STAT3 inhibitors for cancer treatment

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To my family

One never notices what has been done;
one can only see what remains to be done.

Marie Curie

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TABLE OF CONTENTS

ABBREVIATIONS:

Å	angstrom
A	alanine
Ala	alanine
BSA	bovine serum albumine
bp	base pairs
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DN	dominant negative
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
F	phenilalanine
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluoresceinisothiocyanate
GF	growth factor
Gln	glutamine
GTP	guanosine triphosphate
HRP	horseradish peroxidase
IC_{50}	concentration required for 50% inhibition
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
K	lysine
kb	kilobase
kDa	kilodalton
L	leucine
LB	Luria-Bertani medium
Leu	leucine
LIF	leukemia inhibitory factor
Lys	lysine
N	asparagine
NRTK	non-receptor tyrosine kinase
MEM	minimum essential medium
MOI	multiplicity of infection
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pfu	plaque forming units
PIAS	Protein Inhibitors of Activated STATs
Pro	proline
pTyr	phosphotyrosine
Q	glutamine
RNAi	ribonucleic acid interference
SDS	sodium dodecyl sulfate
Ser	serine
SH2	Src homology 2
SOCS	suppressors of cytokine signaling
STAT	Signal Transducer and Activator of Transcription
R	arginine
RT	room temperature
T	threonine
Thr	threonine
Tyr	tyrosine
Tet	tetracycline
Val	valine
VLS	virtual ligand screening
v/v	volume/volume
WB	Western blotting
wt	wild type
w/v	weight/volume
Y	tyrosine
Y*	phosphotyrosine

1 SUMMARY

The critical role of the activation of signal transducer and activator of transcription 3 (STAT3) in the growth and survival of human tumor cells was was one of the subjects of this thesis. It was found that the stable incorporation of v-Src into MCF10A cells, a human immortalized breast epithelial cell line, induced constitutive phosphorylation of STAT3 in these cells. MCF10A-v-Src cells displayed growth factor independence for proliferation and survival, and anchorage independence. However, these cells did not form tumors in nude mice, indicating that they were not fully transformed. Furthermore, inhibition of activated STAT3 in 293 cells decreased growth and induced apoptosis in these cells. The same results were obtained in 293 cells stably transfected with tetracycline-inducible dominant negative (DN) STAT3, and in 293 cells transiently expressing STAT3 RNAi.

Since STAT3 promises to be a good target for cancer treatment, it was attempted to develop inhibitors through rational design. Within STAT3, the SH2-domain was chosen as being the best target site. One limitation of this site turned out to be its similarity to family members in this region, and even to other SH2-domain containing proteins, such as Src, all of which present a highly conserved pocket to bind phosphotyrosines. A medium-throughput assay to measure STAT3 dimerization *in vitro* was established to determine the affinity of compounds to the SH2-domain of STAT3. Peptides of different length derived from the phosphotyrosine-containing motif of STAT3 (AAPY*LKTKF) were tested in the assay. Y*L was found to be the minimal sequence to block dimerization, and out of the tested peptides, PY*LKT had the highest affinity. This was consistent with the observations made on the crystal structure of STAT3, which indicated that besides the phosphotyrosine-binding site, position + 1 and + 3 contributed most importantly to binding. The relevance of position + 1 was further confirmed by substitution of Leu^{+1} by Ala in the peptide Y*LKT, which decreased the affinity more than ten times. Finally, one non-peptidic small molecule inhibitor identified by virtual ligand screening (VLS) proved to be a STAT3 dimerization inhibitor *in vitro*. This is the first small molecule inhibitor of STAT3 identified so far, and might serve to study structure activity relationships (SAR) to optimize the structure and find more potent and bioavailable compounds.

2 INTRODUCTION

2.1 The Signal Transducers and Activators of Transcription (STAT) family

Signal Transducers and Activators of Transcription (STATs) are a family of proteins present latently in the cell, which mediate signals downstream of cytokine and growth factor receptors. STATs were first identified in the 1990s as DNA-binding proteins that attached to the promoters of interferon-inducible genes (Shuai *et al.* 1993). Since then, seven different family members have been cloned and characterized (STAT1 to 4, STAT5A and B, and STAT6), all of which are encoded by different genes. These proteins are considered to have a dual function, acting as signal transducers (transmitting signals from the membrane to the nucleus), and as transcription factors (activating the expression of genes once they bind to their promoters).

Structurally, STATs have several common domains (Fig.1):

- a N-terminal domain involved in the oligomerization of dimers
- a 4-helix bundle
- a β-barrel domain and a connector, involved in DNA-binding
- a Src-homology 2 (SH2) domain that mediates interaction with phosphorylated receptors and dimerization
- a phosphotyrosine containing sequence involved in dimerization
- a C-terminal transactivation domain, which can interact with the transcriptional machinery, transcriptional coactivators, and other regulators of gene expression

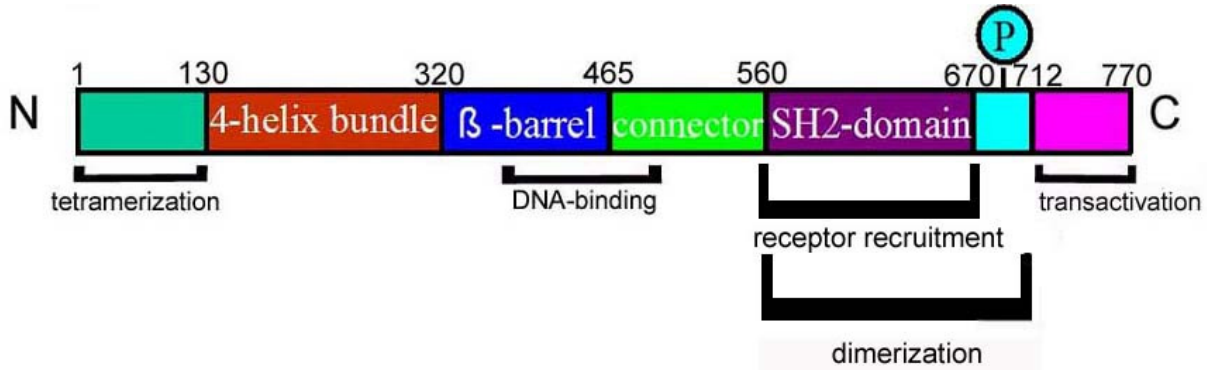


Fig.1. Domain structure of STAT3.

2.2 Activation of STATs

2.2.1 TYROSINE PHOSPHORYLATION

The binding of cytokines to their receptors (Fig.2) induces conformational changes in the receptor, along with their aggregation. This enables the kinases bound to their intracellular domains, the Janus family of kinases (JAK), to phosphorylate each other and become activated. The JAK family has four members: JAK1, JAK2, JAK3, and Tyk2, all of which show significant homology. They constitutively bind to the cytoplasmic tails of a variety of cytokine and hormone receptors, and, when activated, these kinases phosphorylate specific tyrosine sequences in the receptors. The phosphorylated receptors are recognized by the SH2-domains of STATs. This enables their localization to the membrane. In close proximity to the JAK kinases, STATs can then be phosphorylated by these enzymes. In the case of growth factors, such as EGF and PDGF, the intrinsic tyrosine kinase activity of the receptors may directly phosphorylate STATs. Some groups have reported dimerization of STAT proteins prior to phosphorylation. However, these dimers do not translocate to the nucleus, and are also not capable of binding to the DNA and activate gene transcription (Haan *et al.* 2000;Braunstein *et al.* 2003;Schroder *et al.* 2004). This finding could invalidate the current model of STAT activation and support a new model, according to which STATs are already dimerized prior to phosphorylation. Haan *et al.* (1999) observed complex formation of purified STAT3 SH2-domain, and, using fluorescence resonance energy transfer (FRET) in living cells, Kretzschmar *et al.* (2004) found that STAT3 dimers exist in the absence of tyrosine phosphorylation. So, while the direct recruitment of STAT molecules to activated receptors might be the most common, and conceptually certainly the simplest mechanism of activation, the general model of phosphotyrosine-dependent recruitment might not always apply. Phosphorylation of STATs activates them by stabilizing the dimerization of two STAT monomers through reciprocal phosphotyrosine-SH2 interactions. STAT dimers bind to each other through the respective SH2-domains of each monomer. It is thought that dimerization is favored to attachment to the receptor, since the former interaction involves the binding of two different phosphotyrosine and SH2-domains, while the latter takes place through only a single SH2-domain. A nuclear localization sequence

is exposed in STATs upon dimerization. Therefore, upon activation, the monomers detach from the receptors, and the dimers translocate to the nucleus, where they bind to specific DNA-response elements in the promoters of target genes (Seidel *et al.* 1995). Each family member recognizes a slightly different palindromic sequence, and can thereby induce a unique gene expression profile.

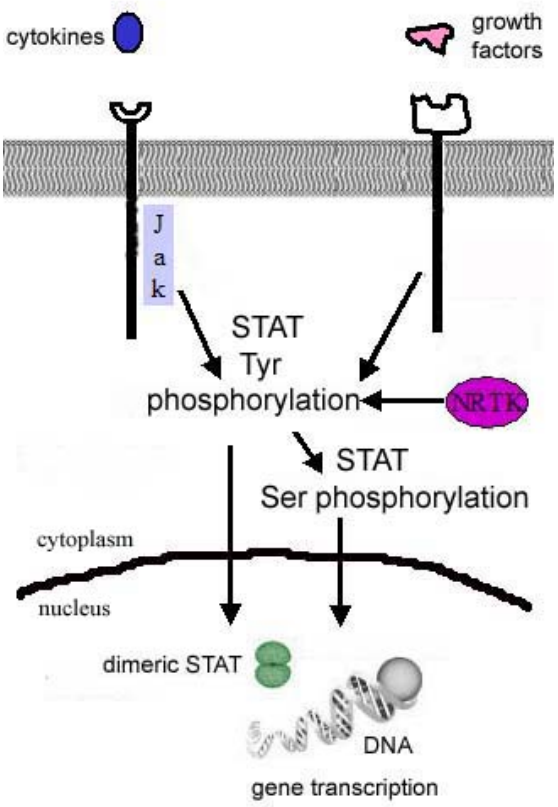


Fig.2. Mechanisms of STAT3 activation. Classical pathway: downstream of cytokine or growth factor signaling, and alternative pathway: involving soluble tyrosine kinases.

2.2.2 SERINE PHOSPHORYLATION

The C-terminal region of STATs contains the transactivation domain, which is required for activation of transcription. Proteins such as the histone acetyl-transferase p300/CREB-binding protein interact with the C-terminal domain of these transcription factors. STAT1 and STAT3, but it has also been reported that STAT4, STAT5A, STAT5B, and STAT6, can be further phosporylated at a Ser at the C-terminus (Ser^{727} in STAT1, Ser^{727} in STAT3, Ser^{721} in STAT4, Ser^{725} and Ser^{779} in STAT5A, Ser^{730} in STAT5B, and Ser^{756} in STAT6). This serine phosphorylation enhances and maximizes their transcriptional activity, but is by itself neither sufficient nor required for their activities. The transcriptional enhancement involves mechanisms that are not fully understood, but may include interactions between STAT coactivator proteins

involved in gene activation. Consequently, both tyrosine and serine phosphorylation are necessary for full activation of STATs, indicating their modulation by different pathways in the cell. STATs could therefore be considered convergence points of different cascades, involving both tyrosine and serine kinases.

However, unlike the better characterized events in STAT activation by tyrosine phosphorylation, the regulation of STATs by serine phosphorylation is less well understood. Mitogen-activated protein kinases (MAPK), including extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and $p38^{MAPK}$ participate in the serine phosphorylation of STAT1 and STAT3 (Chung *et al.* 1997;David *et al.* 1995;Gollob *et al.* 1999;Kuroki and O'Flaherty 1999;Lim and Cao 1999;Ng and Cantrell 1997;Turkson *et al.* 1999). Furthermore, protein kinase C (PKC) plays a role in serine phosphorylation of STATs (Jain *et al.* 1999). Also implicated in the serine phosphorylation are two undefined serine kinases: an H7-sensitive serine kinase, and MAPK kinase (MKK)-dependent/ERK-independent serine kinases (Boulton *et al.* 1995;Ceresa *et al.* 1997;van Puijenbroek *et al.* 1999;Chung *et al.* 1997). Direct phosphorylation of STAT3 and STAT1 *in vitro* by ERKs, p38 and JNK provides evidence that members of the MAPK family can induce STAT serine phosphorylation and regulate their activity physiologically (Chung *et al.* 1997;Turkson *et al.* 1999). Furthermore, the site of serine phosphorylation in both STAT3 and STAT1, Pro-Met-Ser-Pro complies with the MAPK consensus sequence, Pro-X-Ser/(Thr)-Pro (Gonzalez *et al.* 1991;Schaeffer and Weber 1999). Presumably, the relative contribution of each of these serine kinases to STAT signaling in the cell depends on a variety of factors, including cell-type specific expression of the serine kinases, and their interactions with individual STAT members.

2.3 Specificity of the JAK-STAT pathway

The specificity of the JAK-STAT cascade is not driven by the kinases, as is usually the case with other cellular enzymes, but by the recruitment site for STATs in the receptor. The phosphotyrosine and its surrounding sequence in the receptor can only bind proteins with a complementary SH2-domain. For example, the IFNγ (resp IL6, resp erythropoetin) receptor activates mainly STAT1 (resp STAT3, resp STAT5), while both first receptors activate JAK1 and JAK2, and the third only JAK2. However,

the EGF receptor, which also binds JAK1 and JAK2, can activate STAT1, STAT3, and STAT5. Like other phosphotyrosine/SH2-domain interactions that have been characterized in other proteins, e.g. Src kinase, this binding is of high affinity and is highly specific. Hence, the specificity comes from the receptors themselves, and is independent of the kinases involved.

The events described above lead to the activation of STATs very rapidly. Active STATs can be detected in the nucleus only five to fifteen minutes after the addition of cytokines (e.g. IFNα, IFNγ, PDGF, LIF, EGF) in tissue culture (Raz *et al.* 1994). This also demonstrates the direct role of these transcription factors in the cytokine and growth factor responses.

2.4 STATs and gene activation

Once phosphorylated, STATs dimerize and translocate to the nucleus, where they bind to the DNA and modulate gene expression. Each STAT family member activates different genes. For example, STAT3 is known to activate cyclin D1, bcl-xl, $p21^{WAF1/CIP1}$, and c-myc, whereas STAT5 activates Pim1 and oncostatin-M. Each STAT binds to a specific palindromic sequence in the DNA, and activates only the genes that contain this sequence in their promoters (Seidel *et al.* 1995). Studying the binding elements in the promoters of cytokine-responsive genes, it has been found that STATs bind to strongly conserved sequences, with the consensus $TT(N)_{4-6}AA$, where N represents any nucleotide (Seidel *et al.* 1995). Screening of oligonucleotides to find the optimum binding sites for STAT 1, 3, and 4 resulted in the recovery of the consensus oligonucleotide sequence, TTCC(C or G)GGAA (or generically $TT(N)_5AA$) (Horvath *et al.* 1995;Xu *et al.* 1996). Only STAT6, which binds optimally to $TT(N)_6AA$ sequences, prefers different optimal binding sites (Seidel *et al.* 1995;Mikita *et al.* 1996;Schindler *et al.* 1995). However, the natural sites present in the promoters of genes that are regulated in response to particular cytokines show clear preferential binding affinities for a particular STAT or a sopecific set of STATs (Horvath *et al.* 1995;Schindler and Darnell, Jr. 1995;Mikita *et al.* 1996;Xu *et al.* 1996;Schindler *et al.* 1995). Interactions between all the different proteins bound on a specific promoter could influence the DNA-binding stablility of these transcription factors, and this would explain the selective gene activation by each STAT *in vivo*.

2.5 Regulation of the JAK-STAT pathway: the Suppressors Of Cytokine Signaling (SOCS) and the Protein Inhibitors of Activated STATs (PIAS)

The SOCS protein family are negative regulators that bind to the receptors and JAK-family kinases to block STAT activation. Another family of negative regulators are the PIAS, which interact directly with STAT proteins and block their DNA-binding activity. The importance of these physiological negative regulators in cancer is illustrated by the observation that one SOCS family member, SOCS1, is silenced by DNA methylation in multiple myeloma, AML and hepatocellular carcinoma (Galm *et al.* 2003;Coppo *et al.* 2003;Yoshikawa *et al.* 2001). Furthermore, loss of PIAS3 is associated with T-cell lymphoma (Zhang *et al.* 2002a). The inactivation of these two negative regulators might foster the persistent activation of STATs in these tumors. Protein tyrosine phosphatases, such as SH2-containing phosphatases SHP1 and SHP2, are able to negatively regulate STAT signaling by tyrosine dephosphorylation of several components in the pathway. Although all of these STAT regulating proteins are potential candidates for therapeutic development, they are known to regulate other proteins, so that mimics of their function might not be entirely specific to STAT signaling. Additionally, it would be necessary to find activators of these proteins, which is more difficult than finding inhibitors.

2.6 The physiological functions of STATs

STATs regulate cellular processes, including proliferation, differentiation, survival, angiogenesis, haematopoiesis and inflammation (Darnell, Jr. 2002). Each family member has specific functions, which span a wide range of often antagonic activities: from regulation of antiviral host response (Chang *et al.* 2002;Improta and Pine 1997) to the regulation of T-cell development (Kaplan *et al.* 1996;Kaplan and Grusby 1998;Kuo and Leiden 1999;Mohrs *et al.* 2003;Moriggl *et al.* 1999); from stimulation of proliferation and survival (e.g. STAT3 stimulates growth, and is essential for transformation by certain oncogenic tyrosine kinases) to apoptosis in others (e.g.

STAT1 inhibits proliferation and activates apoptosis (Fukada *et al.* 1996,Amin *et al.* 2004;Ning *et al.* 2001;Zhang *et al.* 2002b;Stephanou and Latchman 2003).

The contribution of each STAT to the control of normal cellular processes has been elucidated based on studies of gene knockouts in mice. The family members presenting a lethal phenotype have been studied in tissue-specific conditional knockouts. Homozygous deletion of STAT1 in mice results in deficiencies in their ability to respond to interferons, and these mice become vulnerable to infections from bacterial and viral pathogens (Durbin *et al.* 1996;Meraz *et al.* 1996). Targeted disruption of STAT4 in mice (Kaplan *et al.* 1996;Thierfelder *et al.* 1996) (resp STAT6 (Shimoda *et al.* 1996;Takeda *et al.* 1996)) causes impairments in IL-12 (resp IL-4) induced proliferation of activated T lymphocytes.

Despite their almost identical names, STAT5A and STAT5B have independent genes coded by different chromosomes, and their respective knockouts exhibit different phenotypes: In the case of STAT5A the mice present deficient mammary gland development and lactation during pregnancy (Liu *et al.* 1997), while STAT5B knockouts display sexually dimorphic patterns of liver gene expression (Udy *et al.* 1997). Female STAT5A and B double-knockouts are infertile, and present deficiencies in their immune system (Teglund *et al.* 1998).

In the case of STAT2 (Kimura *et al.* 1996) and STAT3 (Takeda *et al.* 1997), homozygous deletion of each gene is embryonically lethal, indicating an essential role in normal embryonic development. The Cre-Lox recombination system has been used for the development of tissue-specific STAT3 knockouts, in order to circumvent their mortality at day 6.5 to 7 of embryonic development. This has lead to the discovery of the relevance of STAT3 signaling in wound healing (Sano *et al.* 1999), and in IL-6 dependent survival of T lymphocytes (Takeda *et al.* 1998).

2.7 Dominant negative forms of STATs

Naturally occurring splice variants of STATs, such as STAT1β and STAT3β, lack the C-terminal transactivation domain and the serine phosphorylation site. Interestingly, they can often block the function of the full-length proteins (also named α forms) in a dominant negative manner. The physiological role of these isoforms is not completely understood: they are known to be negative regulators of STAT activity in certain

tissues and under specific circumstances, and they might also perform additional and different activities from the α forms. The β forms conserve the DNA-binding capacity, but do not activate genes, which is a logical consequence of their lack of transactivation domain. In biological assays STATβ isoforms are often used as inhibitory tool.

2.8 Constitutively activated mutants of STATs

Artificial mutations of STATs, which render them constitutively active by various mechanisms, demonstrate that persistent activation of STAT3 and STAT5 is sufficient to induce at least some aspects of cell transformation. A constitutively activated STAT3 molecule, STAT3C has been genetically engineered to enable dimerization in the absence of tyrosine phosphorylation. As a result of this enforced dimerization, STAT3C homodimers migrate to the nucleus, bind to STAT3 response elements in promoters and induce gene expression. Moreover, mouse fibroblast expressing STAT3C form colonies in soft agar and induce tumors in nude mice (Bromberg *et al.* 1999). Thus, constitutive activation of STAT3 DNA-binding leads to induction of a STAT3 dependent genetic program that is sufficient to recapitulate some hallmarks of cell transformation in the absence of other signaling pathways that might be activated by TKs. This finding provides further evidence of the causal role of constitutively activated STAT3 signaling in oncogenesis, as opposed to being merely a side effect of TK activity, which also leads to the phosphorylation of many other proteins in the cell. The identification of other STAT3 regulated genes will be important to further define the underlying mechanisms of neoplastic transfomation resulting from constitutive STAT3 signaling.

Expression of constitutively activated STAT5 in an IL-3 dependent mouse pre-B cell line, induces growth factor independent proliferation (Onishi *et al.* 1998). This suggests that STAT5 may also contribute to oncogenesis by promoting cell cycle progression and/or survival.

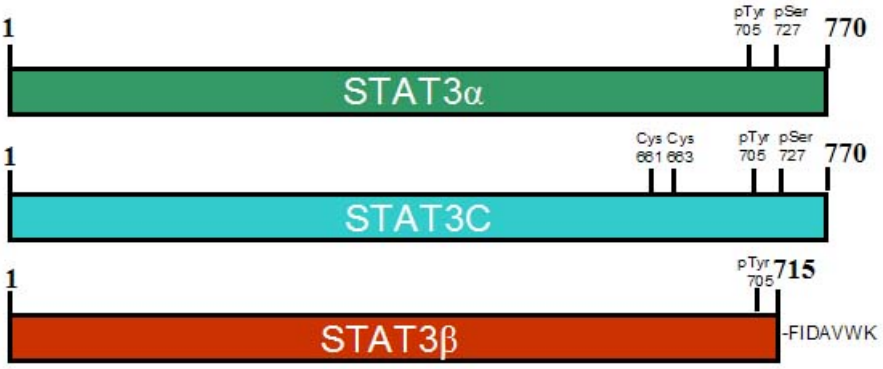


Fig.3. STAT3 variants. STAT3α and β are two naturally occurring isoforms of STAT3, the former being the active variant, and the latter a dominant negative. STAT3C is an artificial mutant with constitutive activity.

2.9 STAT3 in disease

Based on the diverse activities of STATs and on the phenotypes of the deficient mice, it is not surprising that these proteins have been found deregulated in many different kinds of diseases. In particular, STAT3 is constitutively active in inflammatory diseases, such as rheumatoid arthritis (Krause *et al.* 2002;Liu and Pope 2003), Crohn's disease (Atreya *et al.* 2000;Lovato *et al.* 2003), and atherosclerosis (Niculescu and Rus 1999), and also in many cancers. These include solid tumors: head and neck carcinoma (Song and Grandis 2000), breast cancer (Lin *et al.* 2002;Berclaz *et al.* 2001), ovarian cancer (Huang *et al.* 2000), prostate cancer (Barton *et al.* 2004;Campbell *et al.* 2001;Dhir *et al.* 2002;Gao *et al.* 2001;Mora *et al.* 2002), cervical cancer (Page *et al.* 2000), melanoma (Niu *et al.* 2002), and renal carcinoma (Horiguchi *et al.* 2002), as well as leukemias (Lin *et al.* 2000): Acute myeloid leukemia (Schuringa *et al.* 2000;Towatari and Iida 1998;Xia *et al.* 1998), acute promyelocytic leukemia (Dong *et al.* 2003), Hodgkin lymphoma (Kube *et al.* 2001), anaplastic lymphoma kinase-positiveT/Null-cell lymphoma (Zhang *et al.* 2002a), adult T-cell leukemia/lymphoma (Takemoto *et al.* 1997), CML (Coppo *et al.* 2003) and ALL (Spiekermann *et al.* 2001;Schuringa *et al.* 2000;Benekli *et al.* 2002). Persistent STAT3 signaling in tumor cells induces tumor angiogenesis and suppresses anti-tumor immune responses, which seems to further boost tumor progression (Cheng *et al.* 2003;Wang *et al.* 2004). Additionally, it appears that some tumor cells become dependent on constitutive STAT3 activation and are more sensitive to STAT3 inhibitors than normal cells.

2.10 STAT3 as an oncogene

STAT3 in particular can be considered an oncogene, since the constitutively activated mutant, STAT3C, is capable of inducing tumors in nude mice (Bromberg *et al.* 1999). However, since no STAT3 mutation has been detected in human tumors so far, this statement could be challenged. Oncogenes are defined as mutated forms of proto-oncogenes, which are genes whose products are involved in growth stimulation. The mutations can affect the gene's promoter and cause protein overexpression, but they can also affect the coding region and generate a hyperactivated mutant. As yet, none of these have been reported for STAT3. Rather, its activation in malignancies seems to occur downstream of hyperactivated tyrosine kinases and other types of oncogenes. However, STAT3 and STAT5 activate cyclinD1, c-Myc, and bcl-xl, and it is generally accepted in the field that STAT3 promotes cell growth and therefore oncogenic processes. Furthermore, as noted above, a number of different groups have reported constitutive activation of this protein in various tumor types. Hence, even though not being a *bona fid*e oncogene, but because of its direct activation by other oncogenic factors and its role in growth stimulation, STAT3 can still be considered an appropriate target for cancer treatment.

2.11 STAT3 as a target for cancer treatment

Tyrosine kinases are among the most frequently activated oncogenic proteins in cancer cells. Therefore it is not surprising that persistent activation of STAT signaling has been detected in an increasing number of human cancers. STATs often become consistently activated when one or more upstream tyrosine kinases become overactive. Although cancer is characterized by an accumulation of genetic mutations at the DNA level, and oncogenes play an important role in its pathogenesis, the best molecular targets for cancer therapy are not necessarily genetically altered themselves. It has been found that some oncogenic signaling pathways converge at a limited set of nuclear transcription factors, including STAT3. These transcription factors are the final switches that activate gene expression patterns leading to malignancy. It is therefore logical to choose these proteins as anticancer targets,

since targeting a single transcription factor can block the effects of a multitude of upstream genetic aberrations leading to cancer.

A gene-therapy vector that was designed to inhibit STAT3 signaling in a mouse model of murine melanoma has provided proof of concept that human SAT3 is a valid target for cancer therapy (Niu *et al.* 1999). In this study, the expression of a dominant-negative form of STAT3 induced massive apoptosis in mouse melanomas. An unexpected side effect of targeting STAT3 was the accompanying bystander effect: adjacent tumor cells also underwent apoptosis without having undergone gene therapy. These studies were performed in immunocompetent mice using a syngeneic mouse-melanoma cell line. Therefore, this potent bystander effect might involve the immunosuppressive activity of STAT3 (Cheng *et al.* 2003;Wang *et al.* 2004). However, it might also reflect the importance of STAT3 signaling for angiogenesis, or for the release of soluble factors capable of inducing apoptosis. Given the similarities between STAT3 and STAT5, it is worth investigating whether gene therapy of STAT5 dependent tumors triggers a similar bystander effect.

Finally, tissue-specific ablation of STAT3 has shown that non-oncogenic cells lacking STAT3 proliferate and survive well *in vitro* and *in vivo* (Akira 2000). Furthermore, inhibition of STAT3 in mouse fibroblasts does not have deleterious effects in normal cell growth (Niu *et al.* 1999;Turkson *et al.* 1998). The selective response to STAT3 inhibitors in tumor versus normal cells might reflect that tumor cell growth and survival irreversibly depend on high levels of activated STAT3, whereas normal cells might be able to withstand lower levels or use alternative pathways. This is an ideal property of a therapeutic target, since it could imply that STAT3 inhibitors should not affect normal cells.

A specific inhibitor for STAT3 could therefore be very useful for the treatment of cancer. STAT5 is also activated in many tumors (Calo *et al.* 2003) and STAT6 has immunosuppressive activity (Ostrand-Rosenberg *et al.* 2004). Hence, they are also potentially good drug targets for tumor therapy. Additionally, STAT1 is persistently activated in acute leukemias (Spiekermann *et al.* 2001;Calo *et al.* 2003;Gouilleux-Gruart *et al.* 1997). However, STAT1 activation in cancer is probably a side effect of a hyperstimulated cytokine or growth factor signaling pathway that activates different proteins simultaneously. Currently, this STAT family member is considered to have tumor suppressive activity, inhibitig proliferation, and activating apoptosis (Bromberg and Darnell, Jr. 2000). Hence it should not be a target for cancer treatment. Overall,

STAT3 seems to be the most promising candidate of the family, not only due to its activation in many tumors, but also because of its immunosuppressive activity (Cheng *et al.* 2003;Wang *et al.* 2004). Its inhibition could slow down the proliferation of oncogenic cells, as well as stimulate antitumor immunity, which should therefore result in a potent reduction of tumor growth.

2.12 Development of STAT3 inhibitors

STAT molecules could be inhibited through different mechanisms. It is conceivable to target upstream activators of STAT3, in particular the kinases that phosphorylate STATs. This is very useful in the treatment of CML, because in most cases the causative agent of this malignancy is a translocation between chromosomes 14 and 22 that generates the Philadelphia chromosome. This results in the expression of a fusion protein, Bcr-Abl, which is a hyperactivated mutant of Abl. The recent approval of a Bcr-Abl tyrosine kinase inhibitor, STI-571, was a major advance in the treatment of CML. In the case of STAT3, inhibition of its phosphorylation through a blockade at the kinase level has some important limitations, most importantly, the fact that many different kinases can activate STAT3: Hence, it should be expected that only in specific cases inhibition of only one kinase could lead to disappearance of phosphorylated STAT3. Additionally, it would be necessary to develop many different kinase inhibitors to treat all the possible disorders that might present STAT3 activation, since in each case, phosphorylation can be due to other kinases. Finally, kinases do not only phosphorylate one substrate, but they usually many different, and their inhibition could have undesirable consequences due to the blockage of other pathways. Therefore, targeting the kinase or other upstream regulators, such as the cytokine receptors, does not seem to be an optimal way to disrupt STAT3 activation.

Within STAT3, the different functional domains are well characterized, and its structure has been characterized by x-ray crystallography (Becker *et al.* 1998) (Fig.4). This important input allows the rational design of inhibitors. In order to choose the best target site in STAT3, both the structure-function aspects and the physico-chemical properties are relevant. The candidate inhibition sites are the DNA-binding

domain, the dimerization domain, the nuclear localization site, and the transactivation domain.

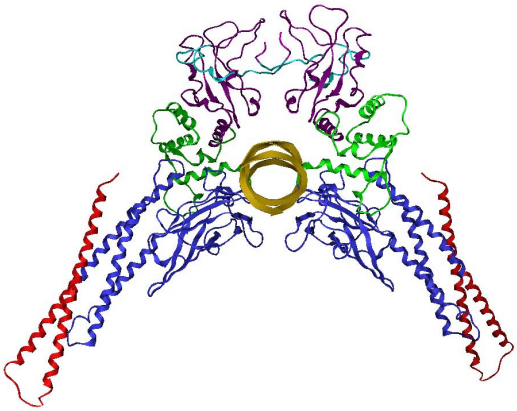


Fig.4. Ribbon diagram of the STAT3 homodimer bound to the DNA: N-terminal-helix bundle (red), β-barrel (blue), connector (green), SH2-domain (violet), phosphotyrosine-containing (turquoise), DNA (gold).

There are many different ways to search for a candidate drug once a specific target site in a molecule is chosen. Typically, drug companies screen more or less randomly created compound libraries for the desired inhibitory activity. Hundreds of thousands of molecules are tested with high-throughput screening procedures in the hope of detecting the desired activity. A soluble and active compound becomes a lead compound when it has repeatedly shown specific inhibition of the target, and is then further modified and tested in order to find more potent candidates. With the recent discovery of the structure of many molecules, due to the high-resolution obtained by x-ray crystallography and nuclear magnetic resonance spectroscopy, another approach to drug screening has been attempted. In this approach, known as rational drug design, the available structural information is used to select compounds to be tested. Additionally, in the case of STATs, since they all have a similarity of 50 to 60%, one can predict with a high degree of confidence the yet unsolved structure of the other family members. Using structure analysis software, one can not only view these proteins in three dimensions, but also select an appropriate target site more easily based upon specific information about the structure and electrostatics of the protein to be targeted. This makes it possible to define a pharmacophore even before active compounds are found, and to select promising compounds to be tested.
In order for a domain to be a good target site for inhibitor development, it has to fulfil certain requirements, namely:

1. Being part of the site involved in the activity of the protein, or having the capacity to influence the conformation of the active site.

2. The domain in particular needs to present a pocket in order to enable attachment of small molecules. Shallow surfaces generally do not allow for strong binding of inhibitors.
3. For intracellular targets, such as STAT3, the site cannot be too polar, so that potential binders have a certain lipophylicity allowing them to pass the cellular membrane.
4. The target site needs to present substantial structural differences from the other proteins in the cell in order to obtain specific inhibitors.

The nuclear localization signal could also be considered for inhibition, however the implicated sequence has not been described for STAT3. There have been reports showing the relevance of an arginine/lysine rich sequence in the nuclear import of STAT1 and 2 by importin alpha (Melen *et al.* 2001;Fagerlund *et al.* 2002;McBride *et al.* 2002), and a nuclear export sequence has also been characterized for STAT1 (McBride *et al.* 2000). However, both are located in the DNA-binding domain, and potential inhibitors would also inhibit DNA-binding. If the nuclear localization signal of STAT3 is also in the same region, studying the DNA-binding domain should also help to find inhibitors of nuclear localization. Another study using N-terminal mutants of several STAT proteins, has implicated the N-terminal domain in nuclear import (Strehlow and Schindler 1998). Hence, with the information published so far, it is not easy to carry out rational design of STAT3 nuclear localization inhibitors.

Another option to inhibit STAT3 activity is to target the DNA-binding domain, which is essential for the activation of gene transcription. Targeting the DNA-binding domain has the benefit that the inhibitors can bind to the dimeric or monomeric form of STAT3, and tumors predominantly have large amounts of the activated dimer. Nonetheless, a strong SH2 inhibitor, which would only bind the monomeric form, and a strong DNA-binding inhibitor, which could target both the monomer and the dimer, should only differ in the kinetic of inhibition: when targeting the dimer, the effects could be expected to take place earlier, while in the case of the monomer, it might be necessary to wait for dephosphorylation of the activated STAT3 molecules for complete inhibition.

The SH2-domain of STAT proteins serves for the recruitment of non-phosphorylated STATs to specific receptors, and is also required for their dimerization. Only phosphorylated and dimerized STATs translocate to the nucleus, bind to the DNA, and activate gene transcription. Blocking the SH2-domain of STATs, these proteins

cannot get phosphorylated, and can also not bind to other STAT monomers. Inhibition of the SH2-domain targets two different and essential events in STAT activation: phosphorylation and dimerization. Therefore, in respect to the first feature required for a good target site, this domain is a promising candidate for the inhibition of STAT3 activity. In 2001 it was found that STAT3 activity could be inhibited using STAT3 phosphopeptides that bound to its SH2-domain (Turkson *et al.* 2001). This group reported XY*L as a minimal peptide to disrupt STAT3 dimers, and further showed that inhibition with a cell permeable PY*LKTK peptide suppressed transformation by the Src oncoprotein (Turkson *et al.* 2001). In 2004, the same laboratory reported peptidomimetics with increased affinity that inhibited cell growth and induced apoptosis, further confirming that SH2-domain inhibitors could be efficient.

3 SPECIFIC AIMS

The aim of the this thesis was two-fold, namely:

1. The study of the role of STAT3 in human tumor development.

As noted in the introduction, oncogenic properties have been ascribed to STAT3, a fact which has been well established in rodent cells. However, it is still not settled whether activated STAT3 can transform human cells. The sufficient and essential role of STAT3 in human tumor development was addressed in the present thesis by way of the following questions:

- Does constitutive phosphorylation of STAT3 lead to transformation of human cells? Do such cells generate tumors *in vivo*?
- Is it possible to establish an inducible knockdown system for STAT3? Does such inhibition of STAT3 lead to growth arrest and apoptosis of human cancer cells?
- Does inducible STAT3 inhibition suffice to cause tumor regression of human xenografts in mice?

2. Identification of drug-like STAT3 inhibitor compounds by rational drug design.

It was mentioned that inhibition of STAT3 has been proposed for the treatment of cancer, and no drug-like STAT3 inhibitor compound has been described so far. The identification of such molecules, by means of rational drug design, using the functional and structural information available, required addressing the following questions:

- What can be a promising target site in STAT3? Is the SH2-domain a good target site in STAT3? Which amino acids are important for the interactions taking place in a STAT3 dimer?
- Is it possible to use the information in STAT3 crystal structure to develop SH2-domain inhibitors through rational design?
- Can the inhibitory compounds be validated by STAT3 specific assays?

4 MATERIALS AND METHODS

4.1 Chemicals, enzymes, and solutions

All commonly used chemicals were purchased from Merck, Sigma Aldrich, Qiagen, Amersham-Biosciences, Bio-Rad or LaRoche Diagnostics. Peptides were synthesized at PolyPeptide Laboratories or at BIOTREND Chemikalien GmbH. Radiochemicals were purchased from Amersham Biosciences.
All solutions were prepared in double deionized water. To prepare sterile solutions, this water was previously autoclaved.

4.2 Antibodies

Penta-His Antibody, BSA-free	Mouse monoclonal antibody recognizing 6xHis tags (Qiagen)
Anti-FLAG M2	Mouse monoclonal antibody recognizing the Flag peptide sequence DYKDDDDK (Sigma Aldrich)
STAT1 p84/p91 (E23)	Rabbit polyclonal antibody recognizing the C-terminal sequence of STAT1 (Santa Cruz)
STAT3 (C-20)	Rabbit polyclonal antibody recognizing the C-terminal sequence of STAT3 (Santa Cruz)
STAT3 (H-190)	Rabbit polyclonal antibody raised against a recombinant protein mapping at amino acids 50 to 240 of STAT3 (Santa Cruz)
STAT3 (F-2)	Mouse monoclonal antibody raised against a recombinant protein mapping at amino acids 50 to 240 of STAT3 (Santa Cruz)

STAT3	Rabbit polyclonal antibody recognizing a sequence of 39 amino acids at the C-terminus of STAT3 (Active Motif)
Phospho-STAT3	Rabbit polyclonal antibody recognizing Tyr^{705} phosphorylated STAT3 (Cell Signaling)
Src	Mouse monoclonal antibody recognizing the amino acids 82 to 169 of $pp60^{Src}$ (Upstate)
HRP-conjugated anti-rabbit	Peroxidase-conjugated AffiniPure F(ab')2 fragment of donkey anti-rabbit IgG (Jackson Immunoresearch)
HRP-conjugated anti-mouse	Peroxidase-conjugated AffiniPure F(ab')2 fragment of donkey anti-mouse IgG (Jackson Immunoresearch)

4.3 Plasmids and baculoviruses

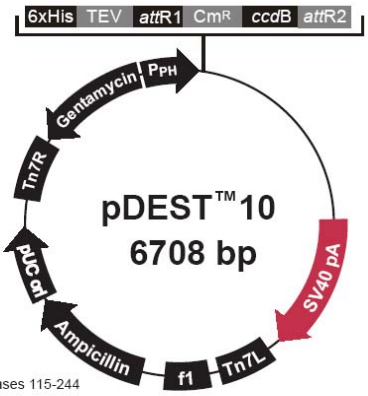
4.3.1 Plasmids

pM/v-src: pp60-v-src eukaryotic expression vector with the Maloney virus promoter and ampicillin resistance (Kmiecik and Shalloway 1987); kind gift of James Turkson

PEF/pGK/puropA eukaryotic expression vector for puromycin-resistance under the control of pGK promoter, and with ampicillin resistance (Huang *et al.* 1997); kind gift of Matthias Grell

PSG5 humanSTAT3ß eukaryotic constitutive expression vector of STAT3ß under the control of SV40-virus promoter, and with ampicillin resistance (Caldenhoven *et al.* 1996); kind gift of James Turkson

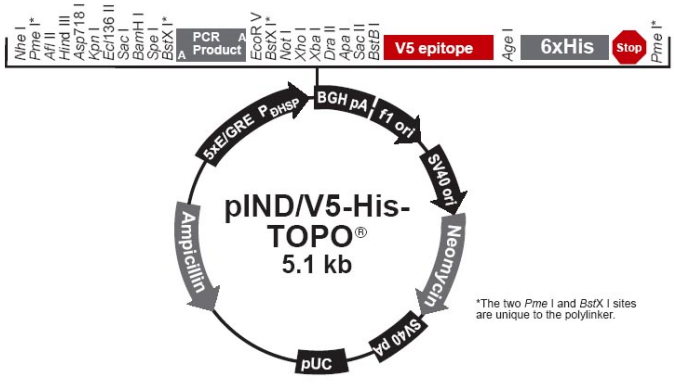
pDEST10 donor plasmid for the generation of recombinant baculoviruses expressing full length STAT3: contains a mini-Tn7 element used for the generation of recombinant bacmids in E. coli DH10Bac, with gentamycin and ampicillin resistance (Invitrogen)



Features of pDEST™10
6708 nucleotides

AcMNPV polyhedrin promoter: bases 115-244
6xHis tag: bases 274-291
TEV cleavage site: bases 313-333
*att*R1 recombination site: bases 337-461
Chloramphenicol resistance gene: bases 711-1370
*ccd*B gene: bases 1712-2017
*att*R2 recombination site: bases 2058-2182
SV40 late polyadenylation signal: bases 2304-2540
Tn7 left arm: bases 2563-2757
F1 intergenic region: bases 2922-3377
bla promoter: bases 3410-3514
Ampicillin resistance ORF (*bla*): bases 3509-4369
pUC origin: bases 4510-5164
Tn7 right arm: bases 5432-5655
Gentamicin resistance ORF: bases 5722-6255 (C)
Prokaryotic promoter: bases 6444-6471 (C)
(C) = Complementary strand

PIND/V5-His-TOPO eukaryotic ponasterone-inducible expression vector, with neomycin and ampicillin resistance (Invitrogen)

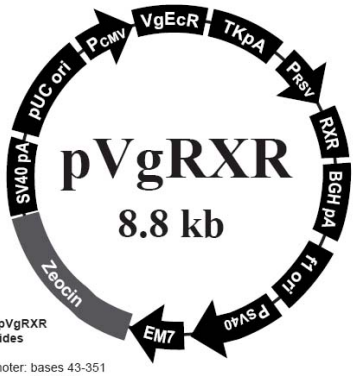


*The two *Pme* I and *BstX* I sites are unique to the polylinker.

Comments for pIND/V5-His-TOPO®
5124 nucleotides

Ecdysone/glucocorticoid Response Elements (5x E/GREs): bases 12-185
Minimal Heat Shock Promoter and 5´ UTR: bases 192-486
Putative Transcriptional Start: base 245
Ecdysone Forward priming site: bases 440-463
Multiple Cloning Site: bases 487-620
TOPO® Cloning site: bases 554-555
V5 epitope: bases 621-662
Polyhistidine tag: bases 672-689
BGH Reverse priming site: bases 712-729
BGH polyadenylation sequence: bases 715-942
f1 origin: bases 988-1416
SV40 promoter and origin: bases 1466-1752
Neomycin resistance gene ORF: bases 1827-2621
SV40 polyadenylation sequence: bases 2797-2927
pUC origin: bases 3310-3983
Ampicillin resistance gene ORF: bases 4128-4988

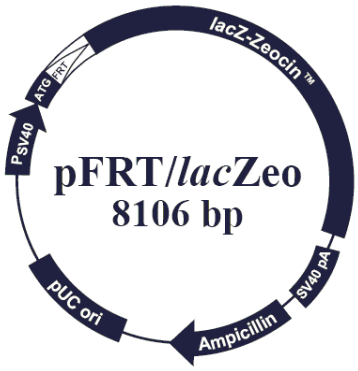
pVgRXR eukaryotic constitutive expression vector of ponasterone receptor, with zeocin resistance (Invitrogen)



Comments for pVgRXR
8803 nucleotides

SV40 early promoter: bases 43-351
EM7 promoter: bases 778-855
Zeocin™ resistance gene (*Sh ble*) ORF: bases 856-1230
SV40 late polyadenylation sequence: base 1294-1424
pUC origin: bases 1533-2206
CMV promoter: bases 2265-2883
VgEcR fusion protein: bases 3035-5275
  modified VP16: bases 3038-3271
  EcR: bases 3302-5275
HSV-1 thymidine kinase polyadenylation sequence: bases 5470-5982
RSV 5′ long terminal repeat: bases 6353-6614
RXR ORF: bases 6637-8025
BGH polyadenylation sequence: bases 8108-8332
f1 origin: bases 8502-8609

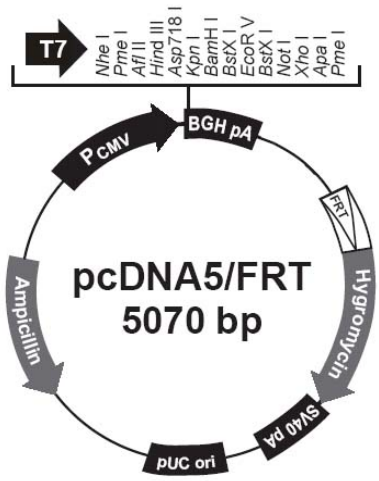
pFRTLacZeo a plasmid containing a Flip-recombination target (FRT) site, and the β-galactosidase reporter gene, with zeocin and ampicillin resistance (Invitrogen). This vector serves the integration of genes, by Flp recombinase-mediated DNA recombination with other plasmids that also have FRT sites (e.g. pcDNA5FRT/FRT/TO). pFRTLacZeo transfected cells can be tested for protein expression with the β-galactosidase reporter. This assures the integration of genes into the genome at a transcriptionally active locus.



Comments for pFRT/*lac*Zeo
8106 nucleotides

SV40 early promoter and origin: bases 278-604
ATG initiation codon: bases 609-611
FRT site: bases 614-661
LacZ-Zeocin™ fusion gene
  LacZ ORF (no ATG): bases 675-3722
  Zeocin™ resistance gene (no ATG): bases 3810-4181
SV40 early polyadenylation signal: bases 5102-5425
bla promoter: bases 6201-6299
Ampicillin (*bla*) resistance gene: bases 6300-7160
pUC origin: bases 7305-7978

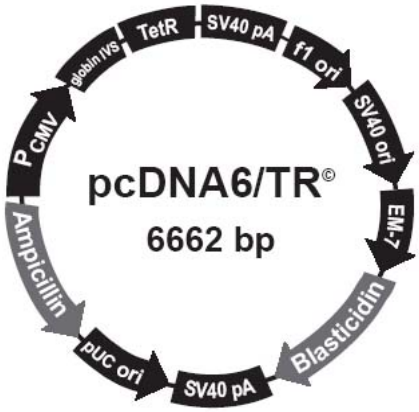
pcDNA5FRT/FRT/TO eukaryotic tetracycline-inducible expression vector, with hygromycin and ampicillin resistance (Invitrogen)



Comments for pcDNA5/FRT
5070 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1028-1252
FRT site: bases 1536-1583
Hygromycin resistance gene (no ATG): bases 1591-2611
SV40 early polyadenylation signal: bases 2743-2873
pUC origin: bases 3256-3929 (complementary strand)
bla promoter: bases 4935-5033 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4074-4934 (complementary strand)

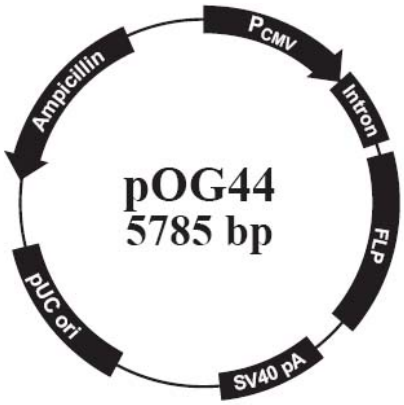
pcDNA6/TR eukaryotic constitutive expression vector of the tetracycline repressor, with blasticidin and ampicillin resistance (Invitrogen)



Comments for pcDNA6/TR©
6662 nucleotides

CMV promoter: bases 232-819
Rabbit β-globin intron II (IVS): bases 1028-1600
TetR gene: bases 1684-2340
SV40 early polyadenylation sequence: bases 2346-2477
f1 origin: bases 2897-3325
SV40 promoter and origin: bases 3335-3675
EM-7 promoter: bases 3715-3781
Blasticidin resistance gene: bases 3782-4180
SV40 early polyadenylation sequence: bases 4338-4468
pUC origin: bases 4851-5521
bla promoter: bases 6521-6625 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5666-6526 (complementary strand)

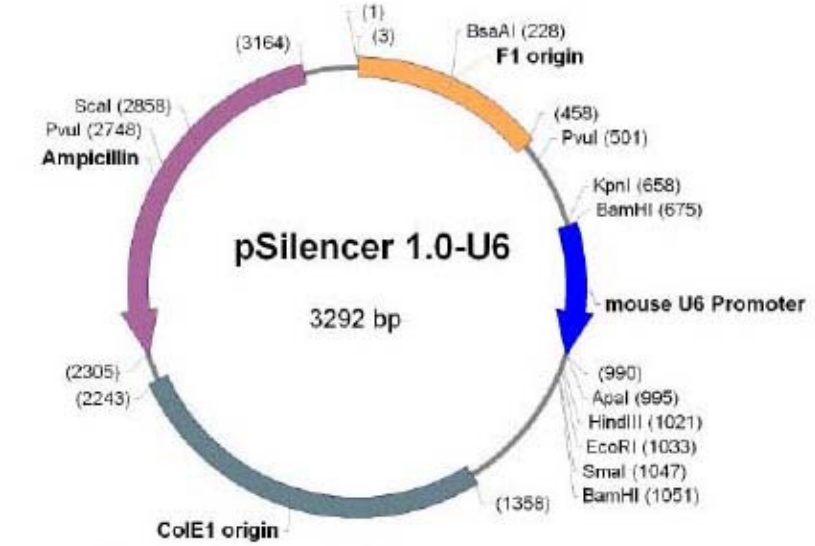
pOG44 eukaryotic constitutive expression vector of FLP recombinase, with ampicillin resistance (Invitrogen)



Comments for pOG44
5785 nucleotides

CMV promoter: bases 234-821
Synthetic intron: bases 871-1175
FLP ORF: bases 1202-2473
SV40 late polyadenylation signal: bases 2597-2732
pUC origin: bases 3327-3993 (complementary strand)
bla promoter: bases 4999-5097 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4138-4998 (complementary strand)

U6/STAT3 RNAi eukaryotic constitutive expression vector of RNAi pairs, with ampicillin resistance; the plasmid expressing the STAT3 siRNA pair: 5'AgUCAggUUgCUggUCAAAdTdT3' and 5'UUUgACCAgCAACCUgACUdTdT3'; kind gift from Ralph Buettner



Sense	Loop	Antisense	
5'-N(19)	TTCAAGAGA	N(19)	TTTTTT-3' (53 bases)
3'-CCGG N(19)	AAGTTCTCT	N(19)	AAAAAATTAA-5' (61 bases)
Apa I			*EcoR I*

4.3.2 Baculoviruses

Viruses expressing full length human STAT3 were generated by Dr. Uwe Hoffmann.

Viruses expressing c-src were a kind gift from James Turkson, and are described in (Zhang *et al.* 2000).

4.4 Cell culture

4.4.1 Mammalian cell lines

All mammalian cells were maintained in a Hereaus 6000 incubator, in a water-saturated atmosphere at 37°C and under 5% CO_2. The cells were maintained in flasks with filter tops, or in 10 cm dishes. When confluent, they were washed with PBS, and treated for approx. 15 min at 37°C with trypsin-EDTA (Invitrogen). When detached, they were replated at 1:10 dilutions or higher. If lower dilutions were required, cells were first centrifuged for 5 min at 150 g, then resuspended and plated in fresh medium.

Serum was always heat inactivated prior to use (20 min at 56°C), in order to inactivate the complement. All media were stored at 4°C and preheated for 15 min at 37°C before dispensing to the cells.

Cell stocks were frozen at $1x10^6$ cells/ml in regular culture medium supplemented with 8% DMSO. Cells were kept at – 80°C for at least 24 h, before storage in liquid nitrogen.

PBS	0.2 M NaCl
	2.5 mM KCl
	8 mM $Na_{2\,h}PO_4$
	1.5 mM KH_2PO_4
	pH 7.4

Cell lines

NIH3T3	Immortalized mouse fibroblasts; ATCC cell line CRL-1658. These cells are cultivated in DMEM supplemented with 10% FBS.
NIH3T3-vSrc-TKS3	Mouse fibroblasts (NIH3T3 cells) transformed by the stable expression of v-Src. They have stable incorporation of the STAT3 reporter construct pLucTKS3 (Turkson *et al.* 1999). These cells are cultivated in DMEM supplemented with 10% FBS and 500 µg/ml Neomycin.
293Trex	Human epithelial kidney cells transformed with adenovirus 5 DNA derived from the ATCC cell line CRL-1573. They have stable incorporation of the pFRT/LacZeo and pcDNA6/TR plasmids. They are designed to generate stable cell lines containing the inducible plasmid pcDNA5FRT/FRT/TO, which can be incorporated into the genome through recombination. These cells are cultivated in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, 100 µg/ml zeocin, and 15 µg/ml blasticidin. When the cells have incorporated pcDNA5FRT/FRT/TO, the zeocin selection has to be stopped and replaced with 100 µg/ml hygromicin. Charcoal treated FBS was used (Biowest, S175F) for these cells in order to avoid the presence of tetracycline in the serum.
MCF10A	Human immortalized breast epithelial cell line; ATCC cell line CRL-10317. These cells are cultivated in DMEM/Ham12 supplemented with 10% Horse Serum, 2 mM L-Glutamine, 20 ng/ml EGF, 100 ng/ml Choleratoxin, and 500 ng/ml Hydrocortison.
DU 145	Metastasized human epithelial prostate cells; ATCC number HTB-81. These cells are cultivated in MEM alpha supplemented with ribonuleosides and deoxiribonucleosides, 10% FBS, and 2 mM L-glutamine.

4.4.1.1 Mycoplasma analysis

While a cell-line was kept in culture, every three months, cells were tested for mycoplasma infection. Since antibiotics inhibit the PCR reaction, they were removed from the culture medium two weeks before the mycoplasma analysis. 5000 cells were resuspended in 1 ml medium (better than PBS, to allow detection of contamination in the medium), and centrifugation at 12000 g for 20 min. The pellet was resuspended in water, and after a second centrifugation (20 min, 12000 g) the cells were resuspended in 20 µl water and lysed at 99°C for 10 min. The lysate was then spinned down shortly, and 2.5 µl of the suspension was used for the PCR reaction. The mycoplasma detection was done according the protocol of the VenorGeM-QP kit (Minerva Biolabs). The real time PCR reaction was performed in a Roche Light Cycler.

4.4.1.2 Expression of proteins in mammalian cells

4.4.1.2.1 Transfection of NIH3T3, 293Trex, and DU145 cells

All these cells were transfected by lipofection, using the Lipofectamine 2000 reagent (Invitrogen). The day before transfection, $4x10^5$ cells/well were seeded onto a 6 well plate. The next morning, 10 µl Lipofectamine 2000 were mixed with 250 µl Optimem (Invitrogen) in an Eppendorf tube, and 4 µl DNA with other 250 µl Optimem in another tube. Both solutions were incubated for 15 min at RT. Then, they were mixed and incubated for 15 min RT. During this incubation, the medium was replaced with 2 ml medium without serum. 750 µl medium without serum were added to the transfection solution, and mixed carefully. Finally, the medium was removed from the cells and 1 ml transfection solution was dispensed to the cells, and then incubated at 37°C. 6 h later, the transfection medium was replaced with 2 ml medium with serum, in order to minimize toxicity. 24 h after transfection, selection medium was added.

4.4.1.2.2 Transfection of MCF10A

These cells were transfected by lipofection, using the Effectene transfection reagent (Qiagen). The day before transfection, $4x10^5$ cells/well were seeded onto a 6 well plate. The next morning, cells were washed with PBS and 1.6 ml full medium dispensed. 4 µg of DNA were diluted in EC Buffer (100 µl total volume), 8 µl enhancer were added and mixed by vortexing for 1 sec. The mixture was incubated at RT for 2-5 min, and spinned down. 25 µl Effectene transfection were dispensed and mixed by pipetting up and down 5 times, and incubated at RT 5-10 min. 600 µl full medium were added, mixed again by pipetting up and down twice, and dispensed dropwise onto the cells, while the plate was gently swirled. 24 h later, selection was started.

4.4.1.2.3 Generation of stable cell lines

In order to determine the antibiotic concentration to be applied for selection, each cell line was tested separately. Untransfected cells were plated on 6-well plates, so as to have them at approx. 90% confluency. The following day, a range of antibiotic concentrations (specific for each antibiotic) was added to the cells, and the percentage of dead cells was monitored over the next two weeks. The concentration chosen for selection was such that it caused the death of >80% of the cells within the first week, and >95% after the second week. An appropriate concentration should not kill all cells within 48 h.

Puromycin	Tested concentrations: 1, 2.5, 5, 7.5, 10 µg/ml.
	MCF10A: 1 µg/ml
Zeocin	Tested concentrations: 50, 125, 250, 500, 750, 1000 µg/ml
	DU145: 500 µg/ml
	MCF10A: 500 µg/ml
	MCF10A v-src Puro B3: 500 mg/ml
	293FlipIn: 100 mg/ml
Blasticidin	Tested concentrations: 0.5, 1, 2.5, 5, 7.5, 10 µg/ml
	DU145: 2.5 µg/ml
	MCF10A: 5 µg/ml

	MCF10A v-src Puro B3: 5 µg/ml
	293FlipIn: 15 µg/ml
Hygromicin	Tested concentrations: 10, 50, 100, 200, 400, 600 µg/ml
	DU145: 100 µg/ml
	MCF10A: 10 µg/ml
	MCF10A v-src B3: 10 µg/ml
	293Trex: 100 µg/ml hygromicin

24 h after transfection with a specific plasmid, the required antibiotic was added at the established concentrations. Cells were monitored over the following weeks. Once large clones had appeared (should be visible with the bare eye around the third or forth week), they were picked up with a pipette tip and transferred to a 96-well plate. The clones were expanded and passed into 24, 12, and then 6 well plates. As soon as cells were available in sufficient number, they were screened for expression of the desired protein, while they were expanded to prepare a stock of the cell line.

4.4.1.2.4 Inducible expression of transgenes in mammalian cells

4.4.1.2.4.1 Ecdysone system

The ecdysone-inducible mammalian expression system is based on the molting induction system of Drosophila, but has been modified for inducible expression in mammalian cells. The system uses the steroid hormone ecdysone analogs, ponasterone A or muristone A, to activate expression of the gene of interest via activation of a heterodimeric nuclear receptor. With this system the protein expression can be induced more than 200-fold.

The gene of interest, STAT3β, was cloned into the inducible expression plasmid, which contains 5 modified ecdysone response elements (E/GREs) upstream of a minimal heat shock promoter and the multiple cloning site (pIND-TOPO-TA vector). Cloning was done by TOPO cloning (Invitrogen).

Along with the inducible plasmid, pVgRXR, a vector expressing the ecdysone receptor subunits, was transfected into the cells. After generation of stable cells, the expression of the gene was induced with 5 µM ponasterone or 10 µM muristone for at least 24 h, and the expression of STAT3β was tested by Western blotting.

4.4.1.2.4.2 Tetracycline system

The Flip-In T-Rex system was used for the generation of stable mammalian cells exhibiting tetracycline-inducible expression of STAT3β from a specific genomic location. To generate these cells, first, independent integration of a plasmid expressing the Tet repressor (pcDNA6/TR) and of a plasmid containing a Flip-recombination target (FRT) site (pFRT/LacZeo) into the genome of the mammalian cell line of choice was required. After cloning and screening of the clones for the LacZ reporter gene expression (see below) to identify clones with integration of the pFRT/LacZeo plasmid into a high-expression region of the genome, STAT3β was introduced into this site, by cotransfection of the pcDNA5FRT/FRT/STAT3β vector with plasmid pOG44.
50 hours after transfection, cells were selected with 15 µl/ml blasticidin and 100 µg/ml hygromicin. One sufficient cells available, they were tested by Western blotting for induction of STAT3β after treatment with 1 µg/ml tetracycline.

4.4.1.2.4.3 Confirmation of gene incorporation and protein expression

In order to verify the incorporation of the plasmid, reporter assays were performed when applicable. Otherwise, whole cell extracts prepared for each clone, and the expression of the desired protein was analyzed by Western blotting.

4.4.1.3 β-Galactosidase *in situ* staining

Cells were washed once with PBS before adding 2 ml fixing solution, and incubated at RT for 15 min. They were then washed twice with PBS, and 1 ml of X-Gal solution was added. Cells were incubated between 15 min and 24 h at 37°C in the incubator. The time of incubation was adjusted depending on the intensity of the blue staining. Cells were then washed twice with PBS, and photographed under the microscope (DMIL Leica), and images were captured using a digital camera (DC 300F, Leica).

Fixing solution	0.25% v/v glutaraldehyde in PBS
Staining solution	A 2% stock solution of X-Gal (Invitrogen) was prepared in dimethyl formamide (DMF). This was then diluted to

0.02% in a solution of 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$.3 h_2O, and 5 mM $K_3Fe_3(CN)_6$ in PBS.

4.4.1.4 Whole cell lysates

Cells were washed with PBS, and 200 to 300 µl RIPA buffer were added to each 6-well dish (or 1 ml for a 10 cm dish). They were then scraped and transferred to an Eppendorf tube. The tubes were rotated at 4°C for 30 to 45 min in a rotating wheel. Finally, they were centrifuged at 14000 rpm and 4°C for 10 min, after which the supernatant, containing the whole cell lysate, was transferred to a new tube.

RIPA	20 mM Hepes pH 7.4
	150 mM NaCl
	5 mM EDTA
	1% NP40 or Triton X 100
	2.5% Deoxycholate
	0.1% SDS
Fresh:	1:500 of protease inhibitor cocktail set III (Calbiochem)
	1 mM sodium orthovanadate
	1 mM DTT

4.4.1.5 Nuclear cell extracts

Cells were placed on ice and washed twice with cold PBS/vanadate. 1 ml PBS/vanadate was added per 10 cm dish, and cells were scraped from the plate. The cells were pipetted into an Eppendorf tube and centrifuged for 5 min at 220 g. All centrifugations were done at 4°C. The cellular pellet was resuspended in twice its volume of Buffer A, and centrifuged for 5 min at 220 g. The supernatant was pipetted into another Eppendorf tube and kept as cytosolic extract, and the pellet was resuspended in once its volume of Buffer C, and incubated on ice for 30 to 45 min. Insoluble material was removed by centrifugation at 15000 g for 20 min. Protein concentrations were determined by the BCA method and extracts were stored at - 80 °C until use.

PBS/Vanadate	0.2 M NaCl
	2.5 mM KCl

	8 mM $Na_{2\ h}PO_4$
	1.5 mM KH_2PO_4 pH 7.4
	100 µM Na_3VO_4
Buffer A	10 mM Hepes-KOH pH 7.8
	1.5 mM $MgCl_2$
	10 mM KCl
	0.5 mM DTT
	0.2 mM PMST
	1 mM Na_3VO_4
Buffer C	20 mM Hepes-KOH pH 7.8
	1.5 mM $MgCl_2$
	420 mM NaCl
	0.2 mM EDTA
	0.2% v/v glycerol
	0.5 mM DTT
	0.2 mM PMSF
	1 mM Na_3VO_4

4.4.1.6 Immunofluorescent staining

Cells were seeded on glass slides and incubated with the biotinylated and cell-permeable peptides. 1 h later, slides were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.2% Triton 100-fold for 5 min, blocked with 5% FBS for 30 min and incubated with a 1:200 dilution of FITC conjugated streptavidin (Jackson ImmunoResearch) for 45 min at RT in the dark. Staining was visualized using a fluorescence microscope (DM LB, Leica), and images were captured using a digital camera (DC 300F, Leica).

4.4.1.7 Proliferation analysis

Cells were plated on a 96-well plate and treated as required. Upon treatment, 5 µl of the oxidized form AlamarBlue were added to 100 µl culture medium. The reduced form of AlamarBlue was measured by fluorescence (excitation λ 560 nm, emission λ

590 nm) during the following 1 to 6 h with a Spectra Magemini fluorescence reader (Molecular Devices).

4.4.1.8 Apoptosis analysis

Caspase activation assay: MCF10A cells were seeded in 50 µl on a 384-well plate and several hours later, when attached, starved overnight. The next morning medium was changed and EGF was added to the treated group, and 24 h later the 50 µl caspase substrate (Asp-Glu-Val-Asp-Rhodamine) dilution to all cells. 3 hours later, the product (rhodamine) was measured by fluorescence (excitation λ 499 nm, emission λ 521 nm) with a Spectra Magemini fluorescence reader.

4.4.1.9 Colony formation in soft agar

A 3% agar (Difco agar noble, BD Bacto Agar, or Biozym Seaplaque agarose) stock solution was prepared in PBS. The solution was autoclaved in a microwave (boiled 3 times), and when clear and well dissolved, it was maintained at 48°C in a water bath, along with the culture medium. The agarose was diluted in the medium, and a 1% agar solution was plated on the wells (1.5 ml/well for 6-well plates). While this underlayer solidified, cells were counted, and 6000 cells were mixed in 1.5 ml 0.5% agar prepared in culture medium. Cells were incubated for 2 h at 37°C to allow for cells to settle down, and the plates were then incubated at RT to allow for solidification of the agar. Once the agar was solid, 500 µl medium were added to each well, and cells were placed into the incubator (37°C, 5% CO_2). The medium was changed every three to four days. 14 days later, colonies were stained with iodonitrotetrazoliumchlorid.

Staining solution: 100 mg iodonitrotetrazoliumchlorid
25 ml water
25 ml methanol

4.4.1.10 Tumor xenografts

Female, inbred SCID mice were purchased at Charles River (Cb-17/lcr/Crl-scidBR). These mice present a severe combined immunodeficiency affecting both B and T lymphocytes. They have normal NK cells, macrophages, and granulocytes.
These mice were kept at 25°C in an incubator with filtered air. Food (standard diet for mouse and rats, Altromin) and sterile water were always available for the animals.
Cells were cultured in the usual conditions, trypsinized, and washed twice in PBS. They were counted as described below and $5x10^6$ cells were resuspended in 100 µl PBS with Mg^{+2} and Ca^{+2} (Biochrom) and the suspension injected subcutaneously into the back of the mice. Six mice of 10 weeks of age, weighing 17 to 20 mg were used for each cell line. At the time of injection, the viability of the cells was controlled as described below. No more than 10% should be dead. After injection of the cells, the weight of the animals and the growth of the protrusion at the site of injection were monitored every third day.

4.4.2 Prokaryotic cells

All bacterial stocks were stored at - 80°C.

E.Coli One Shot TOP10 (Invitrogen)
Chemically competent. Used for chemical transformations:
Cells were thawed on ice, and 20 µl were aliquoted into a sterile Eppendorf tube. Approx. 1 µg DNA was added and mixed by gentle flicking of the tube. The mixture was incubated on ice for 15 min, then at 42°C for 30 s, to heat shock the cells. 500 µl of SOCS medium were added to the cells, transferred to a sterile 14 ml polypropylene (PP) filter top tube and incubated at 37 °C and 200 rpm for 1 h. The bacteria were then distributed onto an agar plate containing the right antibiotic (usually ampicillin, which is applied at 100 µg ampicillin/ml LB-agar). If antibiotic had not been added while preparing the agar plates, it was added before plating the transformed cells, adding the required antibiotic for 20 ml LB medium, and distributing well with the digralski spatula. The plates were then incubated O/N at 37°C.

LB medium	10 g/L tryptone or peptone 5 g/L yeast extract 5.14 g/L NaCl (88 mM final) All the components were dissolved in water and mixed in a bucket. When dissolved, the whole volume was distributed in 500 ml bottles, and autoclaved for 20 min at 121°C (the solution was not left to incubate overnight prior to autoclavation, since potential contaminants could expand in the broth). Prior to being added to the cells it was cooled down to room temperature.
Agar plates	15 g/L agar (1.5% w/v final) were added to a 1 L to a bottle, then 1 L of the non-sterilized LB solution, prepared as described above, was added to the bottle. The mixture was autoclaved immediately (20 min at 121°C), and while still warm and liquid, approx. 20 ml of the solution was distributed to each 10 cm dish. The dishes were placed on a flat surface, while the agar plates cooled down to room temperature and solidified. When solid, they were stored at 4°C and wrapped in plastic to keep in the humidity.

E.Coli DH10B Eletctromax (Invitrogen)

Used for electrotransformation:

The electrocuvettes were placed for 15 min on ice, and the S.O.C. medium (Invitrogen) was prewarmed at 37°C. The bacteria were thawed on ice, and the electroporator (Bio-Rad E.Coli Electroporator) was set to 2.5 kV. 1-5 µl plasmid DNA (1 µg or less), and 20-30 µl bacteria were pipetted into the electrocuvette and mixed with the tip of a Pasteur pipet, placing the mixture at the bottom of the cuvette. The samples were electroporated. The optimal pulse time should be of approx. 5 ms. If electric pulse was appropriate, 500 µl SOC medium were added to the treated bacteria, and mixed carefully. The solution was transferred into a 14 ml polypropylene (PP) filter top tube, and incubated for 1 h at 200 rpm and 37°C. The cells were then plated on agar plates and incubated O/N at 37°C .

E. coli DH10Bac (Invitrogen)
Chemically competent bacteria carrying a baculovirus shuttle vector bMON14272 (bacmid; contains the attachment site for the bacterial transposon, Tn7, i.e. mini-attTn7), and the helper plasmid pMON7124 (encodes the transposase). This allows the preparation of recombinant bacmids by transposition between the shuttle and the pDest10 vectors. These cells were transformed by chemical trasformation as described for E.Coli One Shot TOP10, and were used for the generation of human STAT3 recombinant bacmids .

4.4.2.1 Bacterial culture

The bacteria were picked from the agar plates with a pipet tip, and placed in 2.5 ml LB-Medium with the required antibiotic. They were incubated at 37°C under 200 rpm for 1 h, and then diluted 1:500 to 1:1000 in LB-Medium with antibiotic. The incubation was continued O/N. The following morning, 800 µl of the LB culture were supplemented with 200 µl of sterilized glycerol 100%, and this bacterial stock was stored for approx. 2 years at - 20°C, and for longer periods at - 80°C. The rest of the culture was used for the extraction of DNA.

4.4.2.2 Preparation of plasmid DNA

The plasmids were purified according to the QIAGEN Plasmid Mini-, Midi, and Maxi Kits.

4.4.3 Insect cells

These cells were used for the preparation of viral stocks and the production of proteins. All insect cells were cultivated at 27°C under atmospheric CO_2 conditions, and grown in adherent and in suspension cultures.

Sf9 cells — Sf9 cells are derived from Spodoptera frugiperda. They were cultivated in 1 L Sf-900 II SFM (Invitrogen) supplement 50 U Penicillin/ 50 µg Streptomycin (Invitrogen). The cells were always maintained in the serum-free medium. To store cells, $1x10^7$ cells/ml were frozen in a mixture of 60% Grace's Insect Medium, 30% FBS, and 10% DMSO.

Hi5 cells — High Five cells from Tirchoplusia ni flies. High Five cells are characterized by a higher expression of recombinant proteins than other insect cells. They were cultivated in 1 L Express five SFM (Invitrogen) supplemented with 10 ml L-Glutamine 200 mM (Invitrogen). To store cells, $1x10^7$ cells/ml were frozen in a mixture of 42.5% conditioned Express Five SFM, 42.5% fresh Express Five SFM, 10% DMSO, and 5% FBS.

4.4.3.1 Production of proteins in insect cells

4.4.3.1.1 Suspension culture of Sf9 and Hi5 cells

In order to assess the cell number, 10 µl of the culture were taken and mixed with 10 µl 0.4% trypanblue (Sigma). 10 µl were placed onto a Neubauer chamber, and both the top and bottom corners (each corner contains 16 squares) were counted (Fig.5). Blue (dead) and white (alive) cells were counted separately. The mean of the four corners was calculated, and the number of cells was calculated by:

Cells/ml= mean cell number x 10^4 x 2

Viability (%)= number of living cells x 100/total cell number

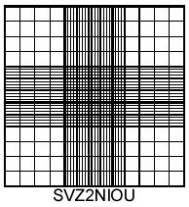


Fig.5. Magnified picture of a Neubauer Chamber.

If the viability of the cells was >90%, cells were adjusted to 4 to 5x10^5 cells/ml with fresh medium, and seeded into a spinning flask (TECHNE/ThermoDUX). They were incubated at 27°C on a cell culture stirrer with 85 rpm spinning (TECHNE/MCS104L4) until they reached 2 to 3x10^6 viable cells (duplication time 18 to 24 h). Every three weeks, cells were pelleted at 100 g for 5 min in order to remove cellular waste and debris.

4.4.3.1.2 Preparation of viral stocks

Viruses were prepared in SF9 cells. 9x10^5 cells/well were seeded in 2 ml Sf900II medium with 1000 U/ml penicillin and 100 µg/ml streptomycin on a 6-well plate (Costar). The plate was placed in a humidifying chamber (i.e. box with humid paper towel), and incubated for 1 h at 27°C in order to allow for cell attachment. For transfection, 5 µl bacmid-miniprep were added to 100 µl Sf900II medium without antibiotics. In another tube, 6 µl Cellfectin (Invitrogen) were mixed with 100 µl Sf900II medium without antibiotics. Both solutions were mixed and incubated for 30 min at RT. 800 µl Sf900II Medium were prepared in a 5 ml polystyrene tube. Cells were washed with 2 ml antibiotic-free medium, and the Cellfectin/DNA mixture was gently mixed with the 800 µl medium. The medium was removed from the cells and replaced with the transfection mixture (1 ml). The plates were incubated for 5 h at 27°C in a humidifying chamber. Medium was then removed, and 2 ml Sf900II with 1000 U/ml penicillin and 100 µg/ml streptomycin added. Cells were incubated at 27°C, and 96 h later, the culture medium was taken and centrifuged for 5 min at 500 g. The supernatant was placed in polypropylene tubes (viral stocks P1), and stocked at 4°C in the dark.

4.4.3.1.3 Virus amplification

In order to increase the amount and titer of the viral stock, it was amplified in a suspension culture of Sf9 cells. 1 ml of virus P1 was added to 10 ml of a 1.5 to 2.0x10^6 viable cells/ml, and incubated for 96 h at 27°C and approximately 100 rpm . The viral supernatant (virus P2) was harvested as described before under preparation of viral stocks. 5 ml of virus P2 were inoculated into 50 ml of a 1.5 to 2.0x10^6 cells/ml culture, and virus P3 harvested after 96 h.

4.4.3.1.4 Plaque assay

Sf9 cells or Hi5 cells were counted as described before. They were adjusted to a concentration of 0.5x 10^6 cells/ml with fresh Sf-900 II SFM with 50 U/L penicillin and 50 µl/L Streptomycin, and 2 ml/well were placed onto a 6 well plate. Cells were incubated for 1 h at 27°C in a humidifying chamber. Viral dilutions (10^{-2} to 10^{-8}) were prepared in Sf-900 II SFM with 50 U/L penicillin and 50 µg/L streptomycin (e.g. 50 µl stock P3 into 5 ml medium…). Usually the virus titer lied between 10^{-6} to 10^{-8}. Under these circumstances, it was sufficient to infect cells with the 10^{-6} to 10^{-8} dilutions. When the cells were attached, the medium was replaced with 1 ml viral dilution and the plates were placed in the humidifying chamber wrapped with alumium. They were incubated at 27°C shaking at approx. 80 rpm. A 4% agarose gel (Invitrogen) was melted at 70°C in a water bath (approx. 1 h required). In two glass bottles, 15 ml of 1.3-fold Sf900 medium with 100 U/ml penicillin and 100 µg/ml Streptomycin (Invitrogen) were prewarmed at 39°C, and 5 ml of 4% melted agarose were added. 4 h after the viral infection, the medium was sucked off and 2 ml/well agarose overlay were carefully dispensed to each well. The plates were incubated 20 min at RT without shaking, and placed in a humidifying chamber for incubation at 27°C. The humidity in the chamber was controlled every day, and between 4 to 8 days later, when no more plaques appeared, the number of plaques were counted (optimal number: 5 to 30 plaques per well) and the virus titer calculated:
pfu/ml = number of plaques/volume virus/dilution factor
(e.g 20 plaques at a viral dilution of 10^{-7}, corresponds to a virus titer of 20/1/10^{-7}= 2 x 10^{-8} pfu/ml)

4.4.3.1.5 Infection kinetic

Cells were counted, and eight separate cultures of Sf9 or Hi5 cells were prepared in fresh medium in order to test the 1,2,5, and 10 multiplicity of infection (MOI=pfu/cell), each at two different harvesting times (40 and 48 h). 1.5 to 2.0x10^6 viable cells/ml were dispensed either into an Erlenmeyer (Duran/Bender & Hobein 391A0285), or into a spinnerflask (TECHNE/ThermoDUX F7608). In order to ensure a good aeration of the culture, it should not occupy more than one tenth of the maximal volume in the Erlenmeyer, or half of the maximal volume in the flask. The viral inocolum was calculated by:
Inoculum (ml)= (MOI(pfu/cell)x number of cells)/titer of viral stock (pfu/ml)
The appropriate virus volume was added to each culture, and the culture was incubated at 27°C and 100 rpm agitation. After 40 h or 48 h, cells were centrifuged at 600 g for 10 min.

4.4.3.1.6 Insect cell lysates

Cells were washed twice with ice-cold PBS, and precipitated at 600 g and 4°C for 5 min. An additional washing step with PBS containing 1 mM sodium orthovanadate was performed when preparing phosphorylated proteins. Cells were resuspended in 1% Nonidet P-40 lysis buffer (10 ml buffer for a 50 ml culture) and lysed on ice for 10 min. The lysate was then centrifuged at 14000 g, 20 min, 4 C. The supernatant was either immediately used, or stored in 500 µl aliquots at - 80°C.

1% Nonidet P-40 lysis buffer	50 mM HEPES (pH 7.9)
	150 mM NaCl,
	1% Nonidet P-401
	water
Fresh:	Complete Mini protease inhibitor tablets (Roche)
	20 mMNaF
	1 mM sodium orthovanadate
	1 mM DTT

STAT3 expression was assessed by Western blotting, as described before.

4.4.3.1.7 Protein expression

The protein expression was performed in suspension culture of either Sf9 or Hi5 cells. The optimal conditions for infection were deduced from the kinetic studies, and scaled up proportionally. The expression of the protein was verified by Western blotting, as described below.

4.5 In vitro assays

4.5.1 Protein analysis

4.5.1.1 Protein quantification

The determination of the protein concentration was done in 96-well plates, (Greiner) using the bicinchoninic acid (BCA) method (Pierce). A standard curve of BSA (1000, 800, 700, 600, 500, 400, 200, 0 µg/ml) was prepared in PBS, supplemented with the same amounts of lysis buffer present in the wells with the samples (1 µl per well). 1 µl of each sample was diluted in 9 µl PBS, and 200 µl of the BCA solution was added to all wells. The plate was incubated for 30 min at 37°C. The absorbance was measured at 562 nm with a Rainbow microtiter plate reader (SLT), and the protein concentration was determined by linear regression.

4.5.1.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Equal amounts of total protein (20 to 60 µg) were prepared, and the volume of all samples was adjusted with PBS, to a maximal volume of approx. 60 µl (resp 30 µl) in the case of an 18-well (resp 26-well) gel. Each sample was supplemented with 4-fold XT Sample Buffer (Bio-Rad), and 20-fold XT Reducing Agent (Bio-Rad). The samples were then boiled for 5 min at 95°C in a heating block. The gels (Criterion XT Bis-tris 10% or 4 to 12% polyacrylamide gels, Bio-Rad) were assembled in the running chamber, and covered with 1-fold XT MOPS running buffer (Bio-Rad). 10 µl molecular weight ladder (Benchmark Invitrogen) and the boiled samples were loaded.

The samples were run at 100-150 V, and the gel was stopped when the dye front arrived at the bottom of the gel.

4.5.1.3 Western blotting

The proteins on the SDS-PAGE gel were transferred onto a 0.2 µm nitrocellulose membrane with the transfer unit (Criterion blotter, Bio-Rad) covered with 1-fold transfer buffer. To avoid overheating the solution, a frozen ice pack was kept inside the buffer. The transfer was performed for 30 min (at most 1 h) at 100 V. The transfer of the proteins to the membrane was checked with PonceauS red staining, and then washed with water. Next, the membrane was blocked with 20 ml blocking solution for 1 h at RT under 100 rpm shaking (alternatively O/N at 4°C with gentle shaking). Then it was washed three times for 3 min with PBST under shaking, and incubated with 20 ml primary antibody solution. All antibody incubations were done for 1 h at RT under 100 rpm shaking (alternatively at 4°C O/N with gentle shaking). After washing four times with PBST, it was treated with 20 ml of secondary antibody, and washed again six times with PBST. Finally, the membranes were incubated for 5 min with the ECL (Enhanced Chemiluminescence) solution and exposed to Hyperfilm ECL chemiluminescence films (Amersham Biosciences).

Running buffer	10-fold XT MOPS running buffer (Bio-Rad) diluted in water
Ponceau red	1.25 g Ponceau S
	52 ml sodium acetate 96%
	448 ml water
Transfer buffer	100 ml 10-fold transfer buffer (i.e. 22.3 g Tris, 120.1 g glycin, up to 1 L water)
	200 ml methanol
	700 ml water
20-fold TBS:	3 M NaCl
	1 M Tris
	pH was adjusted to 7.5 with concentrated HCl

TBST	250 ml 20- TBS
	12.5 ml 20% tween
	up to 5 L water
TBST	24.2 g Tris
	80 g NaCl
	up to 1 L water
	3/4 of the solution were adjusted to a pH of 7.6 with HCl, and the volume was finally adjusted with water
Blocking solution:	5% non-fat milk powder
	1000 ml PBST
1ary antibody	5% BSA
	1 mM NaN_2 (only when reusing solution)
	TBST
2ary antibody	1:2000 antibody
	5% (w/v) non-fat milk powder
	TBST

4.5.1.4 Electrophoretic mobility shift assay (EMSA)

Labeling of radioactive probe:

STAT proteins can bind to the synthetic sis-inducible element (SIE) isolated from the human c-fos promoter. The m67 SIE variant contains a mutation that allows for binding of STAT1 and STAT3 with equal affinity. The radioactive labeling of the double stranded probe was done by addition of [^{32}P]α-dATP to the 5'ends with the Klenow enzyme. Free nucleotides were removed by the Quiaquick Nucleotide Removal kit (Qiagen). The incorporated radioactivity was then measured in a β-counter .

m67SIE: 5'-GATTGACGGGAACTG-3' (STAT binding sequence underlined)

EMSA:

Nuclear extracts (5 µg), containing 0.14 µg/µl poly-(dI-dC) and ^{32}P-labeled double stranded DNA probe were incubated for 20 min on ice. For super-shift assays, the indicated antibody was added, in the other samples the volume was adjusted with water. Samples were electrophoresed in 5% polyacrylamide gel in 0.25% Tris-borate-

EDTA buffer. Gel-shift grade anti-mouse STAT1 and STAT3 (Santa Cruz) polyclonal antibodies were used for supershifts.

4.5.1.5 Enzyme coupled immunosorbent assays (ELISA)

The signal obtained in these assays was measured in the Rainbow microtiter plate reader (SLT).

4.5.1.6 STAT3 DNA-binding ELISA

This assay was performed as described in the TransAM kits for STAT proteins (Active Motive).

4.5.1.7 Dimerization ELISA

4.5.1.7.1 Streptavidin based ELISA

Coating of plates:
Different amounts of biotinylated peptide (AAPY*LKTKFK-biotin and PY*LKTKK-biotin; FKTKLYPAAK-biotin as negative control) were dispensed on streptavidin-coated 96-well plates and incubated at room temperature for 30 min under 100 rpm shaking. Plates were washed four times, and incubated with 10 µg/ml anti-phospho-STAT3 antibody. After one hour incubation at room temperature under 100 rpm shaking, plates were washed three times, and incubated with 1 µg/ml HRP-conjugated anti-rabbit antibody. Signal was developed using ABTS reagent and measured at 405 nm.

Complete assay:
Biotinylated-phospho-STAT3-specific peptides were dispensed on streptavidin-coated 96-well plates and incubated at room temperature for 30 min under 100 rpm shaking. Plates were washed four times, and incubated with Sf9 cell lysates. Upon 1 h incubation with 10 µg/ml anti-STAT3 antibody at RT under 100 rpm shaking, plates were washed 3 times, and incubated with 1 µg/ml HRP-conjugated anti-rabbit antibody. Signal was developed using ABTS reagent and measured at 405 nm.

4.5.1.7.2 Flag-tag based ELISA

A 5 µg/ml anti-flag-antibody solution was prepared in carbonate buffer pH 8.4. 100 µl were dispensed onto a Maxisorb plate (Nunc), and the lids of the plate were sealed with parafilm in order to avoid evaporation. The plates were incubated at 4°C overnight. The following morning, plates were washed four times with PBST, and blocked with 5% BSA in PBST for 1 h at 100 rpm on a rocking shaker. All the shaking was performed at this speed, and from this step on, the assay was performed at RT. In the mean time, 15 µl of non-phosphorylated Sf9-STAT3 cell lysates were either incubated with Flag-tagged peptides (Y*LKTKFGGGGGGDYKDDDDK or KFPYKTLGGGGGGDYKDDDDK as control) on a 96-well plate with v-shaped bottom, and rocked for 30 min. The concentration of peptide was calculated at the final assay volume, which was 100 µl. Flag-tagged peptides were added (30 min at RT and 100 rpm shaking), and, after this incubation, the assay volume was adjusted with water to 100 µl in each well, and then bound to the antibody coated plates (1 h, RT and 100 rpm shaking). Incubation of STAT3 antibody (1 h, RT and 100 rpm agitation) and secondary antibody (1 h, RT and 100 rpm agitation) was done next. Finally, ABTS substrate was added to all wells, and the absorbance measured at 405 nm.

Competition assay:

The assay was done as described above, except that the Sf9-STAT3 cell lysates were preincubated with the compounds in v-shaped 96-well plates for 30 min prior to adding the Flag-tagged peptides. The concentration of the molecules was calculated for the final assay volume, which was 100 µl. In order to minimize the DMSO concentration in the assay, the compounds were prepared as follows: The compounds were dissolved in DMSO at 50 mM. A 7.5 mM dilution of the compound was prepared in Tris-buffer (pH 7.5) 2 µL of this solution were added to the cell lysates

PBST	0.2 M NaCl
	2.5 mM KCl
	8 mM $Na_{2\,h}PO_4$
	1.5 mM KH_2PO_4 pH 7.4
	0.05% Tween 20 in PBS
Tris-buffer	25 mM Tris in water

Carbonate buffer pH 8.4 0.1 M $NaHCO_3$ in water
Set pH to 8.4 with 1 M NaOH

4.5.2 DNA analysis

4.5.2.1 Determination of DNA concentration

The DNA content was calculated by measuring the absorbance at 260 nm (Pharmacia Biotech, Gene Quant). 1 µl of the sample was diluted into 99 µl water, mixed well by vortexing, and pipetted into a quartz cuvette (Perkin Elmer, UV/Vis spectroscopy cell). One unit of absorbance at this wavelength equals 50 µg of double-stranded DNA/mL.

4.5.2.2 Determination of DNA quality

4.5.2.2.1 OD_{260}:OD_{280}

In order to assess the purity of the DNA solutions, the absorbance was measured at a wavelength of λ_1=260 nm and λ_2=280 nm, to measure the DNA and protein concentration, respectively. 1 µl of the DNA sample was diluted into 99 µl deionised water, mixed well by vortexing, and pipetted into a quartz cuvette. The ratio between the absorbances (λ_1/λ_2) was calculated. The DNA purity was considered of good quality with values between 1.6 and 2. Ratios >2 (resp <1.6) indicate a high salt concentration (resp contamination by proteins).

4.5.2.2.2 Electrophoretic separation of DNA:TAE gel electrophoresis

Agarose gels of 1.5% agarose (0.9 g Agarose, 60 ml TAE 1X buffer, and 0.5 µg/ml ethidium bromide, for a 7 well gel) were prepared to separate DNA fragments of around 2000 bp. For very small or much larger fragments the concentration of agarose was adjusted. The solution was boiled three times in the microwave and mixed well before being poured into the gel caster in order to enssure that the agarose was well dissolved. The DNA samples were diluted with 6-fold DNA loading buffer and, when the gel solidified, loaded to the gel, along with 10 µl of DNA ladder

(New England Biolab). In the case of PCR products, 5 µl out of a total of 50 µl were loaded, and in the case of other DNA preparations, approx. 1 µg. The gels were run at 100V.

4.5.2.3 DNA purification

Purification of the DNA samples was done when the ratio OD_{260}:OD_{280} indicated impurities, in order to remove the proteins or ions from the DNA solution.

4.5.2.4 DNA precipitation

3 M sodium acetate pH 4.8 to 5.2 was added to the DNA solution at a proportion of 1/10 of the DNA solution volume, and ethanol 100% at 2.5 times the DNA-volume. The solution was well mixed by vortexing, and incubated for 1 h at - 80°C (alternatively O/N at -20°C). The mix was then centrifuged at 15000 g for 15 min at 4°C.

4.5.2.5 DNA isolation from agarose gels

The DNA sample was run by electrophoresis in an agarose gel, as described before. The band of interest was cut, and the DNA was isolated according to the QIAGEN QIAEXII Gel Extraction Kit.

4.5.2.6 DNA sequencing

To sequence a gene, primers were designed to cover the whole coding sequence at intervals of approx. 800 bp. Primer sequences were selected to have a length of approximately 20 nucleotides. The G/C content was kept at 50%, and the last residue at the 3’ end of each primer was a G or C when possible. Sequencing of plasmids was done at Sequence Laboratories Göttingen GmbH. Sequence analysis was done with the Lasergene software package (DNAStar Incorporation).

4.5.2.7 Gene subcloning

4.5.2.7.1 Primer design

In the case of PCR amplifications, two primers were selected, each starting at different ends of the gene, and both with opposing orientation, such as to amplify the flanked sequence. Primers were designed to have a length of approximately 20 nucleotides, unless a mutation or a tag was to be introduced, in which case they were chosen to be 30 to 50 nucleotides long. The G/C content was kept at 50%, and at 3' ending with a G or C when possible. The annealing temperature for the primers was calculated by

[4 x (#C + #G) + 2 x (#A + #T) – 5]°C

Primers

STAT3β clonation:

Forward: 5'ATGGCCCAATGGAATCAGCT3'

Reverse: 5'AATTCACATGGGGGAGGTAG3'

STAT3β His-tag clonation:

Forward: 5'ATGGCCCAATGGAATCAGCTAC3'

Reverse:

5'TCAGTGATGGTGATGGTGATGTTATTTCCAAACTGCATCAATGAATC3'

All primers were synthesized at Qiagen.

4.5.2.7.2 PCR protocol

PCR mix:	5 µl 10-fold PCR buffer (Qiagen)
	300 µM dNTP (stock containig10 mM of each dNTP)
	1 µM primer 1 (100 µM stock)
	1 µM primer 2 (100 µM stock)
	2 µl polymerase (Proof Start, Qiagen)
	1 ng to 50 ng plasmid DNA
	water up to 50 µl

PCR protocol:
5 min at 95°C (Activation of Proof Start, Qiagen)
30 s at 94°C (denaturation)
30 s at 57°C (annealing)
5 min at 72°C (extension)
Cycles were repeated 29 times starting at the denaturation step.
30 min at 72°C (extension)

The PCR protocol was adjusted according to the annealing temperatures of the primers used for each amplification. When introducing a tag or a mutation in the primers, the protocol was started with 10 cycles at the annealing temperature of the primer regardless of the tag, and followed by 20 cycles at the annealing temperature of the complete primer. For example, for His-tag introduction into STAT3β:

5 min at 95°C (activation of Proof Start, Qiagen)
30 s at 94°C (denaturation)
30 s at 62°C (annealing)
5 min at 72°C (extension)
Cycles were repeated 10 times starting at the denaturation step
30 s at 94°C (denaturation)
30 s at 60°C (annealing)
5 min at 72°C (extension)
Cycles were repeated 20 times starting at the denaturation step with the second annealing temperature.
30 min at 72°C (extension)

4.5.2.7.3 PCR product purification

The PCR product was purified through isolation from agarose gels, as described before.

4.5.2.7.4 Polyadenylation of PCR product

The PCR product was polyadenylated according to the instructions of the QIAGEN A-Addition Kit.

4.5.2.7.5 Topo-TA cloning

4 µl of the PCR product (approx 25 ng) of the polyadenylated DNA product were mixed with 1 µl the linearized TopoTA vector, and 1 µl of a 1:5 dilution in water of the salt dilution provided with the vector. The mix was incubated at RT for 30 min, and transformed into bacteria by electroporation as described before.
The verification of clonation and orientation of STAT3β into pIND/V5-His-TOPO, and STAT3β with his tag into pcDNA5/FRT/TO was done by PCR analysis, using the following primers:
Forward: same forward primer used for the clonation
Reverse: BGH reverse 5'TAGAAGGCACAGTCGAGG3'