 phosphorylation does not affect the expression of IRF1, the expression of GBP2 and TAP1 is highly dependent on this chemical modification. The lack of serine phosphorylation seems to affect basal as well as induced expression levels. The reason why the dependence of individual Stat1 target genes on the S727 phosphorylation varies is not clear yet. Future studies will have to clarify how the promoters of these genes differ and what consequences these differences have for interferon gamma induced Stat1 activation. The fact that the promoters of GBP2 and TAP1 contain ISRE sites in addition to GAS sites, whereas just one single GAS element is located within the IRF1 promoter, might be of importance. Also the proper location of the GAS site in relation to the transcriptional start site may play a role. The presence and influence of other regulatory proteins, such as transcriptional repressors or activators, cannot be

ruled out. In the long run, the proper molecular mechanisms by which Stat1 S727 phosphorylation modulates Stat1 transcriptional activity will have to be clarified. Recent publications proposed a role of Stat1 S727 phosphorylation in the nuclear export of the transcription factor (152). We therefore speculate that the phosphorylation of Stat1 at S727 on one hand triggers the transcriptional machinery, and on the other hand it facilitates a rapid shuttling of the transcription factor back to the IFN receptor, where it can undergo a new activation cycle. By this mechanism, a very sensitive, tightly controlled and fast induction of interferon induced targets genes is possible. Future experiments will clarify whether the hypothesized mechanism described in this work holds true.

## 4.2 Studies on the kinase and kinetics of Stat serine phosphorylation

The second part of my thesis was focusing on the kinase responsible for Stat1 S727 phosphorylation and on the kinetics underlying this phosphorylation event. A former PhD student of Kovarik's lab, Iwona Sadzak, found out that Stat1 has to be chromatin bound to become S727 phosphorylated in interferon responses (75). Members of the lab of Pavel Kovarik further demonstrated in macrophages, derived from bone marrow, that Flavopiridol, a Cyclin-dependent kinase (CDK) inhibitor with a more specific activity towards nuclear CDKs, was able to prevent Stat1 and Stat3 S727 phosphorylation after interferon stimulation. Similar studies in MEFs showed that also in fibroblasts, Stat1 S727 phosphorylation was inhibited by Flavopiridol in interferon gamma responses, without effecting the previously mediated Stat1 tyrosine phosphorylation. Additionally we stimulated MEFs with Anisomycin, which is known to trigger the p38 MAPK dependent serine phosphorylation of Stat1. After Anisomycin administration, Stat1 becomes phosphorylated at S727 without the need of a previous tyrosine phosphorylation. The phosphorylation of Stat1 at serine 727 by p38 MAPK was not affected by the presence of Flavopiridol. We could thereby confirm data claiming that the mechanism underlying Stat1 S727 phosphorylation in interferon responses differs from the one mediated by p38 MAPK (78). The use of Flavopiridol in

HepG2 cells demonstrated that the nuclear CDKs also play a role in Stat1 S727 phosphorylation in human cells in interferon and IL-6 responses.

All members of the Stat family except Stat2 and Stat6 were reported to contain a serine residue inside their transactivation domain, located in a conserved consensus site, PMSP (Stat1, Stat3 and Stat4) or PLSP (Stat5) (90). Since the phosphorylatable serine is located within a quite conserved motif across the Stats, it stands to reason that the same kinase phosphorylating Stat1 at S727 is also able to phosphorylate other Stat members at the same residue. HepG2 cells stimulated with IL-6 showed an abolished Stat3 S727 phosphorylation if pretreated with the inhibitors Flavopiridol and UO126. The constant growth factor mediated signaling to Stat3 through the ERK pathway made the use of a Mek1/2 inhibitor (UO126) mandatory. The tyrosine phosphorylation of Stat3 by its cells surface receptor was not disturbed by Flavopiridol in IL-6 responses. Together these data indicate that Stat1 and Stat3 are phosphorylated by a similar subset of nuclear kinases at serine 727 in interferon and IL-6 responses and that this kinases can be specifically inhibited by Flavopiridol. The fact that Flavopiridol targets nuclear CDKs is consistent with the finding that Stat1 has to be chromatin bound to become phosphorylated at S727 (75). However, inhibitor studies are of pleiotropic nature and therefore specific siRNA-mediated gene silencing experiments, targeting CDK7, CDK8 and CDK9, are required. Furthermore, the dependence of Stat4 and Stat5 on nuclear CDKs to become serine phosphorylated has to be investigated. In vitro kinase assays will help answering the question, which nuclear CDK is able to phosphorylate Stat proteins at the conserved PMSP motif.

Previous studies demonstrated that Stat1 has to become tyrosine phosphorylated and has to translocate into the nucleus, where it is assembled into transcriptional complexes, in order to become phosphorylated at serine 727. Apart from these prerequisites, not much is known about the kinetics underlying Stat serine phosphorylation. To shed light on this phosphorylation event, chromatin immunoprecipitation assays where performed. Mouse embryonic fibroblasts expressing wild-type Stat1 molecules were stimulated with IFN-γ and subsequently

pS727-Stat1 and Stat1-C were selectively immunoprecipitated. The promoters of three well known Stat1 target genes in interferon responses, GBP2, TAP2 and IRF1, were subsequently analyzed for pS727-Stat1 and Stat1 binding. All three promoters displayed Stat1 binding to the upstream located GAS element after 5 min of interferon stimulation. Maximal Stat1 recruitment to the GBP2, TAP1 and IRF1 promoter was reached after 10 to 20 min of interferon stimulation. Serine phosphorylated Stat1 was detectable after 10 to 20 min of interferon stimulation and stayed at the promoters of the observed genes for at least one hour. To summarize, this data shows that Stat1 binding to its target promoters precedes the appearance of serine phosphorylated Stat1. This finding is consistent with publications stating that Stat1 gets serine phosphorylated once it is bound to chromatin. Future experiments should aim to address the questions in which ways polymerase recruitment is timely related to Stat1 and pS727-Stat1 appearance on the different promoters, and if CDK7, CDK8 or CDK9 are recruited at all. Since the dependence of individual Stat1 target genes on the S727 phosphorylation varies, it will be interesting to find out how the recruitment of Stat1 and pS727-Stat1 differs between individual genes. Chromatin immunoprecipitation assays in Stat1 wild-type and S727A mutant cells will hopefully further clarify the role of Stat1 serine phosphorylation on Stat1 transcriptional activity.

# 5 Materials and Methods

## 5.1 Cell culture

### 5.1.1 Culture conditions:

All cell culture work was performed under sterile conditions. Cells were grown at 37°C with 5% $CO_2$ and 95% humidity.

### 5.1.2 Cultivation of cell lines

3T3 immortalized MEFs as well as HepG2 cells were grown in a DMEM High glucose (4.5 g/l) medium containing 10% fetal calve serum, Penicillin (100 U/ml) and Streptomycin (100µg/ml). Passaging was realized by removing the medium, washing the cells one time with sterile 1x PBS (4°C) and a subsequent incubation with 1 ml 1x Trypsin-EDTA per 10 cm tissue culture dish for several minutes. Cells were then resuspended in warm full DMEM medium and split in the required ratio.

### 5.1.3 Freezing of cells

Cells were washed once with sterile PBS after medium removal and harvested by trypsinization with Trypsin-EDTA for several minutes. Trypsinization was stopped by adding 10 ml of full DMEM per 10 cm tissue culture dish. The cell suspension was centrifuged at 1200 rpm for 5 min at room temperature, the medium removed and the pellet resuspended in FCS containing 10% DMSO. Cells were then immediately transferred into a cryotube and stored at -80°C. After at least one day at -80°C, cells can be transferred to liquid nitrogen for long time storage.

### 5.1.4 Thawing of cells

Cells were thawed quickly in a water bath at 37°C. After thawing, cells were immediately resuspended in 10 ml of warm full DMEM and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cells were resuspended in an aliquot

of warm full DMEM. The suspension was then transferred into a 10 cm tissue culture dish containing warm full DMEM.

### 5.1.5 Cell counting

Cells were counted using a Neubauer Chamber. After trypsinization cells were resuspended in warm full DMEM medium. 50µl of cell suspension was mixed with 50 µl of trypan blue solution (0.4%) and the cell number was assessed under the light microscope.

| **1000 x Penicillin/Streptomycin (10 ml)** | |
|---|---|
| Penicillin | 0.6 g |
| Streptomycin | 1 g |
| $ddH_2O$ | up to 10 ml |
| Sterile-filter and store aliquots at -20°C | |

## 5.2 mRNA level analysis in 3T3 immortalized mouse embryonic fibroblasts (MEFs)

3T3 immortalized Stat1 wt and Stat1 S727A MEFs were seeded the day before the experiment on 6 cm tissue culture dishes. On the day of the experiment, cells displayed 70 - 80% confluency. To induce the expression of Stat1 depended genes, cells were stimulated with mouse IFN-γ (5 ng/ml) according to experimental setup.

### 5.2.1 RNA isolation using Isol-RNA lysis reagent (5Prime)

Cells were grown on 10 cm (6 cm) tissue culture dishes and treated according to the experimental setup. Dishes were washed once with ice cold 1x PBS after removing the medium on ice. Thereafter, 750 µl (300 µl) Isol-RNA lysis reagent was added to the culture dishes and cells were harvested using a rubber policeman. The crude cell lysate was transferred into a safe-lock tube (Eppendorf) and incubated for 5 min at room temperature. 200 µl (80 µl) of chloroform ($CHCl_3$) was added and the extract was incubated for 15 min at room temperature after being shortly vortexed. The tubes

were then centrifuged for 20 min at 12.000 rpm at 4°C. The upper aqueous layer was carefully transferred into a fresh tube and 500 µl (220 µl) of 2-Propanol was added and the two layers were mixed gently. The lysates were centrifuged for 15 min at 12.000 rpm at 4°C after being incubated at room temperature for 10 min. The supernatant was discarded and the pellet was washed once with 1 ml of 75% ethanol. After 5 min centrifugation at 12.000 rpm at 4°C the remaining liquid was removed and the pellet was dried for 15 min at room temperature with the lid of the tube being open. The pellet was resuspended in 40 µl (25 µl) of RNase-free dd$H_2O$ on ice and stored at -80°C.

### 5.2.2 Reverse transcription of RNA (cDNA synthesis)

1 µg of isolated RNA was mixed with 1 µl of Oligo dT (100 pmol/µl) and RNase-free dd$H_2O$ was added until a final volume of 11 µl was reached. After 5 min of incubation at 70°C, the tube was kept on ice and 4 µl of 5x m-MuLV RT Buffer (Fermentas), 2 µl of 10mM dNTP-mix and 2 µl of dd$H_2O$ were added. The tube was incubated again for 5 min at 37°C and 1 µl of RevertAid (200 U) reverse transcriptase was added while the tube was in the 37°C heating block. After 60 minutes incubation at 42°C, the reaction was stopped by incubating the tube for 10 min at 70°C. cDNA was stored at -20°C.

### 5.2.3 Quantitative RT-PCR using SYBR Green

cDNA was diluted to a final volume of 200 µl with d$H_2O$ and 5 µl of cDNA was used as a template for triplicates or at least duplicates. The standard dilutions (dilutions from 1:2 to 1:16) were prepared from the sample that should derive the highest reading. This setup was prepared for each individual primer pair being analyzed as well as for HPRT, serving as an internal housekeeping control. To each well of the 96-well PCR plate containing a sample, 20 µl of a freshly prepared master mix with the appropriate primers were added. To eradicate contaminations of the master mix or contaminations of RNA with genomic DNA, one well was filled with 5 µl of d$H_2O$ and one with 1 µl of RNA and 4 µl of d$H_2O$, respectively. qRT-PCR was then performed using a master cycler detecting the fluoresce signal emitted from SYBR Green.

| Master mix | |
|---|---|
| | final concentration |
| 2,5 µl 25 mM $MgCl_2$ | 1.5 mM |
| 2,5 µl 10x Taq buffer with KCl | 1x |
| 0,5 µl 10 mM dNTP | 200 µM |
| 0,075 µl 100 µM rev primer | 300 nM |
| 0,075 µl 100 µM fwd primer | 300 nM |
| 1 µl SYBR Green (1:300) | |
| 0,2 µl Taq DNA Polymerase | 1 U |
| 13,15 $ddH_2O$ | |
| total 20 µl | |

### 5.2.4 qRT-PCR primers and conditions

| Primer | Sequence |
|---|---|
| mIRF1-fwd | 5'- CCG AAG ACC TTA TGA AGC TCT TTG-3' |
| mIRF1-rev | 5'- GCA AGT ATC CCT TGC CAT CG-3' |
| mHPRT-fwd | 5'- GGA TTT GAA TCA CGT TTG TGT CAT-3' |
| mHPRT-rev | 5'- ACA CCT GCT AAT TTT ACT GGC AA - 3' |
| mGBP2-fwd | 5'- TGC TAA ACT TCG GGA ACA GG - 3' |
| mGBP2-rev | 5'- GAG CTT GGC AGA GAG GTT TG - 3' |
| mTAP1-fwd | 5'- CTG GCA ACC AGC TAC GGG T - 3' |
| mTAP1-rev | 5'- TGA GAA AGA GGA TGT GGT GGG - 3' |

| Repeats | Temperature | Time |
|---|---|---|
| 1x | 95,0°C | 5 min |
| 40 cycles | 95,0°C | 15 sec |
| | 60,0°C | 15 sec |
| | 72,0°C | 20 sec |
| 1x | 95,0°C | 15 sec |
| 1x | 60,0°C | 15 sec |
| 1x | 20,0 – 95,0°C | 20 min |
| 1x | 95,0°C | 15 sec |
| 1x | 20,0°C | 2 min |

## 5.3 Chromatin Immunoprecipitation (ChIP) in 3T3 MEFs

For this experiment Stat1 3T3 MEFs, stably reconstituted with wild-type Stat1, were used. Prior to the assay, cells were tested for Stat1 expression using immunofluoresecnce microscopy. The day before stimulation, cells were seeded on 15 cm tissue culture dishes containing 20 ml of full DMEM medium. The day of the experiment cells displayed 70 - 80% confluency. Cells were treated with mouse IFN- γ (10 ng/ml) according to the experimental setup.

### 5.3.1 Immunofluorescence microscopy

Cells were grown on 3,5 cm tissue culture dish containing a glass cover slip. Cells were stimulated with mouse IFN- γ (5 ng/ml) for 30 min and afterwards immediately fixed for 5 min at room temperature by adding 37% formaldehyde to the medium (final concentration 1,85%). Cover slides were then briefly washed twice with 1x TBST. Cell membranes were permeabilized by incubating slides in 1% Triton X-100 in 1x TBST for 1 min at room temperature, followed by washing them twice with 1x TBST for 5 min to remove the remaining Triton X-100 solution. After blocking unspecific binding with 3% BSA in 1x TBST for 30 min at room temperature, cells were incubated with primary

antibody solution (antibody diluted in 1% BSA in 1x TBST + 0,05% $NaN_3$) for 1 hour. Unbound primary antibody was removed by washing slides three times for 5 min with 1x TBST. Fluorescent labelled secondary antibody solution (antibody diluted in 1% BSA in 1x TBST) was put on slides for 60 min in the dark at room temperature. Subsequently the slides were washed three times for 10 min with 1x TBST to remove unbound secondary antibody. Cover slips were mounted on glass slides using Dako Fluorescence Mounting Medium and analyzed using a fluorescence microscope.

| Specificity | Species | Dilution | Company |
|---|---|---|---|
| α-Stat1 N-terminus | Mouse | 1:1000 | BD Transduction Laboratories |
| α-mouse Alexa 488 | Goat | 1:20000 | Invitrogen |

### 5.3.2 Chromatin Immunoprecipitation (ChIP) assay

#### 5.3.2.1 ChIP assay – Day 1

Cells were treated according to the experimental setup followed by crosslinking proteins and DNA by adding 540 µl of 37% formaldehyde (final concentration 1%) to the 15 cm tissue culture dishes containing 20 ml of full DMEM medium. After 10 min incubation at room temperature on a shaker, the crosslinking reaction was stopped by the addition of 1 ml of 2,5 M Glycine (final concentration 125 mM) for 5 min at room temperature on a shaker. Dishes were then put on ice and cells were washed three times with ice cold 1x PBS. To harvest the cells, 5 ml cold 1x PBS containing Protease Inhibitor cocktail (Roche) and 1 mM phenylmethanesulfonylfluoride (PMSF) was added and cells were scraped into a 15 ml falcon tube using a rubber policeman. Cells were centrifuged at 1500 rpm for 5 min at 4°C, the liquid was removed and the pellet washed with 5 ml of WASH I Buffer containing Protease Inhibitor cocktail (Roche) and 1 mM PMSF. Falcon tubes were then kept on ice for 15 min before being centrifuged at 1500 rpm for 5 min at 4°C. The buffer was removed and the pellet was washed with 5 ml of WASH II Buffer (Protease Inhibitor cocktail (Roche) and 1 mM PMSF). Cells were

incubated on ice for 15 min and afterwards centrifuged for 5 min at 1500 rpm at 4°C. After properly removing the liquid, the pellets were resuspended in 1 ml of SDS Lysis buffer (Protease Inhibitor cocktail (Roche) and 1 mM PMSF) and the extracts were transferred into round-bottomed 2 ml Eppendorf tubes. The tubes were then shaken for 1 hr on a turning wheel at 4°C and thereafter kept at 4°C over night.

#### 5.3.2.2 ChIP assay – Day 2

Extracts were taken from 4°C and put at room temperature until SDS precipitates dissolved completely. Samples were sonicated seven times for 15 sec using an intensity of 45% and a duty cycle of 90%. Afterwards the lysates were centrifuged twice for 15 min at 13200 rpm at 8°C and each time the supernatant was transferred into a fresh tube. 30 µl of aliquots for sonication control were taken and stored at 4°C. The amount of DNA inside the extracts was measured using a NanoDrop ND1000 spectrophotometer, blanked on SDS Lysis Buffer. 25 µg of chromatin were taken as input and sonication control and stored at 4°C. For every specific antibody and for the IgG control, 50 µg of chromatin was diluted 1:10 in Dilution Buffer (Protease Inhibitor cocktail (Roche) and 1 mM PMSF) in a volume of 300 – 2000 µl and pre-cleared with 20 µl of blocked Sepharose A Beads for 1 hr on a turning wheel at 4°C. The tubes were then centrifuged for 5 min at 2800 rpm and the supernatants were transferred into fresh tubes. Specific antibodies as well as IgG control antibodies were added and the lysates were incubated over night on a turning wheel at 4°C.

#### 5.3.2.3 ChIP assay – Day 3

Tubes were taken from the turning wheel and immune complexes were captured by adding 45 µl of blocked Sepharose A Beads to the extracts. Lysates containing beads were then incubated for 3 hrs on a turning wheel at 4°C, before being centrifuged for 5 min at 2800 rpm. The supernatant was discarded and beads were washed with 1 ml of the following buffers: RIPA Buffer, High Salt Buffer, LiCl Buffer and two times TE Buffer. Each time the supernatant was discarded, 1 ml of the given buffer was added. The tubes were then centrifuged for 5 min at 2800 rpm at 4°C, after shaking for 10 min on

a turning wheel at 4°C. Immune complexes were subsequently eluted by adding 200 µl elution Buffer to the beads and keeping the tubes for 15 min at room temperature in a thermo block at 1000 rpm. Tubes were then centrifuged for 5 min at 2800 rpm at room temperature and the supernatants were transferred into fresh tubes. The elution was repeated twice to rescue all immune complexes bound to the beads, ending up with 400 µl of eluted complexes for each antibody. To the aliquots taken for input (25 µg of chromatin) and sonication control (25 µg of chromatin) on the previous day, elution buffer was added to a final volume of 400 µl. Reverse crosslinking was achieved by adding 20 µl of 4M NaCl to all samples and incubating the tubes at 65°C over night.

#### 5.3.2.4 ChIP assay – Day 4&5

Samples were taken from 65°C and centrifuged shortly. To digest proteins, 8 µl of 0,5 M EDTA, 16 µl of 1 M Tris pH 6,5 and 2 µl of Proteinase K (20 mg/ml) were added to each de-crosslinked eluate. Samples were then incubated in a thermo block for 1 hr at 55°C, shaking at 600 rpm. After Proteinase K digestion, tubes were shortly centrifuged and eluates were transferred into 1,5 ml Phase Lock Gel Heavy tubes (5 PRIME). Subsequently, 450 µl of phenol chloroform isoamyl alcohol was added and the two layers were mixed by inverting the tubes several times. Samples were then centrifuged for 2 min at 1200 rpm at 16°C and the upper aqueous layer was carefully transferred into a fresh tube. To precipitate DNA, 1 ml of 96% Ethanol, 40 µl of Sodium acetate and 1,5 µl of Glycogen were added. The tubes were shortly vortexed and incubated at -20°C over night.

The next day, samples were centrifuged for 45 min at 13200 rpm at 4°C and the pellets were washed once with 1 ml of 70% Ethanol. Ethanol was discarded and the pellets were air-dried for 15 min at room temperature. DNA was resuspended in 200 µl of TE buffer and incubated for 10 min at 55°C before being stored at -20°C. The following day, PCR and qRT-PCR analysis were performed.

### 5.3.2.5 Bead preparation for ChIP analysis

Protein A Sepharose beads (Protein A Sepharose™ CL-4B, GE-Healthcare) were washed twice with 20 ml of sterile $dH_20$ in a 50 ml Falcon tube by shaking gently. The Falcon tube was put on ice until beads sank down due to their own weight. After discarding the $dH_20$, beads were resuspended in 10 ml of sterile TE Buffer by shaking gently. The beads were kept on ice until they sank down on their own and subsequently the supernatant was discarded. Beads were then resuspended in an equal amount of fresh, sterile TE buffer. The beads, properly dissolved in TE Buffer, were then aliquoted into 1,5 ml Eppendorf tubes (1 ml of dissolved beads consisted of 0,5 ml of beads and 0,5 ml of TE Buffer). The tubes were then centrifuged for 1 min at 3000 rpm at 4°C, the supernatants were discarded and 500 µl of Bead Blocking Solution was added. The beads were resuspended by flipping the tube gently and left on a turning wheel over night at 4°C. Blocked beads can be stored up to 5 month at 4°C.

### 5.3.2.7 ChIP antibodies

| Specificity | amount/50 ug chromatin | Company |
|---|---|---|
| α-Stat1 C-terminus | 4 µl | BD Transduction Laboratories |
| α-phosphoS727-Stat1 | 4 µl | Kovarik et al., 1998 |
| IgG control – TTP preimmune serum | 1 µl | Kovarik et al., |

### 5.3.2.6 ChIP buffers and solutions

If not stated otherwise, sterile filtering is required.

| WASH I Buffer | |
|---|---|
| 10% Triton X-100 | 10 ml |
| 0,5 M EDTA | 8 ml |
| 0,5 M EGTA | 0,4 ml |
| 1 M HEPTES | 4 ml |
| $ddH_2O$ | up to 400 ml |

| WASH II Buffer | |
|---|---|
| 4 M NaCl | 20 ml |
| 0,5 M EDTA | 0,8 ml |
| 0,5 M EGTA | 0,4 ml |
| 1 M HEPTES | 4 ml |
| $ddH_2O$ | up to 400 ml |

| Dilution Buffer | |
|---|---|
| 4 M NaCl | 16,704 ml |
| 1 M Tris pH 8,0 | 6,688 ml |
| 0,5 M EDTA | 0,96 ml |
| 10% Triton X-100 | 44 ml |
| 10% SDS | 0,4 ml |
| $ddH_2O$ | up to 400 ml |

| TE Buffer | |
|---|---|
| 1 M Tris pH 8,0 | 4 ml |
| 0,5 M EDTA | 0,8 ml |
| $ddH_2O$ | up to 400 ml |

| RIPA Buffer | |
|---|---|
| 4 M NaCl | 15 ml |
| 1 M Tris pH 8,0 | 20 ml |
| 10% NaDOC (fresh) | 20 ml |
| 10% NP40 | 40 ml |
| 10% SDS | 4 ml |
| $ddH_2O$ | up to 400 ml |

| High Salt Buffer | |
|---|---|
| 4 M NaCl | 40 ml |
| 1 M Tris pH 8,0 | 20 ml |
| 10% NP40 | 40 ml |
| 10% SDS | 4 ml |
| $ddH_2O$ | up to 400 ml |

| LiCl Wash Buffer | |
|---|---|
| 1 M LiCl | 100 ml |
| 1 M Tris pH 8,0 | 20 ml |
| 10% NP40 | 40 ml |
| 10% NaDOC (fresh) | 20 ml |
| $ddH_2O$ | up to 400 ml |

| Lysis Buffer | |
|---|---|
| 10% SDS | 1 ml |
| 1 M Tris pH 8,0 | 0,5 ml |
| 0,5 M EDTA | 0,2 ml |
| $ddH_2O$ | up to 10 ml |

| Elution Buffer | |
|---|---|
| 10% SDS | 2 ml |
| 1M $NaHCO_3$ | 1 ml |
| 1 M DTT | 0,1 ml |
| $ddH_2O$ | up to 10 ml |

| Bead Blocking Solution | |
|---|---|
| Salmon Sperm DNA | 10 µl |
| 10% BSA (Fraction V, MP Biomedicals) in $dH_2O$ | 50 µl |
| Sodium Azid | 25 µl |
| TE Buffer | 415 µl |

### 5.3.3 PCR conditions and Primers

#### 5.3.3.1 Gbp2 and Tap1/Lmp2 promoter

Input samples were diluted 1:10 prior to PCR amplification. PCR products were separated using a 2% agarose gel.

| Primer | Sequence |
|---|---|
| mGbp2_distal_ChIP-fwd | 5'- TGA TTT CCC AGC ATT TGA CA - 3' |
| mGbp2_distal_ChIP -rev | 5'- AGG GTG AAA AGG GTG TGG TT - 3' |
| mTap1/Lmp2_ChIP -fwd | 5'- GCT GCT AGG CAG AAC TCC AA - 3' |
| mTap2/Lmp2_ChIP -rev | 5'- GAG CTG GTG GAG CTG ACT AGA - 3' |

| Repeats | Temperature | Time |
|---|---|---|
| 1x | 94,0°C | 2 min |
| 30 cycles | 94,0°C | 30 sec |
| | 60,0°C | 30sec |
| | 72,0°C | 30 sec |
| 1x | 72,0°C | 5 min |
| 1x | 20,0°C | ∞ |

| Master mix | |
|---|---|
| Template DNA | 5 µl |
| Redmix | 12,5 µl |
| 100 µM rev primer | 0,6 µl |
| 100 µM fwd primer | 0,6 µl |
| dd$H_2O$ | 6,3 µl |
| total 25 µl | |

#### 5.3.3.2 DNA agarose gel electrophoresis

2 g of agarose were diluted in 100 ml 1x TAE Buffer and properly dissolved using a microwave. The liquid gel was cooled down to approximately 50°C, 7 µl of ethidium bromide (5 mg/ml) was added and the gel was poured into a gel chamber and left to solidify.

| **50x TAE Buffer, pH 8,0** | |
|---|---|
| Tris | 242 g |
| $CH_3COOH$ | 57,1 ml |
| 0,5M EDTA | 100 ml |
| $ddH_2O$ | up to 1 l |

#### 5.3.3.3 IRF1 promoter

Input samples were diluted 1:20 prior to qRT-PCR amplification. qRT-PCR was performed using KAPA SYBR FAST qPCR Universal mastermix (PEQLAB) according to the manufacturer's manual

| **Repeats** | **Temperature** | **Time** |
|---|---|---|
| 1x | 95,0°C | 5 min |
| 40 cycles | 95,0°C | 15 sec |
| | 61,0°C | 20 sec |
| | 72,0°C | 20 sec |
| 1x | 95,0°C | 15 sec |
| 1x | 20,0 – 95,0°C | 20 min |
| 1x | 95,0°C | 15 sec |

| Primer | Sequence |
|---|---|
| mIRF1_ChIP-fwd | 5'- CCC ACT CGG CCT CAT CAT T- 3' |
| mIRF1_ChIP –rev | 5'- GGA GCA CAG CTG CCT TGT ACT T - 3' |

## 5.3 Chemical inhibition of nuclear CDKs in Stat1 and Stat3 signaling

Immortalized Stat1 deficient 3T3 MEFs, stably reconstituted with wild-type Stat1, as well as HepG2 cells were seeded on 6 cm tissue culture dishes the day before the experiment. Cells were 70% confluent when treated with cytokines and inhibitors. Immortalized 3T3 MEFs were pretreated with the inhibitor Flavopiridol (500 nM) for 30 min. After stimulation with mouse IFN-γ (5 ng/ml) or Anisomycin (100 ng/ml) according to the experimental setup for 40 min, whole cell extracts were prepared. Human HepG2 cells were pretreated with Flavopiridol (500 nm) and/or the MEK 1/2 inhibitor UO126 (10 µM) for 30 min. Cells were subsequently stimulated with human IL-6 (10 ng/ml) and human IFN-γ (10 ng/ml) for 40 min according to the experimental setup.

### 5.3.1 Western Blot analysis

#### 5.3.1.1 Preparation of whole cell extracts

After cells were treated with inhibitors and cytokines for the appropriate time spans, 6 cm tissue culture dishes were transferred on ice to immediately stop cellular functions. Cells were washed twice with ice cold 1x PBS and the remaining liquid was properly removed using a pump. 100 µl of lysis buffer (4°C) was added and cells were scraped afterwards using a rubber policeman. Cell suspensions were then transferred into 1,5 ml Eppendorf tubes. Whole cell extracts were cleared by centrifugation at 10 000 rpm for 5 min. To the remaining supernatant 20 µl of 6x SDS sample buffer was added. Protein extracts were then denatured for 5 min at 95°C and stored at -20°C.

#### 5.3.1.2 SDS polyacrylamide gel electrophoresis (SDS – PAGE)

The gel pouring apparatus was assembled using 0,75 mm spacers. A 10% separation gel was prepared, the still liquid solution was poured immediately into the apparatus and covered with 1 ml of isopropanol. After 10 min of polymerization, the isopropanol was removed. The stacking gel solution was then poured into the upper edge of the apparatus and a comb was inserted. The stacking gel was left to polymerize for 10 minutes and subsequently the gel was assembled into the gel electrophoresis equipment. The comb was removed and the apparatus filled with 1x Running Buffer. Slots were filled with 15 µl of protein extract. One unused slot was filled with 7 µl of prestained protein marker, while every other empty lane was filled with 15 µl of SDS Sample Buffer. The gel was run at 20 mA/gel until the blue dye front completely ran off.

#### 5.3.1.3 Semi-dry Blotting

The SDS polyacrylamide gel was taken out of the gel electrophoresis equipment, rinsed with $ddH_2O$ and put into a dish filled with Cathode Buffer. A wet nitrocellulose membrane (Schleicher & Schuell) soaked in Anode II Buffer was put on top of the gel. Three filter papers soaked in Anode II Buffer followed by 5 filter papers immersed with Anode I Buffer were stacked on top. After turning the whole assembly upside down, additional 6 filter papers soaked in Cathode Buffer were put directly on the gel. Air bubbles were removed and the blot was run for 90 min at 25 V and 60 mA/gel.

| TOP | |
|---|---|
| - | 6x filter paper in Cathode buffer |
| ↓ | Gel in water |
| | Membrane in Anode II buffer |
| | 3x filter paper in Anode II buffer |
| + | 5x filter paper in Anode I buffer |
| BOTTOM | |

#### 5.3.1.4 Ponceau S staining

After blotting, the membrane was rinsed once with $ddH_2O$ and subsequently incubated for 1 min in 2 ml of Ponceau S solution. The spare dye was washed away with $ddH_2O$, after which the protein bands were clearly visible. The dye was removed completely from the membrane using 1x TBST.

#### 5.3.1.5 Immunostaining, stripping, signal detection

Membranes were blocked with 20% low fat milk powder in 1x TBST or 3% BSA in 1x TBST for at least 60 min at room temperature on a shaker. Spare milk powder solution was washed away three times for 15 min using 15 ml of 1x TBST at room temperature on a shaker. The membrane was then incubated over night at 4°C on a shaker with a primary antibody solution, consisting of antibodies diluted in 1x TBST containing 1% BSA and 0,05% NaN3. The following day, the primary antibody was collected (can be reused if kept at 4°C) and the membrane was washed three times for 15 min with 1x TBST. The freshly prepared secondary antibody solution (secondary antibody diluted in 1x TBST) was put on the membrane and after 30 min of incubation in the dark, the membrane was washed three times for 15 min with 1x TBST. The fluorescence signal was detected and quantified using the Odyssey Infrared Imaging System (LI-COR).

For the reincubation of a membrane with a different primary antibody, the nitrocellulose membrane was washed once with $ddH_20$ and subsequently incubated with Stripping Buffer for 5 min at room temperature on a shaker. The Stripping Buffer was then washed away completely from the membrane with the help of 1x TBST. The membrane was then blocked again so that the immunostaining with a different primary antibody can be repeated.

#### 5.3.1.6 Western Blot antibodies

Primary antibodies

Primary antibodies were diluted in 1x TBST containing 1% BSA and 0,05% $NaN_3$.

| Specificity | Species | Dilution | Company |
|---|---|---|---|
| α-Stat1 N-terminus | Mouse | 1:1000 | BD Transduction Laboratories |
| α-Stat1 C-terminus | Rabbit | 1:1000 | Kovarik et al., 1998 |
| α-phosphoS727-Stat1 | Rabbit | 1:1000 | Kovarik et al., 1998 |
| α-phosphoY705-Stat3 | Rabbit | 1:1000 | Cell signaling |
| α-Stat3 C-terminus | Rabbit | 1:1000 | Cell signaling |
| α-phosphoS727-Stat3 | Mouse | 1:500 | Cell signaling |

Secondary antibodies

Secondary antibodies were diluted in 1x TBST.

| Specificity | ID | Dilution | Company |
|---|---|---|---|
| α-rabbit | IRDye700 | 1:20000 | Invitrogen |
| α-rabbit | IRDye800 | 1:20000 | Rockland |
| α-mouse | Alexa Fluor 680 | 1:20000 | Rockland |
| α-mouse | IRDye800 | 1:20000 | Rockland |

### 5.3.1.7 Buffers and Solutions

| 10x PBS Phosphate buffered Saline, pH 7.3 | |
|---|---|
| KCl | 25 mM |
| NaCl | 1.4 M |
| $Na_2HPO_4$ x 2 $H_20$ | 81 mM |
| $KH_2PO4$ | 15 mM |
| $ddH_2O$ | |
| adjust pH 7.3 | |

| Frackelton Buffer, pH 7.1 | |
|---|---|
| Tris | 10 mM |
| NaCl | 50 mM |
| NaF | 50 mM |
| $Na_4P_2O_7$ | 30 mM |
| Triton X-100 | 1% |
| $ddH_2O$; | |
| adjust pH 7.1; do not autoclave; store 4°C | |

| Lysis Buffer (fresh!) | |
|---|---|
| Frackelton buffer | 1 ml |
| $Na_3VO_4$ | 100 µM |
| DTT | 1 mM |
| Protein-Inhibitor (Complete, Roche) | 1x |

| SDS Sample buffer (10 ml) | |
|---|---|
| 10% SDS | 2 ml |
| Glycerol | 1.25 ml |
| 0,5M Tris-HCl pH 6,8 | 2,5 ml |
| Beta-mercaptoethanol | 1 ml |
| 0,05% bromphenol blue | 0,1 ml |
| dd$H_2O$ | 3,15 ml |

| Ponceau S | |
|---|---|
| Ponceau | 0,1% |
| Trischloraceticacid (TCA) | 3% |
| dd$H_2O$ | |

| Separation Gel (10% gel) | |
|---|---|
| 10% SDS | 40 µl |
| 1,5 M Tris-HCl, pH 8,8 | 1 ml |
| 50% acrylamide solution | 0,8 ml |
| 20% APS | 12 µl |
| Temed | 8 µl |
| dd$H_2O$ | 2,2 ml |

| Stacking Gel (4% gel) | |
|---|---|
| Stock (5% acrylamide; Tris 0,125 M; 0,1% SDS, ddH2O; pH 6.8) | 3 ml |
| Temed | 6 µl |
| 20% APS | 9 µl |

| Cathode Buffer | |
|---|---|
| Methanol | 20% |
| SDS | 0,01% |
| 6-aminocaproic acid | 0,04 M |
| $ddH_2O$ | |

| Anode I Buffer | |
|---|---|
| Methanol | 20% |
| Tris | 0,3 M |
| $ddH_2O$ | |

| Anode II Buffer | |
|---|---|
| Methanol | 20% |
| Tris | 2,5 mM |
| $ddH_2O$ | |

| 1x TBST | |
|---|---|
| Tris | 10 mM |
| NaCl | 150 mM |
| Tween20 | 0,01% |
| $ddH_2O$; adjust pH 8,0 | 2,2 ml |

| Stripping Buffer pH 2,5 | |
|---|---|
| Glycine | 200 mM |
| NaCl | 150 mM |
| Tween20 | 0,01% |
| $ddH_2O$; adjust pH 2,5 | 2,2 ml |

| 10x running Buffer | |
|---|---|
| Tris | 0,25 M |
| SDS | 1% |
| Glycine | 1,92 M |
| $Na_4P_2O_7$ | 30 mM |
| $ddH_2O$ | |

## 5.4 GST-tagged protein pulldown

### 5.4.1 Heat shock transformation of BL21 cells

Competent BL21 cells were thawed on ice. An aliquot of 50 µl was gently mixed with 2 µl of isolated plasmid and incubated for 5 min on ice. After incubation for 2 min at 42°C, cells were kept on ice for 5 min. Bacteria were subsequently plated on LB-ampicillin agar dishes and incubated over night at 37°C.

### 5.4.2 Inoculation of bacterial culture

A single clone was picked form a LB-ampicillin agar dish and inoculated in 25 ml LB-medium, containing 100 mg/ml ampicillin.

### 5.4.3 GST pulldown

200 ml of LB-medium containing 100 mg/ml of ampicillin were inoculated with 2,5 ml over night bacterial culture. Cells were grown at 37°C until an $OD_{600}$ of 0,4 was reached. To induce protein expression, 100 µ of IPTG as well as 100 mg/ml of ampicillin were added. Bacteria were subsequently incubated on a shaker for 3 hrs at 24°C. Cells were harvested with a 5 min - 6000 rpm - 4°C centrifugation step. The pellet was then washed once with cold 1x PBS and centrifuged for 10 min at 6000 rpm. The supernatant was discarded and cells were resuspended in 1.6 ml Lysis Buffer (see chapter 2.3.1.7). The cell suspension was then sonicated six times for 20 sec using 40%

intensity and a duty cycle of 40%. After a 10 min centrifugation step at 12 000 rpm at 4°C, the supernatant was transferred into a fresh Eppendorf tube. To pull down GST-tagged proteins, 65 µl packed Glutathione Sepharose 4B beads (Amersham Bioscience), washed twice with 1x TBST, were added. The samples were then incubated over night on a turning wheel at 4°C. The previous day, beads were washed three times with 1x TBST and twice with kinase buffer. Both buffers contained 1x Protease Inhibitor cocktail (Roche) and 1 mM phenylmethanesulfonylfluoride (PMSF). Proteins were eluted twice with 200 µl GST Elution Buffer for 45 min, shaking at 350 rpm at room temperature. To check the pulldown efficiency, 5 µl eluate were mixed with 10 µl SDS sample buffer and analyzed by SDS – PAGE (see chapter 5.3.1.2). The gel was then stained with Coomassie Blue and visualized using the Odyssey Infrared Imaging System (LI-COR).

### 5.4.4 Buffers and Solutions

| **LB agar (1 L)** | |
|---|---|
| Tryptone | 10 g |
| NaCl | 10 g |
| Agar | 20 g |
| Yeast extract | 5 g |
| $ddH_2O$ | Up to 1l |
| ph 7,0; autoclave | |

| **Kinase buffer** | |
|---|---|
| Tris | 25 mM |
| $MgCl_2$ | 10 mM |
| $ddH_2O$ | |

| LB liquid high salt (1 L) | |
|---|---|
| Tryptone | 10 g |
| NaCl | 10 g |
| Agar | 20 g |
| $ddH_2O$ | Up to 1l |
| ph 7,0; autoclave | |

| GST Elution Buffer | |
|---|---|
| Glutathione | 40 mM |
| Triton X100 | 0,5% |
| $MgCl_2$ | 10 mM |
| DTT | 1 mM |
| Tris | 25 mM |
| $ddH_2O$; adjust pH to 7,5 | |

| Coomassie Staining Solution | |
|---|---|
| Coomassie Blue G-250 | 0,05% |
| methanol | 50% |
| acetic acid | 10% |
| $ddH_2O$ | |

| Coomassie Destain Solution | |
|---|---|
| methanol | 40% |
| acetic acid | 7,5% |
| $ddH_2O$ | |

## 5.5 Stable transfection of Stat1-S727A MEFs with inducible shCDK8 pTRIPZ

### 5.4.1 Transfection of Stat1-S727A cells

Immortalized Stat1 deficient 3T3 MEFs, stably reconstituted with S727A Stat1, were seeded on three 10 cm tissue culture dishes and six 3,5 cm tissue culture dishes, containing a glass cover slip for immuno fluorescence microscopy, the day before the transfection. For each of the three used plasmids, one 10 cm dish and two 3,5 dishes were transfected. Cells reached around 70% confluence the day of the experiment. The transfection was achieved by adding 350 µl and 116,6 µl freshly prepared transfection mix to the 10 cm tissue culture dish (containing 6 ml full DMEM) and the 3,5 cm tissue culture dishes (containing 2 ml of full DMEM), respectively. After 8 h of transfection the medium was changed to normal full DMEM and the cells were left to regenerate over night. To test the transfection efficiency for each plasmid, construct transcription was activated by the addition of doxycycline (1 µg/m) to one of the two 3,5 cm dishes over night. The next day, 3,5 cm tissue culture dishes were fixed with 1,8% formaldehyde and the expression of the shRNAmir marker, the Red Fluorescence Protein, was visualized using immunofluorescence microscopy.

| **Transfection mix 10 cm dish** | |
|---|---|
| Plain DMEM | 300 µl |
| Turbofect | 40 µl |
| Plasmid | 15 µg |
| Incubate 20 min at RT | |

| **Transfection mix 3,5 cm dish** | |
|---|---|
| Plain DMEM | 100 µl |
| Turbofect | 13 µl |
| Plasmid | 5 µg |
| Incubate 20 min at RT | |

### 5.5.2 Selection of positive clones with puromcyin

The day after transfection, 3 µg/ml puromcyin was added to each 10 cm dish. For two weeks, the medium (containing 3 µg/ml puromcyin) was changed every two days to remove dead cells. The selection process was considered finished after cells reached 80% confluency.

### 5.5.3 Testing of bulk culture for construct expression

The percentage of cells that had stably integrated the expression construct was assessed by fluorescence microscopy. For each construct, cells were seeded on two 3,5 cm tissue culture dishes containing a cover slip. For each plasmid, one of the two dishes was treated with doxycycline (1 µg/m) over night. The next day, cells were fixed with 1.8% formaldehyde and the percentage of positive cells was estimated using a fluorescence microscope.

### 5.5.4 Limiting dilution cloning and clone testing

To obtain cell clones derived from one single cell, bulk culture dishes were trypsinized and the cells were then diluted with condition medium to cell suspensions containing 10 cells/ml, 20 cells/ml, 30 cells/ml and 60 cells/ml. Each cell suspension was then seeded in 100 µl/well aliquots into separate 96 well plates. As soon as one well on a 96 well plate or on a 24 well plate was fully confluent, cells were transferred to 24 well plates or 3,5 cm dishes, respectively. Full 3,5 cm dishes were tested for stable construct integration using fluorescence microscopy (see chapter 5.5.3). Positive clones (more than 80% of the cells expressing the construct) were expanded on 10 cm tissue culture dishes and frozen as soon as possible.

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Conditioned medium

Immortalized Stat1 deficient 3T3 MEFs, stably reconstituted with S727A Stat1, were seeded on 10 cm tissue culture dishes. The next day, the medium was removed, sterile filtered and diluted 1:3 with fresh full DMEM. The so produced condition medium can be stored up to 2 weeks at 4°

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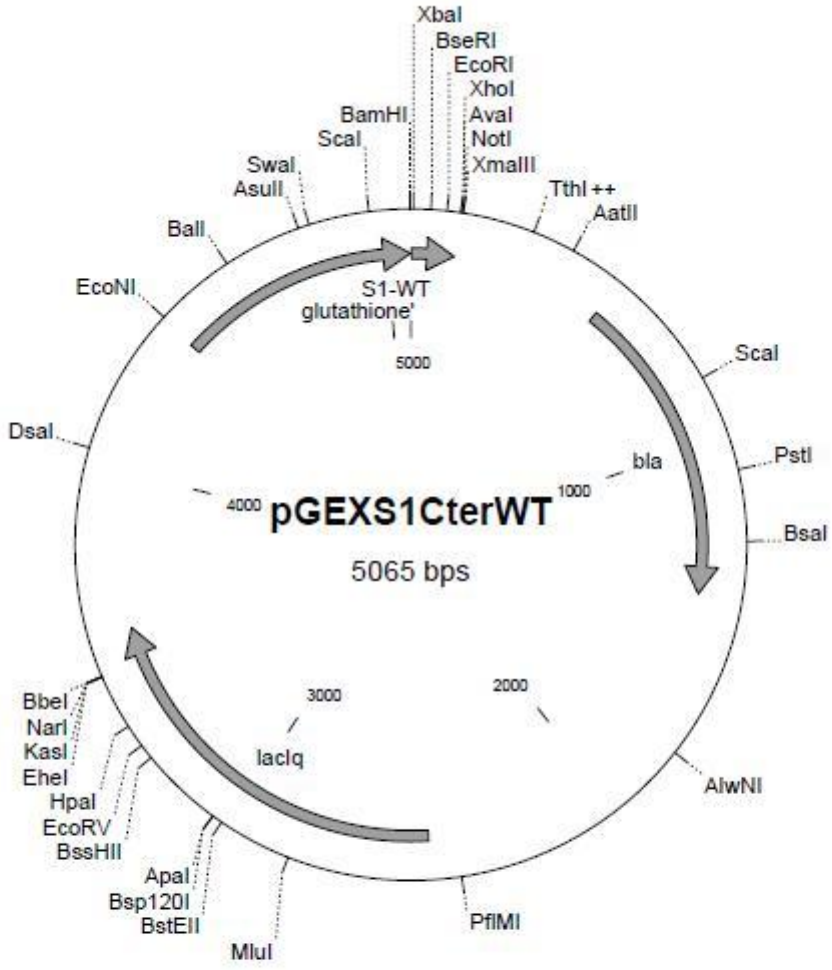
# 7 Appendix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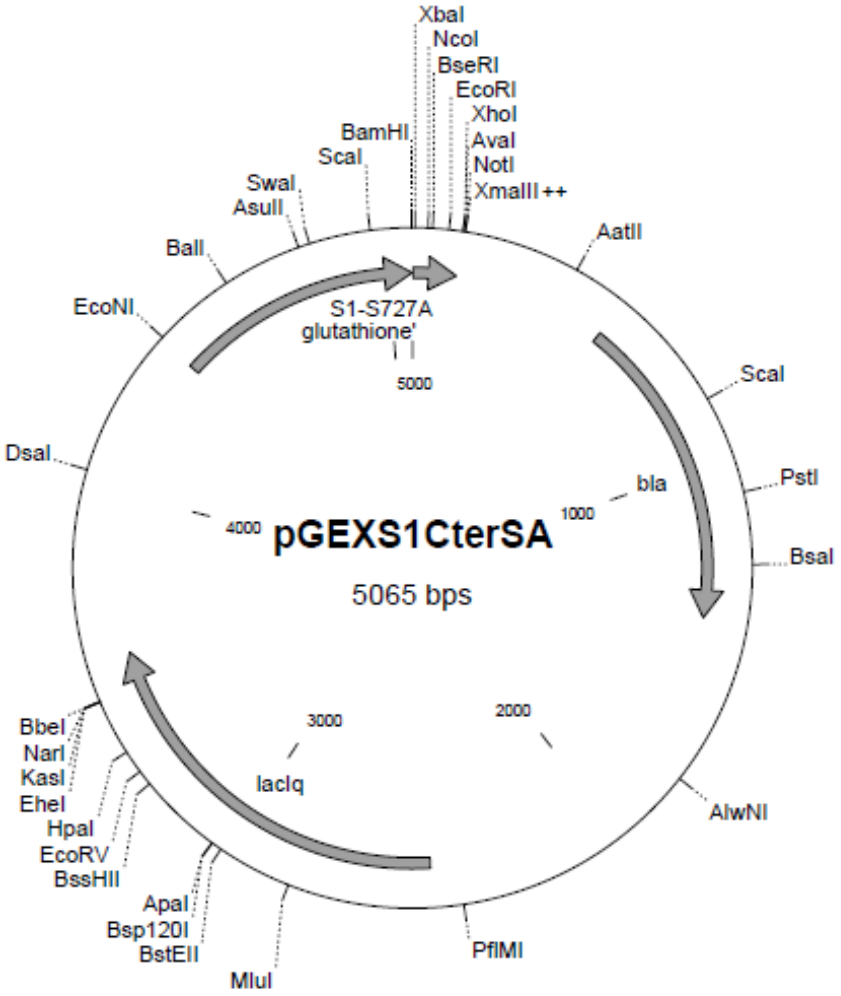
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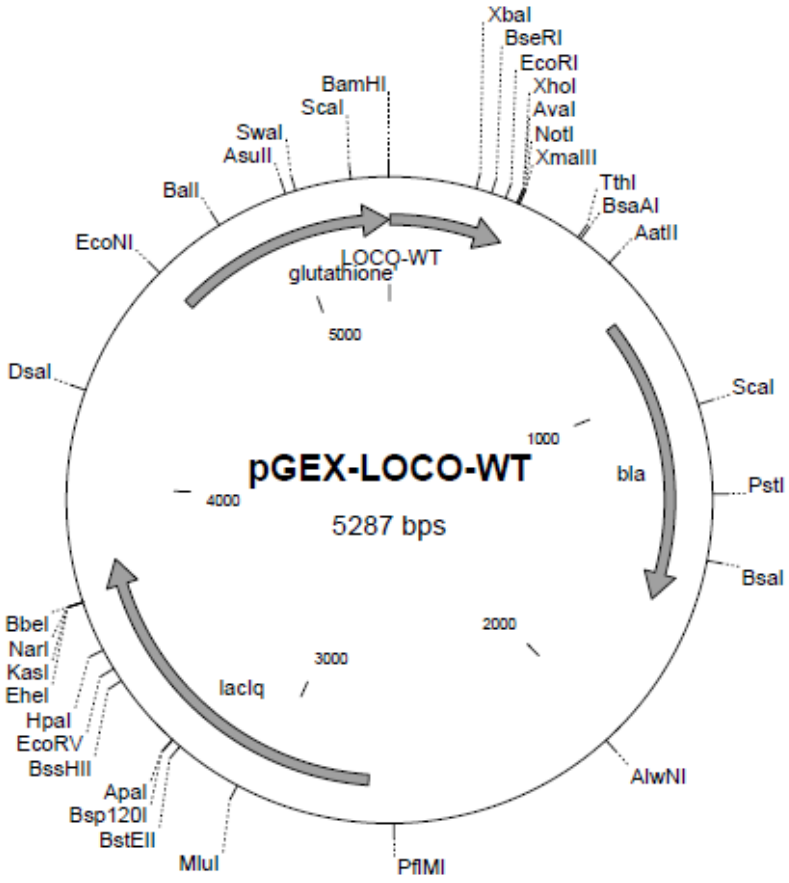
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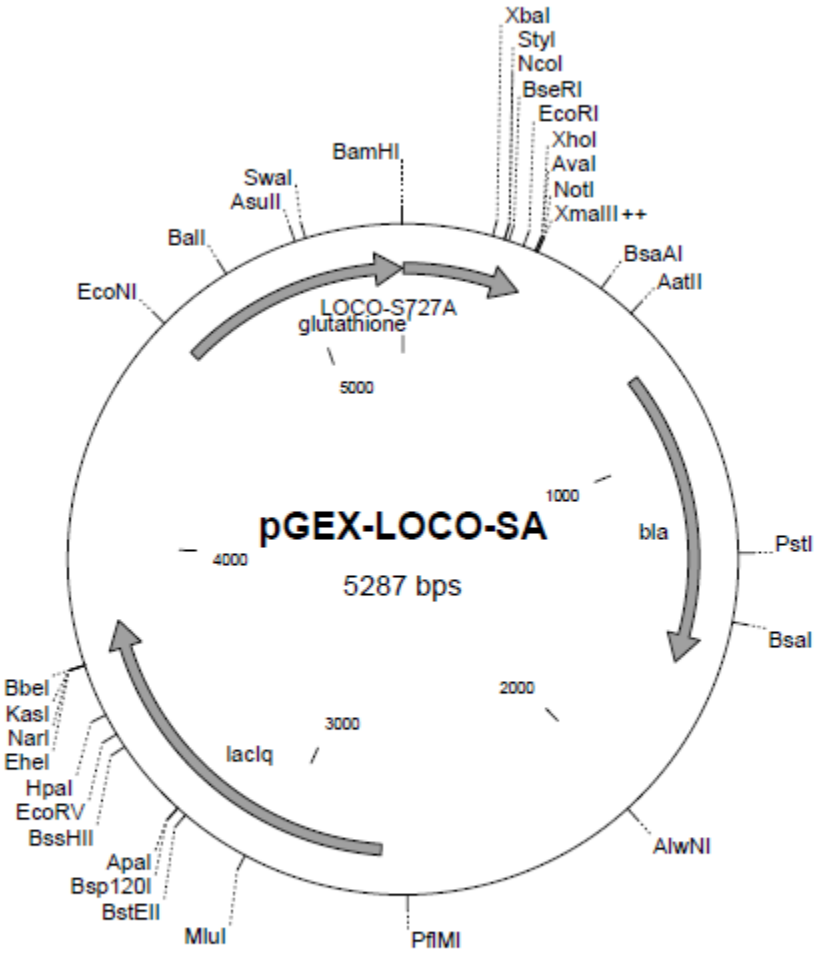
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# 8 Curriculum vitae

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## Education

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| 2006 – 2008 | study of biology at the University of Vienna |
| 2001 – 2005 | Private academic high school St. Ursula, Vienna |

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