Stat3 and Tumor Cell Proliferation

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Nicole Schick

aus Mannheim, Deutschland

Leiterin der Arbeit: Prof. Dr. Nancy E. Hynes

Friedrich Miescher-Institute, Basel

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Basel, den 3.02.2004

Prof. Dr. Marcel Tanner
Dekan

To my family and friends for their support, encouragement and love.

The more you know, the harder it is to take decisive action. Once you become informed, you start seeing complexities and shades of gray. You realize that nothing is as clear and simple as it first appears. Ultimately, knowledge is paralyzing.
Bill Watterson *(1958 -), Calvin & Hobbes (THERE'S TREASURE EVERYWHERE)*

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I. SUMMARY

In the more prosperous countries of the world cancer is the cause of death of roughly one person in five; the five most common cancers are those of the prostate, breast, lung, colon/rectum and the uterine cervix. Cancer is caused by the stepwise acquisition of mutations that allow: limitless replicative potential, insensitivity to anti-growth signals, evasion of apoptosis, self-sufficiency in growth signals, tissue sustained angiogenesis and invasion and metastasis. Two obvious targets for cancer drug development are the two cooperating conditions that permit cell expansion; deregulated cell proliferation and inhibition of apoptosis

Stat3, a member of the Signal transducers and activators of transcription (STAT) protein family of transcription factors, seems to play a role in cancer progression since it is constitutively activated in a wide variety of human malignancies. In addition, constitutively active Stat3 is involved in growth promoting and apoptosis inhibiting functions in tumor cells. On the other hand cytokine induced activation of Stat3 can lead to growth inhibition and apoptosis in some normal and cancerous cells. The precise contribution of Stat3 to these different scenarios in cancer cells could not be elucidated so far, which makes it difficult to target Stat3 in cancer therapy. The aim of this thesis was to investigate the mechanism by which Stat3 controls proliferation of tumor cells that are proliferatively inhibited by IL-6 type cytokine treatment. Dimerization of IL-6 type cytokine receptors, upon ligand binding, leads not only to activation of the JAK-STAT pathway, but also to activation of the mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol dependant kinase (PI3K) signaling cascade. To investigate the participation of Stat3 in IL-6 induced anti-proliferative effects without the influence of the other pathways, we have designed an inducible Stat3 construct, in which the entire Stat3 coding sequence was fused to the estrogen receptor ligand-binding domain (ER-LBD) that can be dimerized by addition of 4-hydroxytamoxifen (4HT).

Stable expression of this construct in A375 melanoma cells and stimulation with 4HT showed that Stat3 activation alone is sufficient for the anti-proliferative effects induced by IL-6 type cytokine stimulation. Surprisingly, treatment of cells with a

combination of OSM and 4HT, led to strong and prolonged Stat3 activity, and induced cell cycle arrest and apoptosis. These results suggest that Stat3 activation in cancer cells, in which IL-6 has anti-proliferative effects, leads to inhibition in tumor cell proliferation, whereas strong and prolonged activation of Stat3 induces apoptosis. Therefore, for cancer therapy it might be desirable to induce Stat3 activation in these specific cases.

Additionally, we identified the Stat3 target genes that play a role in the observed biological effects. Using oligonucleotide microarray analysis and small interfering (si) RNA targeting, we showed that the transcription factor C/EBPδ, a Stat3 target, is a mediator of Stat3 anti-proliferative effects. Furthermore, using the same techniques, we found a novel Stat3 target, the transcription factor TEL (ETV6), which we identified as a negative regulator of Stat3 activity and Stat3 induced biological functions. The mechanism whereby TEL inhibits Stat3 activity was further investigated. Stat3 inhibition by TEL requires histone deacetylase (HDAC) activity since addition of Trichostatin A (TSA), a general HDAC inhibitor, blocked TEL mediated repression. The pointed domain of TEL was identified as being essential for Stat3 inhibition. Interestingly, the DNA binding domain mutant of TEL was still able to repress Stat3 activity. Moreover, TEL and Stat3 interact since TEL was detected in immunoprecipitates of Stat3.

Taken together our data show that TEL is a newly identified Stat3 target that represses Stat3 transcriptional activity by interacting with Stat3 and recruiting HDACs to the transcriptional complex. Therefore, we suggest that TEL might be part of a novel negative feedback loop in the Stat3 signaling cascade.

II. ACKNOWLEDGMENTS

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III. Abbreviations

AML	Acute myeloid leukemia
APRF	Acute-phase response factor
AR	Androgen receptor
CAMK2	Ca^{2+}/calmodulin-dependent kinase II
CDK	Cyclin Dependent Kinase
C/EBP	CCAAT/enhancer Binding Protein
CBP	CREB Binding Protein
CNTF	Ciliary neurotrophic factor
CRM1	Chromosome Region Maintenance 1
EGFR	Epidermal Growth Factor Receptor
EPO	Erytropoietin
EPOR	Erytropoietin Receptor
ERK	Extracellular Signal-Regulated Kinase
ETS	E-Twenty-SIX
GAS	Gamma interferon activated sequence
Grb2	Growth Factor Binding Protein 2
GRIM-19	Gene associated with retinoid-interferon-induced mortality-19
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
4HT	4-hydroxytamoxifen
IL	Interleukin
IMP	Importin
IFN	Interferon (
IRF	Interferon Regulatory Factor
ISG15	Interferon-stimulated gene 15
JAK	Janus Kinase
JNK	c-Jun N-Terminal Kinase
LMB	Leptomycin B
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
Mcm	Minichromosome Maintenance
MEKK1	MAPK/ERK Kinase Kinase 1
MSK1	Mitogen and stress response kinase-1
NES	Nuclear Export Signal
NLS	Nuclear Localisation Signal
Nmi	N-Myc Interactor
NPC	Nuclear pore complex
O- GlcNAc	O-linked N-acetylglucosamine
PDGFR	Platelet Derived Growth Factor Receptor
PIAS	Protein Inhibitor of Activated STAT
PI3K	Phosphatidylinositol Dependant Kinase
PKCδ	Protein Kinase C δ
PP2A	Protein phosphatase 2A

PRMT-1	Protein Arginine Methyltransferase-1
PTPase	Protein tyrosine phosphatase
PTPεC	PTPepsilonC
RTK	Receptor tyrosine kinase
SCID	Severe combined immunodeficiency
SCID	Severe combined immune deficiency
SH2	Src Homology Domain 2
SHP2	Src homology domain 2 protein tyrosine phosphatase
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
Stat3DN	Dominant negative Stat3
SUMO	Small ubiquitin-related modifier
TAD	Transactivation domain
TC-PTP	T cell protein tyrosine phosphatase
Tip 60	Tat-interactive protein, 60kDa
TPPII	Tripeptidyl peptidase II
TSA	Trichostatin A
Ub	Ubiquitin
Y	Tyrosine

IV. Introduction

STATs in signal transduction

Signal transducers and activators of transcription proteins (STATs) are a family of latent cytoplasmic transcription factors that are activated in response to extracellular stimuli. They have first been discovered in interferon (IFN) regulated gene transcription, specifically Stat 1 and Stat2 (Schindler et al., 1992b). Today seven STAT members have been identified in mammalian cells: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 ranging in size from 750-850 amino acids resulting in proteins of 90 –115 kDa that are abundantly present in many cell types. As a result of series of duplication processes, the STAT family is genetically localized to three chromosomal regions, (Table 1) (Copeland et al., 1995).

Family member	Chromosomal localization	
	Murine	Human
Stat1	1	2q32.3
Stat2	10	12q13.3
Stat3	11	17q21.2
Stat4	1	2q32.2
Stat5a	11	17q21.2
Stat5b	11	17q21.2
Stat6	10	12q13.3

Tabel 1: STAT chromosomal localization. Adapted from (Benekli et al., 2003)

STAT Structure

The analysis of crystallographic studies: of Stat1 and Stat3 core amino acids (residues ~130-712; lacking an N-terminal and C-terminal domain) bound to DNA, of the NH_2 terminus of Stat4 (Fig.1), as well as mutagenesis studies revealed several regulatory

common domains of the STAT proteins (Becker et al., 1998; Chen et al., 1998; Darnell, 1997; Vinkemeier et al., 1998).

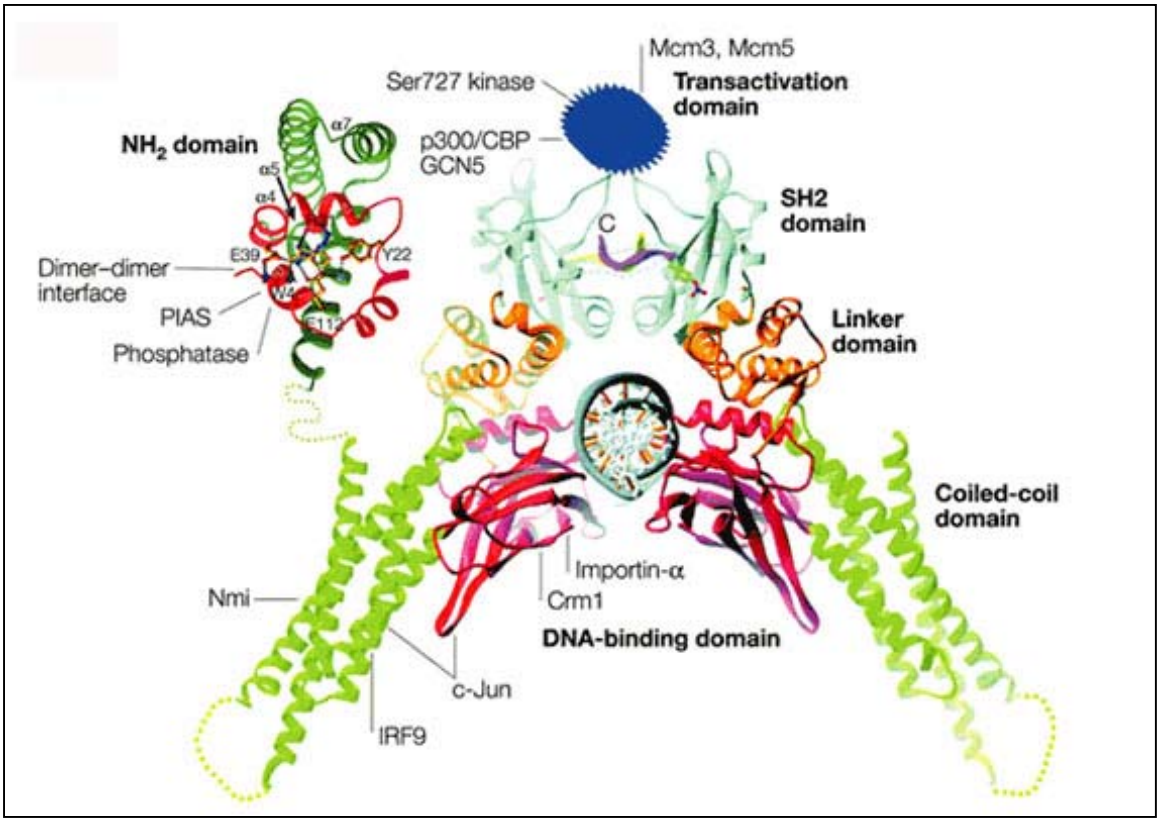

Fig. 1: Three-dimensional structure of STAT 1 proteins. The core structure (amino acids 130–712) shows binding of a STAT1 dimer to DNA and the location of binding sites of various proteins in various domains. The N-terminal structure, the placement of which in the intact structure is undefined, also interacts with various partners, as does the C-terminal transactivation domain, the structure of which is unknown. Adapted from (Levy and Darnell, 2002).

The STAT structure is illustrated in (Fig. 2). The N-terminal region of STAT is important for protein-protein interactions and for dimer-dimer interactions to form tetrameric STAT molecules. Many natural STAT binding sites are in close proximity of each other (~ 20 base pairs apart) and are occupied by tetramers (dimer–dimer pairs) (Darnell, 1997). Deletion of the amino terminus leads to STAT binding to single sites, tetramers are not formed. In addition, it has been shown that tetramer formation is necessary for a strong STAT–DNA interaction at adjacent sites and is important for maximal transcriptional

stimulation (John et al., 1999; Vinkemeier et al., 1996; Xu et al., 1996; Zhang and Darnell, 2001). Contiguous to the oligomerization domain is a four-stranded helical coiled coil domain from residue ~130-320, which provides an extensive surface to interact with other proteins, for example CBP/p300 (Zhang et al., 1996). Additionally it has been shown that the N-terminus is involved in receptor recognition, phosphorylation, nuclear translocation, and dephosphorylation (Murphy et al., 2000; Strehlow and Schindler, 1998).

The DNA binding domain, between residues 320 and 490, contains several .β-sheets and determines DNA sequence specificity of individual STATs. Following the DNA binding domain is a linker domain (residues 490 to 580) with a highly conserved structure but to date unknown function. Mutations within this domain inhibit Stat1 driven transcription after IFN-γ stimulation due to rapid on-off-rates of Stat1 on the DNA, which results in reduced binding times (Yang et al., 2002a; Yang et al., 1999).

The Src- homology 2 (SH2) domain that extends between residues 580 and 680 is a well known common structural motif, which mediates dimerization via SH2-phosphotyrosyl peptide interactions (Shuai et al., 1994). The critical STAT tyrosine residue (.700) that is phosphorylated upon activation and required for dimerization via SH2 is near the SH2 domain. A Transactivation domain (TAD) at the COOH-terminal end of the molecule, 38 to 200 residues in length, depending on the various STAT proteins, is involved in interaction with transcription complexes. The C-terminus contains one more interesting feature, in the case of Stat1, Stat3, Stat4 and Stat5, a serine phosphorylation site, which has been shown to contribute to transcriptional activation and seems to be important for protein-protein interactions (Decker and Kovarik, 2000).

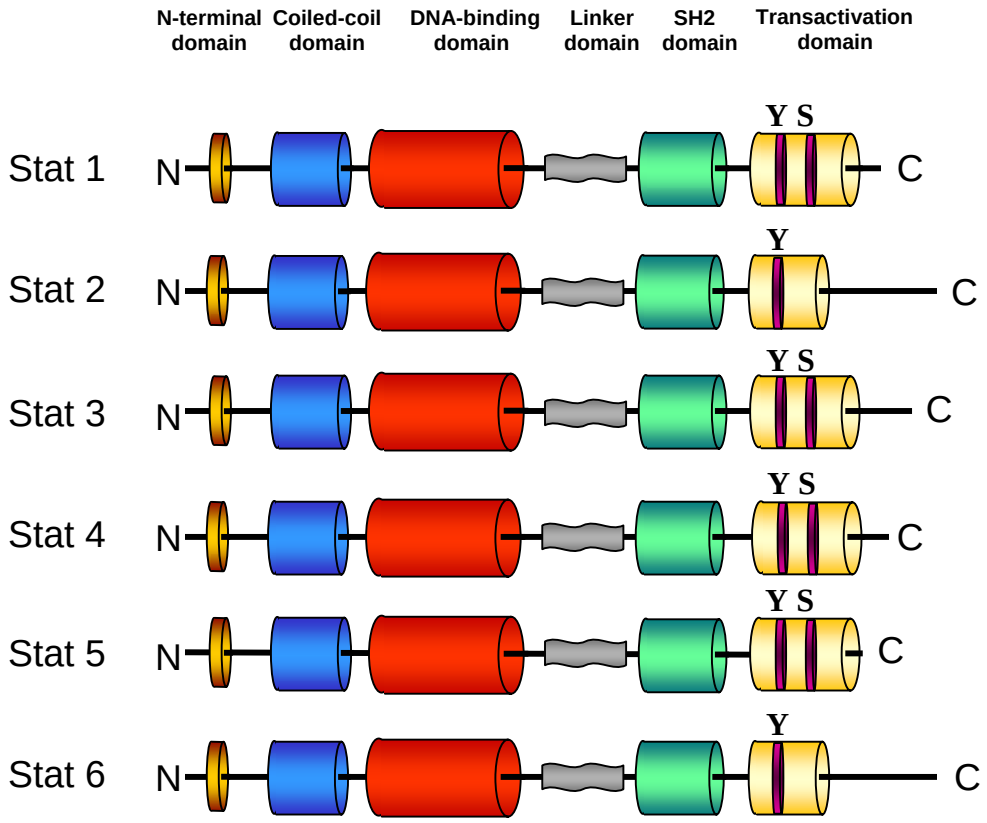

Fig. 2: Domain structures of STAT proteins**.** Adapted from (Bowman et al., 2000)

STAT isoforms

Naturally occurring splice variants of STATs lacking regions of the C-terminal transactivation domain, including the serine residue, have a competitive dominant-negative (DN) effect on gene induction, mediated by the STAT pathway, counteracting the signaling of the full length STAT.

The truncated isoforms still get tyrosine phosphorylated, dimerize and bind DNA where they exert their dominant negative effect by blocking the DNA-binding sites in STAT responsive gene promoter elements. So far splice variants of Stat1, Stat3 and Stat5 have been identified, named Stat1β, Stat3β, and Stat5β, that can be generated by two distinct mechanisms: alternative mRNA splicing and proteolytic processing (Caldenhoven et al., 1996; Moriggl et al., 1996; Sasse et al., 1997; Schaefer et al., 1997; Schindler et al., 1992a; Wang et al., 1996). Splicing joins the coding sequences (exons) by removing the

intervening noncoding sequences (introns) from primary transcripts. Thus, alternative splicing can lead to a vast repertoire of functional diversity by producing multiple RNAs and proteins from a single gene. Compared to wild-type Stat3, Stat3β has seven new amino acids and lacks an internal domain of 50 base pairs from the C terminal of Stat3 (Fig 3). This splice product is a naturally occurring isoform of Stat3 and encodes a 80-kDa protein which also lacks the Ser 727 phosphorylation site (Caldenhoven et al., 1996). In the case of proteolytic processing, Stat5 has been reported to be truncated at the transcriptional activation domain (Azam et al., 1997; Lee et al., 1999). STATβ splice variants function as negative regulators of transcription and are therefore widely used to study the role of STAT proteins.

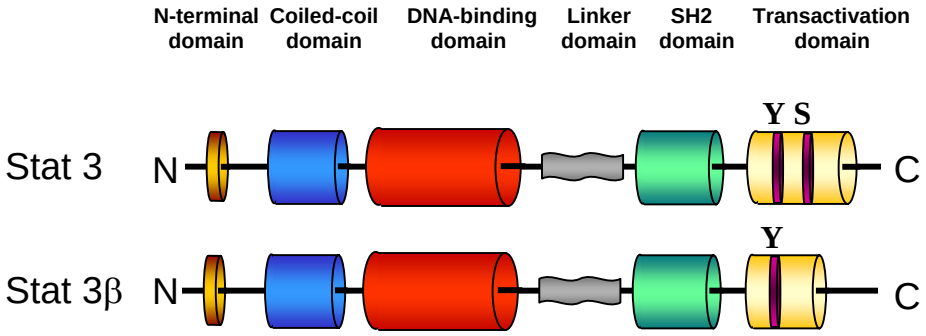

Fig. 3: Domain structures of Stat3 isoforms**.** Adapted from (Bowman et al., 2000)

Mechanisms of STAT activation and regulation

STATs are activated by over 40 different polypeptides binding cytokine receptors, G-protein-coupled receptors, receptor tyrosine kinases like epidermal growth factor receptor and platelet derived growth factor receptor (EGFR and PDGFR) and by numerous non-receptor tyrosine kinases (e.g. Src and Abl) (Fig. 4).

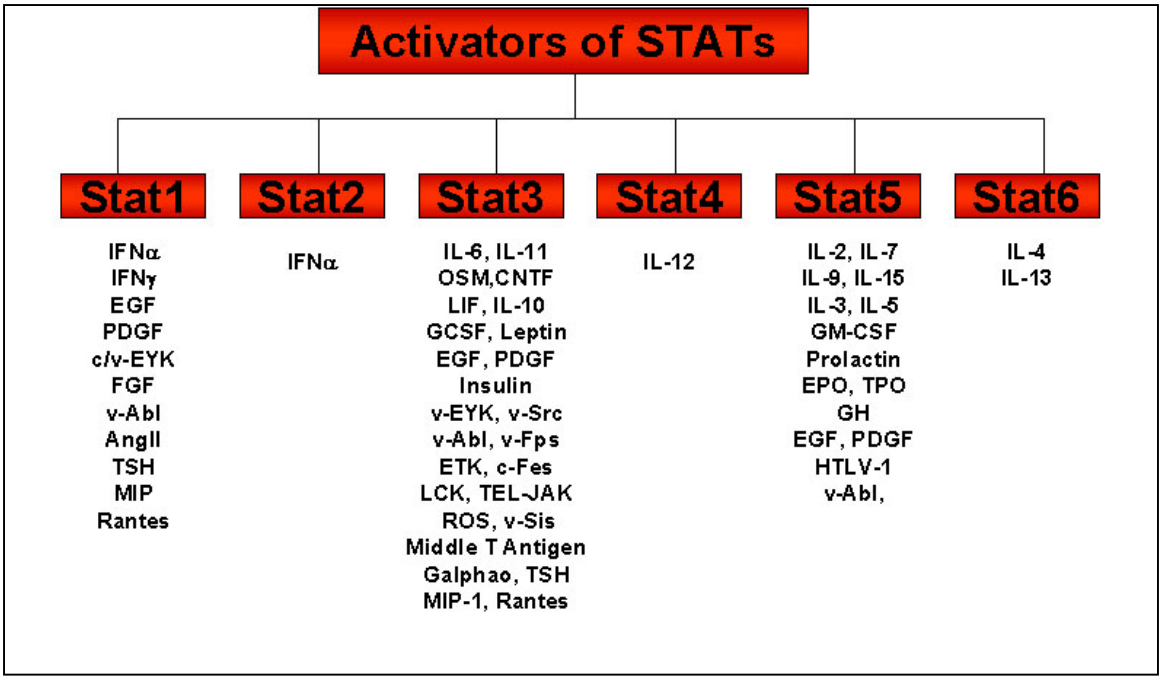

Fig. 4: Activators of STAT proteins**.** Adapted from (Bromberg, 2001)

For cytokine receptors it has been shown that receptor associated JAKs (Janus Kinases) phosphorylate STATs (Greenlund et al., 1995; Madamanchi et al., 2001; Marrero et al., 1995; Park et al., 2000b). In the case of growth factors such as the EGF and PDGF, that bind to receptor tyrosine kinases (RTKs), STAT activation can take place either directly (for example Stat1 activation by PDGF) or via other associated non-receptor tyrosine kinases (e.g. JAK and Src) (Leaman et al., 1996; Olayioye et al., 1999; Vignais et al., 1996; Wang et al., 2000b) (Fig. 5). In addition, non-receptor tyrosine kinases such as activated Src and ABL can directly phosphorylate STAT proteins in the absence of ligand induced receptor activation (Fig. 5) (Cao et al., 1996; Danial and Rothman, 2000; Yu et al., 1995). Phosphorylation of STATs results in homodimerization or heterodimerization through reciprocal interaction between SH2 domains and phosphotyrosines. Stat1, Stat3, Stat4, Stat5a and Stat5b form homodimers. Stat1 and Stat2 and Stat1 and Stat3 can also form heterodimers. As a result, STAT dimers translocate to the nucleus where they bind to specific STAT DNA-binding elements, originally termed the GAS (gamma interferon activated sequence) element ($TTN_{5-6}AA$), in the promoter of target genes and activate transcription (Bromberg et al., 1999; Levy and Darnell, 2002; Seidel et al., 1995).

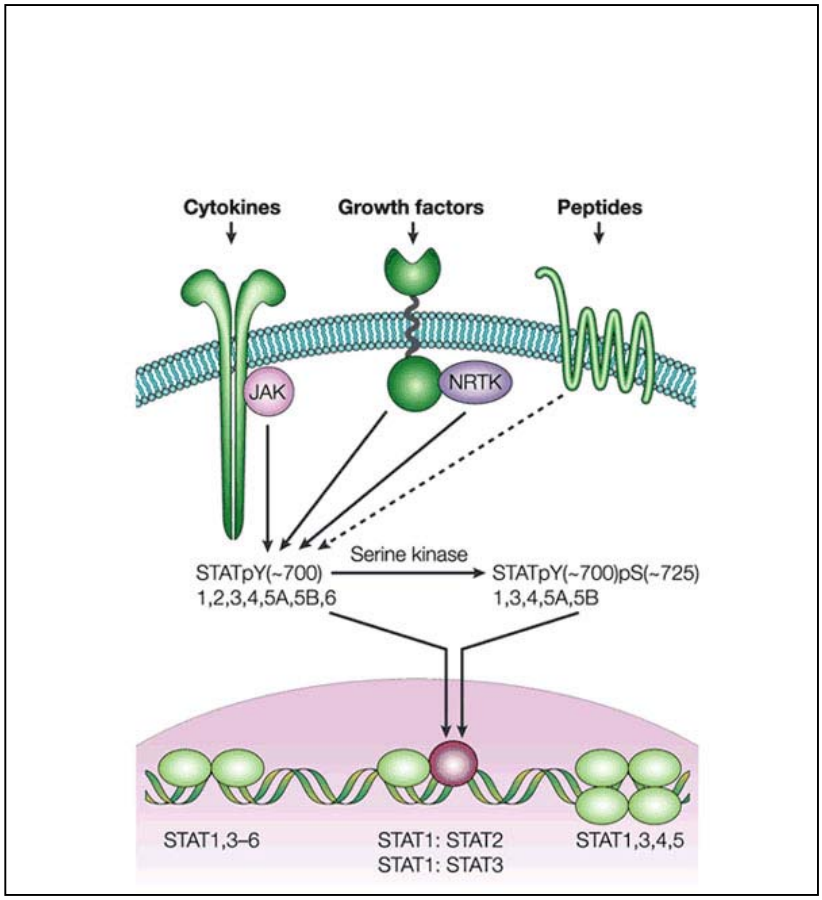

Fig. 5: Overview of STAT signaling. STATpY, tyrosine-phosphorylated STAT; pS, serine-phosporylated. Adapted from (Levy and Darnell, 2002)

Perhaps the best-studied pathway for STAT activation is through the JAKs otherwise knows as the JAK-STAT pathway (Fig. 6). Binding of IL-6-type cytokines to their specific receptors leads to hetero-oligomerization with the common signal transducer gp130, resulting in phosphorylation and activation of the receptor associated Janus kinases (Jaks). Subsequently five tyrosines in the cytoplasmic tail of gp130 get phosphorylated. The phosphorylated tyrosines serve then as docking sites for two major signal-transducing molecules; SHP2 (Src homology domain 2 protein tyrosine phosphatase) binds at Tyr759 and interacts with adaptor molecules such Gab 1/2, p85 and Grb2 mediating the activation of intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol-3 phosphate (PI3K)

pathways (Qu, 2002). Binding of Stat3 at Tyr767, 814, 905, and 915 of gp130 (Heinrich et al., 1998; Schmitz et al., 2000a) is followed by JAK-induced phosphorylation of STATs.

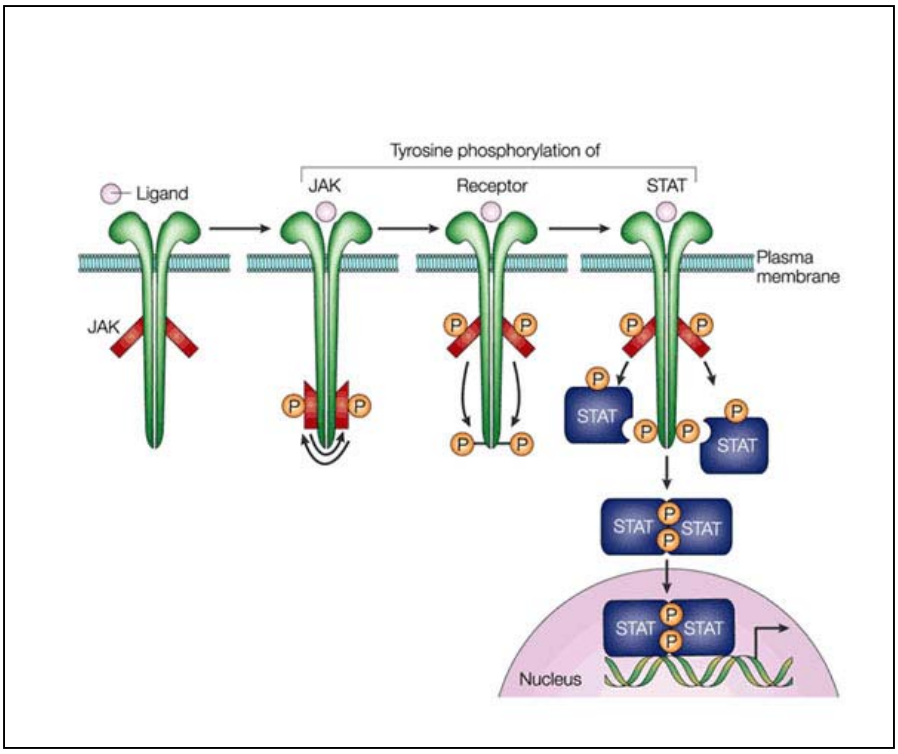

Fig. 6: JAK–STAT pathway. Adapted from (Levy and Darnell, 2002)

JAK family kinases

There are four mammalian members of the JAK family of tyrosine kinases: JAK1, JAK2, JAK3, and Tyk2 known. They range in size from 120 to 130 kDa and expression studies indicate that JAK1, JAK2 and TYK2 are ubiquitously expressed. JAK3 is expressed predominantly in cells of hematopoietic origin (Leonard and O'Shea, 1998). The chromosomal location in humans has been mapped to 1p13.3 for JAK1, 10q23-q24 for JAK2, 19p13.2 for TYK2 and 19p13.1 for JAK3 (Firmbach-Kraft et al., 1990; Ihle et al., 1995; Kumar et al., 1996; Pritchard et al., 1992; Riedy et al., 1996). JAKs consist of seven conserved JAK homology (JH) domains (Fig. 7). The most interesting feature of the JAK proteins is the presence of two domains in the C-terminal portion of these molecules including a distinctive pseudokinase domain (JH2) and a tyrosine kinase domain (JH1), although only the JH1 domain appears to be functional. The JH2 domain, which shows considerable homology to the tyrosine kinase domain, lacks certain critical amino acids required for a functional kinase. However, an important regulatory function in regards to kinase activity was attributed to the JH2 domain (Luo et al., 1997; Saharinen et al., 2000). Additionally Fujitani *et al* (1997) described the JH2 domain as an interaction site with Stat5 providing a different way of activation of Stat5 (Fujitani et al., 1997). Interestingly, SH2 or SH3 domains are missing in JAKs, it has been shown that the JH3 domain shares homology with SH2 domains, however, binding of phosphotyrosine to this domain has not been reported. The amino-terminal JAK homology domains, JH7–JH3, follow a non-conserved amino terminus of ~ 30-50 amino acids, constituting a FERM (four-point-one, ezrin, radixin, moesin) domain which mediates association with receptors.

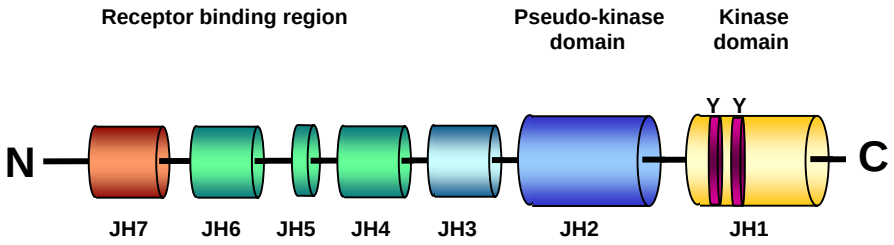

Fig. 7: Domain structure of JAK. JH: JAK homology

JAK kinases are essential for the action of type I and type II interferons (IFN), while they are also activated by many other cytokines (Table 2) (Imada and Leonard, 2000).

Cytokines	JAK-kinase
IFNs	
IFNα/β, IL-10	JAK1, TYK2
IFNγ	JAK1, JAK2
Cytokines whose receptors share γc	
IL-2, IL-4, IL-7, IL-9, IL-15, IL-21	JAK1, JAK3, JAK2, TYK2
Cytokines whose receptors share βc	
IL-3, Il-5, GM-CSF	JAK2
Cytokines whose receptors share gp130	
IL-6, IL-11, OSM, CNTF, LIF, CT-1	JAK1, JAK2, TYK2
IL-12	JAK2, TYK2
Cytokines whose receptors are homodimers	
Growth hormone, Prolactin, EPO, TPO	JAK2

Table 2: Activation of JAKs by cytokines. Adapted from (Imada and Leonard, 2000)

Although specific JAKs are activated through each cytokine receptor and may partially contribute to specificity, the JAK kinases by themselves are not an absolute determinant of the specificity in cytokine signaling, since many different cytokines activate the same JAKs. Nevertheless, JAK gene targeting studies have identified characteristic signaling defects (Table 3). These studies indicate that *JAK1*$^{-/-}$ mice exhibit a perinatal lethal phenotype but lack other abnormalities. They are small at birth and fail to nurse, lymphopoiesis but not myelopoiesis is severely impaired. They fail to respond to cytokines that bind three distinct families of cytokine receptors, including IFNs (type II cytokines), γc-dependent cytokines, and gp130 dependent cytokines (Rodig et al., 1998). The JAK2 knockout mice exhibit embryonic lethality (i.e., day 12.5), due to the absence of definitive erythropoiesis; additional immunological impairments caused by impaired cytokine signaling are observed (Neubauer et al., 1998; Parganas et al., 1998). Tyk2

knockout mice exhibit increased pathogen susceptibility caused by impaired responses to interferon and IL-12 (Karaghiosoff et al., 2000; Shimoda et al., 2000). The most relevant knockout for human disease is that of *JAK3*, whose product exhibits a relatively exclusive association with the γcommon receptor chain. Mutations in the common cytokine receptor chain, which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, cause severe combined immunodeficiency (SCID) in humans (Leonard, 1996; Noguchi et al., 1993). Likewise, *JAK3*$^{-/-}$ mice demonstrate profound reduction in thymocytes, B cells and T cells similar to (SCID); due to failure of cytokine signaling from γc-containing receptors (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995). Indeed mutations in JAK3 have been found to lead to severe combined immunodeficiency disease (SCID) in some patients (Macchi et al., 1995; Russell et al., 1995).

Aberrant JAK kinase activity has also been implicated in various human malignancies, such as leukemia (Verma et al., 2003). For example, chromosomal translocation of the short arm of chromosome 9, containing the kinase domain of JAK2, to the short arm of chromosome 12, containing the pointed domain of TEL, results in a fusion protein TEL-JAK2 with an overactive kinase. TEL-JAK2 fusion proteins have been found in lymphoid and myeloid leukemia (Lacronique et al., 1997). Ectopical overexpression of TEL-JAK2 in hematopoietic cell lines leads to constitutive activation of Stat1, Stat3 and Stat5 and induces growth factor independent proliferation (Schwaller et al., 1998).

Gene	Phenotype of null mice
JAK1	Viable but perinatal lethality due to neurological defects, SCID
JAK2	Embryonic lethality, due to failure in erythropoiesis, impaired cytokine signaling
JAK3	Viable and fertile, SCID
TYK2	Viable and fertile, impaired IFN and IL-12 response, increased pathogen susceptibility

Table 3: Phenotype of JAK knockout mice. Adapted from (O'Shea et al., 2002)

Posttranslational Modifications of STAT proteins

In addition to tyrosine phosphorylation, STATs are also subject to other posttranslational modifications such as serine phosphorylation, methylation, acetylation, ISGylation, ubiquitylation, sumoylation and glycosylation (Table 4).

Post-translational modifications of STATs	Biological function
tyrosine phosphorylation	Activation of STATs
serine phosphorylation	Enhances activation of STATs
acetylation	Activation of Stat6
ISGylation	Positive feedback loop of Stat1
arginine methylation	Increased DNA binding of Stat1
glycosylation	Activation of Stat5
ubiquitylation	Degradation of STATs
sumoylation	No clear function

Table 4: Post-translational modifications of STATs

Serine phosphorylation has been described for Stat1 and Stat3 at serine 727, Stat4 at serine 721, Stat5a at serine 725, Stat5b at serine 730 and for Stat6 at unknown serines in the region between residues 719-789 (Beuvink et al., 2000; Pesu et al., 2000; Visconti et al., 2000; Wen et al., 1995; Wick and Berton, 2000; Yamashita et al., 1998). Different kinases have been reported to participate in serine phosphorylation of STATs contingent on the cell type and the cytokine/growth factor studied (Table 5).

Kinase	Reference:
Ca^{2+}/calmodulin-dependent kinase II (CAMK2)	(Nair et al., 2002)
c-Jun N-terminal kinase (JNK)	(Decker and Kovarik, 2000)
extracellular signal-regulated kinase (ERK)	(Chung et al., 1997b)
MAPK/ERK kinase kinase 1 (MEKK1)	(Lim and Cao, 2001)
mitogen and stress response kinase-1 (MSK1)	(Wierenga et al., 2003)
p38 MAPK	(Uddin et al., 2002; Xu et al., 2003)
Pak1	(Wang et al., 2003)
PI3K	(Fung et al., 2003)
protein kinase C δ (PKCδ)	(Jain et al., 1999; Schuringa et al., 2001; Schuringa et al., 2000; Uddin et al., 2002)

Table 5: Potential serine/threonine kinases of STATs.

Serine phosphorylation enhances transcriptional activity of Stat1, Stat3 and Stat4 (Decker and Kovarik, 2000; Visconti et al., 2000; Wen et al., 1995). More recent data shows on the other hand that serine phosphorylation enhances the ability of Stat1 to drive expression of some, but not all target genes (Kovarik et al., 2001). There seems to be no consequence of serine phosphorylation on nuclear translocation of Stat1/Stat3 and Stat5a/Stat5b (Decker and Kovarik, 2000; Yamashita et al., 1998). However, serine phosphorylation might be important for the duration of stimulation, since a Stat5a serine 725 mutant displayed sustained DNA binding activity compared with DNA-binding of wild type Stat5a (Beuvink et al., 2000).

Methylation of arginine 31 in the N-terminal domain, which is conserved in STATs, by protein arginine methyltransferase-1 (PRMT-1), has been reported in the case of Stat1. This methylation is independent of tyrosine or serine phosphorylation and does not

require cytokine stimulation. Interestingly, methylation leads to a weaker interaction of Stat1 with protein inhibitor of activated STATs (PIAS) 1, resulting in increased DNA-binding of Stat1 (Mowen et al., 2001). PRMT has been associated with other elements of the JAK/STAT pathway, as PRMT-1 interacts with the IFNα/β receptor 1 and PRMT-5 interacts with JAKs (Abramovich et al., 1997; Pollack et al., 1999).

Acetylation of Stat6 by histone acetyltransferase CREB-binding protein (CBP)/p300 is required for transcriptional activation of the 15-lipoxygenase-1 (15-LOX-1) by IL-4 (McDonald and Reich, 1999; Shankaranarayanan et al., 2001).

Recent studies have demonstrated a positive regulatory role for ISGylation in the JAK/STAT signaling pathway. ISGylation of JAK1 and Stat1 is achieved by interferon-stimulated gene 15 (ISG15) a ubiquitin-like protein that conjugates to numerous proteins in cells treated with IFNα or lipopolysaccharide (LPS). (Malakhov et al., 2003; Malakhova et al., 2003).

Interestingly, new results show the essential function of O-linked N-acetylglucosamine (O- GlcNAc) on threonine 92, as a posttranslational modification, which seems to be important for the transcriptional activity of Stat5. O-linked glycosylation has only been seen on nuclear Stat5 following cytokine stimulation, however, glycosylation seemed not to be a requirement for nuclear translocation of Stat5. Mutational analysis demonstrated that Stat5, which cannot be glycosylated was not able to activate a reporter gene construct. The authors concluded this failure to the impaired interaction of un-glycosylated Stat5 with CBP. Since glycosylation was also observed on other STATs, it seems likely that this posttranslational modification might be essential for interaction with co-activators following cytokine stimulation (Gewinner et al., 2003). Ubiquitylation and sumoylation will be described in the section “Negative Regulators of STATs”.

Nuclear Import and Export of STAT proteins

STAT proteins translocate to the nucleus upon Tyr phosphorylation and dimerization and translocate back into the cytoplasm after dephosphorylation in the nucleus. This processes are widely studied by various groups and dependent on active transport through the nuclear pore complex (NPCs), since STAT dimers are too large (> 50kD) for passive diffusion through the NPC. Expression of a nuclear localization signal (NLS) is essential for active transport of molecules through the nuclear pore. NLS signals are usually either single stretches or double stretches of residues, which have a high content of basic arginine and lysine amino acids, spaced by around 10 nonconserved residues (Dingwall and Laskey, 1991). Members of the importin α (impα) family, which includes 6 family members and each member features 8 to 10 Armadillo (Arm) repeats, bind to NLS signals in proteins that are targeted for nuclear transport. Importin β then binds to impα and carries impα and its cargo protein, via binding to nucleoporins in the NPC, through the NPC into the nucleus where it associates with Ran-GTP, which leads to the release of impα and its load. Nuclear export, similar to the nuclear import, requires also the presence of a special signal, a hydrophobic nuclear export signal (NES), as well as soluble carriers named exportins. There are specific exportins such as CAS, which is important for export of impαs, while there are also more general exportins like CRM1 (chromosome region maintenance 1). CRM1 identifies NESs, which consists of a short sequence with hydrophobic amino acids rich in leucine, and binds the NES carrying cargo together with Ran-GTP to form a stable ternary complex. The complex travels through the NPC and dissociates in the cytoplasm after the hydrolysis of Ran-GTP. Leptomycin B (LMB), an antibiotic, binds irreversibly to CRM1, blocks its ability to bind to the NES and therefore inhibits its activity (Fig.8) (McBride and Reich, 2003)

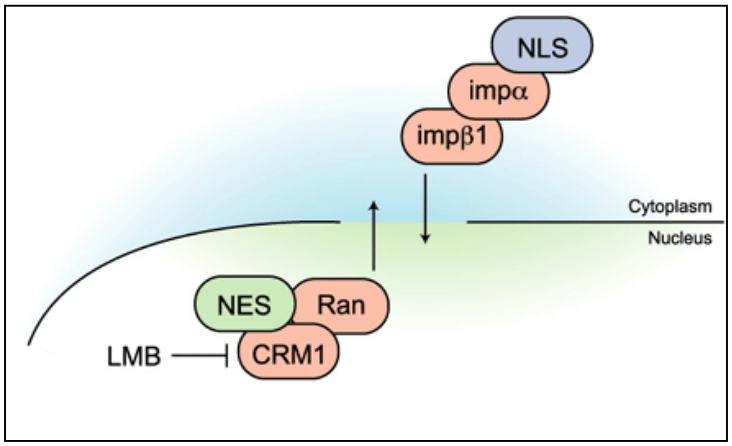

Fig. 8: Depiction of nuclear trafficking. Adapted from (McBride and Reich, 2003).

Recent studies indicate a NLS signal, including leucine 407 and lysines 410 and 413 within the DNA binding domain of Stat1 and interaction of impα5 with tyrosine-phosphorylated Stat1 (Fagerlund et al., 2002; McBride et al., 2002; Melen et al., 2001). Stat1 nuclear export is achieved via a NES, residues 399-410, within the DNA binding domain, and CRM1 binding to this area (McBride et al., 2000). A second NES domain, a leucine-rich helical segment, has been described in the N terminus of Stat1 (Begitt et al., 2000). However, CRM 1 binding to this region has not been reported yet, which leads to the assumption that this region participates in a different manner in nuclear export. Interestingly the NES of Stat1 seems to be hidden, when Stat1 is bound to the DNA and it is therefore essential to dislodge Stat1 from the DNA for tyrosine dephosphorylation, which leads to the accessibility of the NES by CRM1 and the nuclear export of Stat1 (Meyer et al., 2003). Potential protein tyrosine phosphatases will be described later under "Protein tyrosine phosphatases (PTPases)".

Bild et all. showed that Stat3 is associated with endocytotic vesicles during the transport through the cytosol arguing that receptor-mediated endocytosis is a requirement for Stat3 shuttling from the plasma membrane to the perinuclear region (Bild et al., 2002). Nevertheless, several points speak against this observation since constructs such as Stat3ER and Stat3-C have been shown to translocate to the nucleus without receptor activation (Bromberg et al., 1999; Milocco et al., 1999) in addition, two NLS have been reported recently for Stat3. One in the in the alpha-helix 2 region of the coiled-coil

domain, where arginines 214-215 have been shown to include a novel Stat3 NLS, which can be stimulated by epidermal growth factor as well as by interleukin-6. Of great surprise is that truncation mutants containing only the N-terminal and coiled-coil domain of Stat3 are localized to the nucleus without ligand stimulation (Ma et al., 2003). In addition to the area in the coiled-coil domain a NLS has been identified in the DNA binding domain, arginines 414-417, which correspond to lysines 410-413 of Stat1, a known Stat1 NLS (Fagerlund et al., 2002; Ma et al., 2003). On the other hand, leucine 411 of Stat3, corresponding to leucine 407 of Stat1, a necessary residue for Stat1 nuclear transport, is not essential for Stat3 nuclear import (Ma et al., 2003).

Nuclear export of Stat3 is achieved via three nuclear export signal (NES) elements. Two of these elements, 306-318 and 404-414, corresponde to those recently identified in Stat1 399-410, and a third, Stat3 524-535, is novel (Bhattacharya and Schindler, 2003; McBride et al., 2000). It is also possible to block nuclear export of Stat3 by leptomycin B as it is the case for Stat1 (Bhattacharya and Schindler, 2003; Nakayama et al., 2002). In contrast to Stat1 LMB treatment not only blocks the export of Stat3 from the nucleus back to the cytoplasm after activation, but also promotes nuclear accumulation of Stat3 in resting cells, leading to the assumption that there might be non tyrosine phosphorylated monomer Stat3 in the nucleus.

Similar to Stat3 Stat5b demonstrates monomer shuttling between the nucleus and the cytoplasm, since LMB treatment in the absence of cytokine, led to the accumulation of Stat5B in the nucleus (Zeng et al., 2002). The authors further support this concept with the observation that a mutated Stat5b, which has the Tyr 699 important for phosphorylation and dimerization mutated, also accumulates in the nucleus after LMB treatment (Zeng et al., 2002). Thus Stat3 and Stat5b appear to have constitutive NLS and NES function that promotes shuttling of monomeric STATs. This might happen in a different way than the shuttling of tyrosine phosphorylated dimerized STATs. Growth hormone-induced nuclear import of Stat5b seems to rely on a NLS in the DNA-binding domain, 466-469, (Herrington et al., 1999). Furthermore, deletion of the region up to residue 104 inhibit translocation of factor stimulated Stat5b, whereas region between residues 138 and 165 is essential for cytokine independent import of Stat5b. A putative

NES is located in the region between residues 578-723 of Stat5b, this export is LMB sensitive (Zeng et al., 2002). Given that Stat2 is not able to form homodimers and the fact that Stat2 is constitutively associated with interferon regulatory factor-9 (IRF-9), it was shown that Stat2 localizes to the nucleus as part of a Stat1-Stat2-IRF-9 complex (Lau et al., 2000; Martinez-Moczygemba et al., 1997; McBride et al., 2002). So far there are no reports about NLS or NES in Stat4 and Stat6. Nevertheless, it is of interest that STAT molecules that dimerize without tyrosine phosphorylation are able to translocate to the nucleus, as it is shown for Stat1, Stat3, Stat5 and Stat6 (Bromberg et al., 1999; Milocco et al., 1999). This suggests that dimerization alone is sufficient to unmask a latent STAT nuclear localization sequence and induce nuclear translocation, sequence-specific DNA binding, and transcriptional activity.

Negative Regulators of STATs

The transient nature of STAT activation suggests that nature has also invented sophisticated mechanisms to turn off the signaling pathway. In fact, recent studies show that STAT signaling is negatively regulated at different points in the signaling cascade including by dephosphorylation and endocytosis of the receptors, dephosphorylation of the JAKs and STATs (Fig. 9a, 9c), proteolytic degradation of: the receptors, the STAT proteins or the JAKs and via two different protein families; the suppressors of cytokine signaling family (SOCS) and proteins that inhibit activated STAT proteins family (PIAS) (Fig. 9b, 9d). In addition, inhibition is achieved by splice variants (see section “STAT isoforms”) (Fig. 9e). The different mechanisms of inhibition will be explained in the following sections.

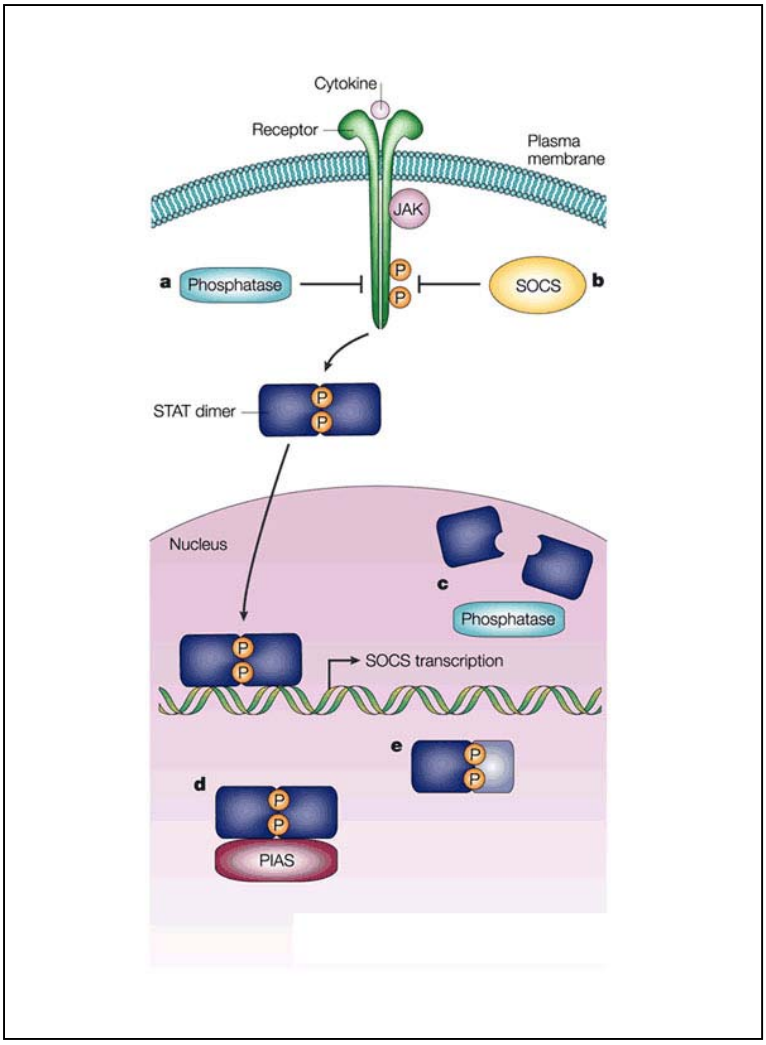

Fig. 9: Negative regulators of STAT molecules (Levy and Darnell, 2002).

Receptor mediated endocytosis

The function of the plasma membrane is to separate the cytoplasm of a cell from the extracellular environment. The coordination of the exit or entry of small and large molecules via regulating mechanisms plays an important role. Small molecules for example amino acids, sugars and ions, can enter the cells via the aid of integral membrane protein pumps or channels. Endocytosis is a term for a process where macromolecules cross the plasma membrane in endocytic vesicles. A small portion of the plasma membrane, that encloses the macromolecule and then invaginates and pinches-off forms the vesicles. Two main types of endocytosis occur, phagocytosis ("cellular

eating"), which involves the ingestion of large particles, such as microorganisms or dead cells and pinocytosis ("cellular drinking"), the uptake of fluid and solutes. Phagocytosis is typically restricted to specialized mammalian cells, whereas pinocytosis occurs in all cells by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Fig. 10) (Conner and Schmid, 2003).

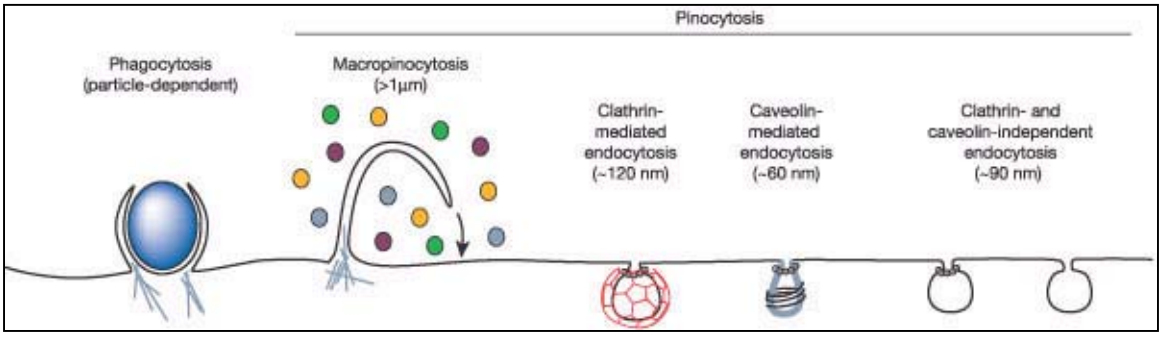

Fig. 10: Multiple portals of entry into the mammalian cell. Adapted from (Conner and Schmid, 2003)

An important function of endocytosis in cell signaling is to downregulate signal responses by internalizing receptors either constitutively or upon ligand binding. This provides an excellent mechanism to inhibit many signaling pathways by constitutively internalizing a pool of receptors from the plasma membrane and via switching off signaling after ligand binding. Signal transduction by the receptor would otherwise occur indefinitely once it had been initiated. Recruitment of clathrin to internalization signals within cytosolic tails of receptors at the plasma membrane leads to invagination of the receptors. Evidence of receptor mediated endocytosis as a negative regulation of STAT signaling originate from studies that indicated that the IL-6R is downregulated by its ligand (Zohlnhofer et al., 1992). Recent data demonstrate that the common signal transducer gp130 carries a di-leucine internalization motif within the intracellular domain (Dittrich et al., 1994). In addition, mutation of serine 139 to an alanine of gp130 reduces the internalization rate by 50 % suggesting that serine phosphorylation upon IL-6

stimulation leads to a conformation change and exposure of the internalization signal to the endocytotic machinery (Dittrich et al., 1996).

Targeted Degradation

Ubiquitylation, the conjugation of proteins with a protein called ubiquitin (Ub), is essential for the degradation of proteins whose levels have to be regulated either constitutively, or in response to extracellular stimuli and changes in the cellular environment. Ubiquitylation is a multistep process involving at least three types of enzymes (Fig. 11).

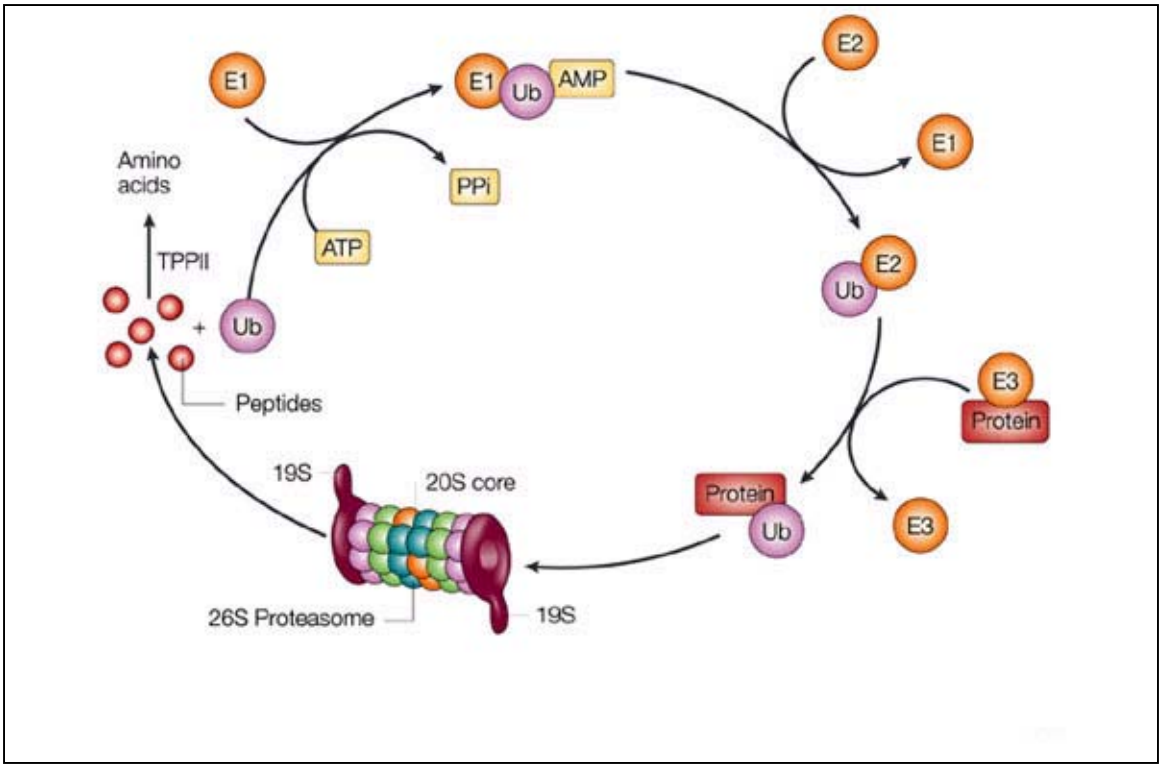

Fig. 11: The ubiquitin pathway. Adapted from (Tisdale, 2002).

Free ubiquitin is activated in an ATP-dependent manner, by the activity of an ubiquitin-activation enzyme (E1), leading to the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin (glycine 76). Subsequently, the ubiquitin group is transferred to one of many distinct ubiquitin-conjugating enzymes (E2). Finally, a

ubiquitin protein ligase (E3) catalyses the transfer of Ub from the E2 enzyme to the target protein. Specificity arises from the different enzymes that are involved in mediating ubiquitylation as well as the types of the ubiquitin complex that are formed, multiubiquitylation serves mainly to label the substrate for degradation whereas monoubiquitylation regulates numerous processes, for instance endocytosis, DNA repair and transcriptional regulation (Aguilar and Wendland, 2003). Degradation of ubiquitylated substrates is performed by the 26S proteasome, which is composed of the two terminal 19S regulatory subcomplexes bound to the proteolytic 20S core. Substrates destined for degradation are labeled with polyubiquitin (Ub_n), which is recognized by the 19S complex. Short oligopeptides are released from the 26S proteasome after degradation and are then further degraded into tripeptides by the protease tripeptidyl peptidase II (TPPII). Ubiquitin-proteosomal degradation is important for downregulation of the JAK/STAT signaling pathways. It was shown with the use of proteasome inhibitors that JAK1, JAK2 and JAK3 are targets for degradation (Ungureanu et al., 2002; Yu and Burakoff, 1997) as well as tyrosine phosphorylated Stat1 Stat4, Stat5 and Stat6 with only marginal effects on Stat2 and Stat3 (Kim and Maniatis, 1996; Ulane et al., 2003; Wang et al., 2000a). In addition, a C-terminal domain in Stat5 promotes degradation, since deletion of this domain resulted in a protein that was more stable compared to the wildtype protein (Wang et al., 2000a).

Protein tyrosine phosphatases (PTPases)

Dephosphorylation is an important way of inhibition of the STAT signaling pathway and can occur in the cell cytoplasm, where the receptor, the JAKs and the STATs are targets, as well as in the nucleus where dephosphorylation of the STATs takes place. Today several PTPs have been identified that negatively regulate the JAK/STAT pathway (Table 6).

The first cytoplasmic tyrosine phosphatases found to be involved in inhibiting JAK activity were the closely related SH2 containing phosphatases (SHP), SHP1 and SHP2 (David et al., 1995; Jiao et al., 1996; Klingmuller et al., 1995; Symes et al., 1997; Wu et al., 2002; You et al., 1999). SHP1 is mainly expressed in hematopoietic tissues, whereas

SHP2 is ubiquitously expressed. All tyrosine-phosphorylated signaling molecules such as receptors, JAKs or STATs have to be considered as possible targets for SHP2. However, direct interaction has only been shown with SHP1, JAK1 and JAK2 (Jiao et al., 1996) and for SHP2 with JAK1 and Stat5 (Chen et al., 2003; Chughtai et al., 2002; Yin et al., 1997). More phosphatases may play a role in cytoplasmic deactivation of the STATs but they have not been found yet. The transmembrane PTPase CD45 is highly expressed in all haematopoietic lineages and binds and dephosphorylates JAKs (Irie-Sasaki et al., 2001). Other PTPases, expressed in haematopoietic cells, are PTPepsilonC (PTPεC) and the T cell protein tyrosine phosphatase (TC-PTP). The inhibitory effect of PTPεC is selective for IL-6- and IL-10-induced JAK-STAT signaling (Tanuma et al., 2000; Tanuma et al., 2001). JAK1 and JAK3 are physiological substrates of TC-PTP and phosphorylation of Stat5 following interleukin (IL)-2 stimulation is abrogated in T cells that overexpress TC-PTP (Ibarra-Sanchez et al., 2000; Simoncic et al., 2002). A cytosolic phosphatase PTP1B has been shown to dephosphorylate JAK2 and TYK2, but not JAK1, in response to interferon stimulation. Additionally, PTP1B dephosphorylates prolactin (PRL) activated Stat5a and Stat5b, thereby negatively regulating the PRL-mediated signaling pathway. (Aoki and Matsuda, 2000; Myers et al., 2001). The PRL-mediated signaling pathway can also be inhibited by PTP-PEST, since it was shown that PRL activation of JAK2 was dramatically reduced in HC11 cells pretreated with EGF, and that PTP-PEST, which is constitutively associated with JAK2, can be upregulated in response to EGF treatment (Horsch et al., 2001). Interestingly, the serine/threonine protein phosphatase 2A (PP2A) is associated with Jak2 and Stat5 in an IL-3-dependent manner. PP2A is tyrosine phosphorylated by Jak2, resulting in inhibition of phosphatase activity. Interaction of PP2A and Stat5 tends to result in decreased tyrosine phosphorylation and decreased nuclear translocation of Stat5 (Yokoyama et al., 2001).

As discussed earlier nuclear export of STAT proteins is dependent on tyrosine dephosphorylation, which leads to the accessibility of the NES by CRM1 and the nuclear export of STAT proteins. Potential nuclear phosphatases for STAT dephosphorylation are SHP2 for Stat1 (Wu et al., 2002) and the nuclear isoform of TC-PTP (TC45) for Stat1 and Stat3. Embryonic fibroblasts (MEFs) that lack TC45 fail to dephosphorylate IFN

induced tyrosine-phosphorylated Stat1 and dephosphorylation of Stat3, but not Stat5 or Stat6, is also affected (Haspel and Darnell, 1999; ten Hoeve et al., 2002) In the contrary overexpression of TC45 results in dephosphorylation of Stat3, Stat5a and Stat5b (Aoki and Matsuda, 2002; Yamamoto et al., 2002). The identification of further PTPs that are involved in dephosphorylating specific STATs in the nucleus will be of great interest.

cytoplasmic tyrosine phosphatases	Targets
SHP1	JAK1 and JAK2
SHP2	JAK1 and Stat5
CD45	All JAKs
PTPεC	JAK1 and TYK2
TC-PTP	JAK1, JAK3
PTP1B	JAK2, TYK2 and Stat5
PTP-PEST	JAK2
nuclear tyrosine phosphatases	**Targets**
SHP2	Stat1
TC45	Stat1, Stat3 and Stat5

Table 6: Cytoplasmic and nuclear tyrosine phosphatases of the JAK/STAT pathway

The SOCS family of inhibitors

The suppressors of cytokine signaling (SOCS) are a family of intracellular proteins that play a crucial role in negatively regulating the response of the immune system to cytokines. These inhibitors have been variously named by independent discoverers as suppressor of cytokine signalling (SOCS), JAK-binding proteins (JABs), STAT-induced STAT inhibitors (SSIs) or cytokine-induced SH2 (CIS) proteins. The eight proteins that

belong to the SOCS family include cytokine-inducible SH2 domain protein (CIS) and SOCS1-SOCS7. SOCS proteins contain a central SH2 domain, an amino-terminal domain of variable length and a divergent sequence that carries in the case of SOCS1 and SOCS3, a kinase inhibitory region and a carboxy-terminal 40 amino-acid module known as the SOCS box (Fig. 12) (Krebs and Hilton, 2000). The SOCS box can bind to elongins B and C, which are known components of a ubiquitin E3 ligase complex suggesting that SOCS proteins may target signaling molecules like JAK2, to the proteasome for degradation (Kamura et al., 1998; Ungureanu et al., 2002; Zhang et al., 1999a). Interestingly, Kamizone et al. demonstrated that the SOCS box of SOCS1 interactes with Cullin-2 and promotes ubiquitylation of TEL-JAK2 (Kamizono et al., 2001).

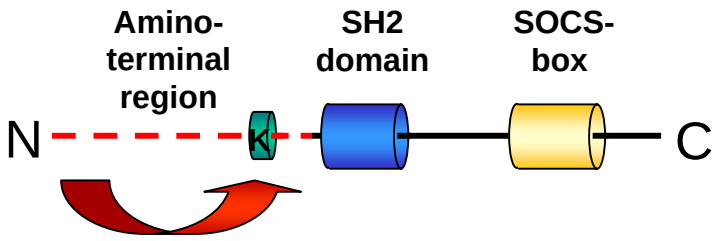

Fig. 12: Domain structure of SOCS. Red arrow: variable N-terminal domain; K: kinase inhibitory region. Adapted from (Krebs and Hilton, 2000).

SOCS proteins are generally expressed at low levels in unstimulated cells and expression is largely increased upon cytokine, insulin and EGF stimulation (Alexander, 2002; Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). The fact that they subsequently inhibit JAK/STAT signaling leads to the conclusion that they act as classical feedback loop inhibitors (Matsumoto et al., 1997; Yoshimura et al., 1995). Posttranslational modifications of SOCS3 have also been shown after stimulation with cytokines and growth factors. SOCS3 becomes Tyr phosphorylated at Y204 and Y221, in the conserved SOCS box, by JAKS and other receptor tyrosine kinases (Cohney et al., 1999). Haan et al. showed that phosphorylation of SOCS3 at Y204 and Y221, inhibits the SOCS3-elongin C interaction and activates proteasome-mediated SOCS3 degradation. Mutation of these two tyrosines lead to stabilization of SOCS3 (Haan et al., 2003). This suggests

that cytokines are regulating JAK/STAT feedback inhibition via two conflictive ways; upregulation of SOCS proteins and increasing the degradation of SOCS proteins.

The mechanism of inhibition varies between the different SOCS proteins (Fig: 13). SOCS1 binds directly via its SH2 domain to tyrosine phosphorylated JAKs, as a consequence JAK activity is directly inhibited. SOCS3 uses a different mechanism for inhibition of JAKs, it binds to the activated receptor directly (Nicholson et al., 1999; Sasaki et al., 1999). Binding to gp130 occurs at the Y759 motif that also binds SHP2 (De Souza et al., 2002; Nicholson et al., 2000; Schmitz et al., 2000b). With the help of experiments allowing the recruitment of only SHP2 or SOCS3 to gp130 the independent negative regulatory function of each inhibitor has been shown, suggesting that SHP2 and SOCS3 trigger two distinct inhibitory mechanisms (Lehmann et al., 2003). CIS does not affect the activity of JAKs, compared to SOCS1 and SOCS3, it competes with STATs for binding sites at the receptor and therefore inhibits STAT phosphorylation (Verdier et al., 1998).

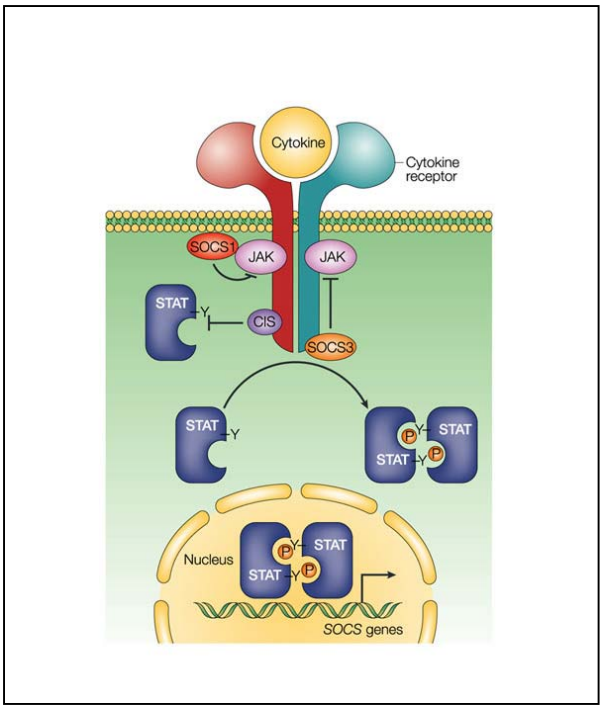

Fig. 13: Inhibition of JAK/STAT pathway by SOCS proteins. Adapted from (Shuai and Liu, 2003)

Gene targeting studies demonstrated the essential function of the SOCS proteins in the regulation of the immune system (Table 7) (Krebs and Hilton, 2000; Levy and Darnell, 2002).

Gene	Phenotype of null mice
Cis	Normal
SOCS1	Perinatal lethality, IFN-γ-overproduction, liver degeneration, hypersensitivity to LPS, increased apoptosis in lymphoid organs.
SOCS2	Gigantism due to dysregulated growth hormone and Igf1 signaling.
SOCS3	Embryonic lethality, placental and hematopoietic defects.

Table 7: Effects of SOCS proteins as shown in knockout mice. Adapted from (Levy and Darnell, 2002).

Conditional gene targeting studies to generate mice lacking SOCS3 in macrophages showed that the SOCS proteins have specificity for cytokines but not for JAKs or STATs. For example, Stat3 activation was prolonged after IL-6 stimulation but not after stimulation with IL-10, which both activate Stat3, in SOCS3 $^{-/-}$ macrophages, illustrating a new specificity for SOCS3 mainly targeting gp130 dependent signaling pathways (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003).

The PIAS family of inhibitors

Five members belong to the family of proteins that inhibit activated STAT (PIAS) PIAS1, PIAS3, PIASxα, PIASxβ and PIASy. A RING-finger-like zinc-binding domain (RLD) in the central region of PIAS is the most conserved domain. In addition they have a highly acidic region, which carries a putative small ubiquitin-related modifier1 (SUMO1) interaction motif (SIM), a serine/threonine (S/T) region at the C-terminus as well as a SAP (scaffold attachment factor A and B) domain at the N-terminus (Fig. 14). The SAP domain mediates interactions between nuclear receptors and their co-regulators. PIASy does not bear the SIM motif nor the S/T region.

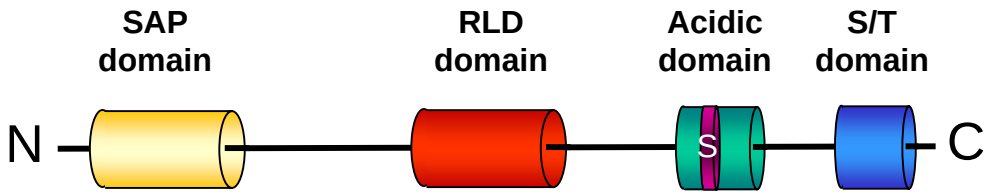

Fig. 14: Domain structure of PIAS. S: SIM motif.

PIAS1 has first been identified by yeast two-hybrid screens with Stat1β as a bait (Liu et al., 1998). The authors also identified PIAS1 as a novel negative regulator of Stat1 that blocks DNA-binding and consequently inhibits Stat1 mediated transcriptional activity after IFN stimulation. The inhibitory effect was specific for Stat1 and required interaction of PIAS1 with the Stat1 dimer, but not unphosphorylated Stat1 monomer (Liao et al., 2000; Liu et al., 1998). The specific regions for interaction are a region near the C-terminus of PIAS1 (amino acids 392-541), which interacts with the N-terminus (amino acids 1-191) of Stat1 (Liao et al., 2000). Interaction of PIAS1 to methylated Stat1 is reduced as described under "Posttranslational Modifications of STAT proteins" (Mowen et al., 2001).

PIAS3 was found via searching of the expressed sequence tag (EST) database for other members of the family and showed to be an inhibitor of IL-6 activated Stat3 or prolactin activated Stat5 (Chung et al., 1997a; Rycyzyn and Clevenger, 2002). The mechanism of inhibition is identical to the mechanism of PIAS1 inhibition of Stat1 also requiring Stat3 phosphorylation. The zinc finger protein Gfi-1 is able to rescue the PIAS3 induced inhibition of Stat3 via direct interaction with PIAS3, though the exact mechanism is not known (Rodel et al., 2000). Interestingly PIAS1 as well as PIAS3 have been shown to enhance the transcriptional activity of androgen receptor (AR), while PIASy on the other hand repressed AR-mediated gene activation in prostate cancer cells (Gross et al., 2001). Other members of the family have been found to inhibit STATs, PIASy inhibits Stat1 and PIASx inhibits Stat4 after IFN or IL-12 stimulation, respectively (Arora et al., 2003; Liu et al., 2001). However, DNA binding activity of Stat1 or Stat4 is not changed (Arora et al., 2003; Liu et al., 2001). In fact, PIASx is present in the DNA-binding complex and the inhibitory activity of PIASx on Stat4 mediated gene activation is eliminated by the

histone deacetylase inhibitor trichostatin A (TSA) (Arora et al., 2003). Other evidence for the importance of histone deacetylases (HDACs) in the PIAS induced mechanism comes from the description of association of PIAS with HDAC molecules; PIASxβ associates with HDAC3 and PIASy interacts constitutively with HDAC1 (Long et al., 2003; Tussie-Luna et al., 2002). A further characteristic of PIAS proteins is that they exhibit E3-small ubiquitin-related modifier (SUMO) ligase activity (Johnson and Gupta, 2001; Kotaja et al., 2002; Sachdev et al., 2001; Schmidt and Muller, 2002).

Similar to ubiquitylation, sumoylation, the covalent attachment of SUMO to its substrate, requires a set of enzymes (E1-E3) for its conjugation to the target that are different from the enzymes acting on ubiquitin. The SUMO E1 activating enzyme is a heterodimer composed of the proteins Aos1 and Uba2, whereas the ubiquitin E1 is a single protein, Uba1. Furthermore, sumoylation requires only one E2 conjugating enzyme Ubc9, compared to the vast number of E2 enzymes available in the ubiquitin pathway. The ligating E3 enzyme, which performs an adaptor function between the E2 enzyme and the substrate, has recently been identified as PIAS, RAN-binding protein 2 (RanBP2) or the polycomb protein (PC2). The E3 enzymes are all sumoylated themselves. All proteins, which are targeted for sumoylation share a common motif, a short consensus sequence, the ΨkxE motif. Lastly, Ulp is a protein that has a dual function in maturation of the SUMO pre-protein and in the removal of SUMO from the target (Fig. 15) (Seeler and Dejean, 2003).

Sumoylation influences various processes, for example, subcellular localization or prevention of ubiquitylation of the substrates. Protein targeting effects of SUMO imply nuclear import or export as well as sequestration of transcription factors to specific cellular compartments. Both functions have been observed with the transcriptional repressor TEL, which will be discussed later.

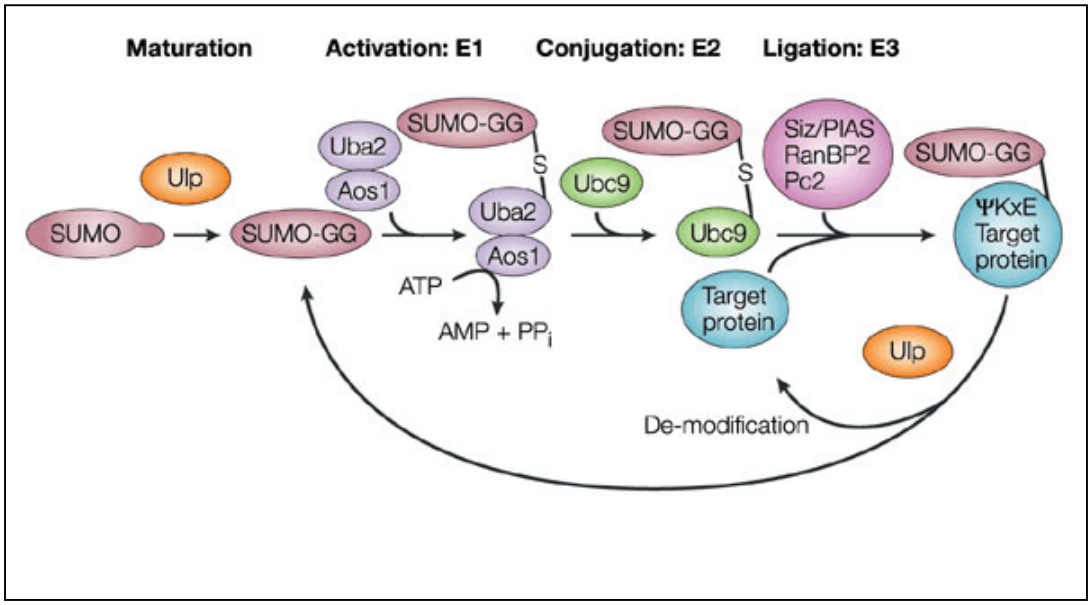

Fig. 15: The SUMO pathway. Adapted from (Seeler and Dejean, 2003)

As mentioned above, it was previously known that PIAS proteins exhibit E3 SUMO ligase activity but it has been shown only in recent times that Stat1 is sumoylated by PIAS family members. Sumoylation occurs at a single, evolutionary conserved amino acid residue, lysine 703, and mutation of this lysine abolishes sumoylation. The function of sumoylation is not clear since Rogers et al. do not see an effect on Stat1 activation while other experiments show an increase in IFN stimulated Stat1 activation after sumoylation (Rogers et al., 2003; Ungureanu et al., 2003). Taken all results together this data implies that the mode of inhibition of STAT proteins via the PIAS family can include different ways via reduced DNA-binding (Fig. 16a), by recruitment of histone deacetylases (HDAC) to the transcription complex (Fig. 16b), or via sumoylation (Fig. 16c). Further studies will be necessary to completely unravel the mechanisms by which different members of the PIAS proteins are able to negatively regulate the STAT signaling pathways.

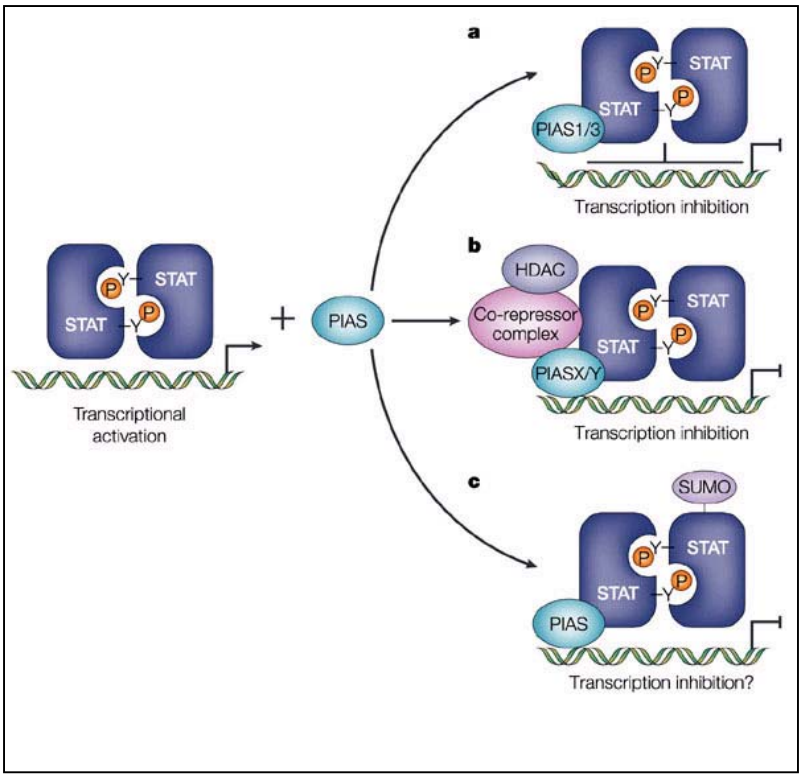

Fig. 16: Possible mechanisms for inhibition of the JAK/STAT signaling pathways by PIAS. Adapted from (Shuai and Liu, 2003)

STAT interacting proteins (co-activators)

It becomes increasingly clear that transcription factors rarely operate alone, but rather work together with other transcription factors or co-activators to enhance or repress each other. Most transcription factors interact with histone acetyltransferases (HATs) enzymes, which link acetyl groups to histones, and co-activators. The TADs of Stat1, Stat2, Stat3, Stat5 and Stat6 interact with the CREB-binding protein (CBP/p300) (Hiroi and Ohmori, 2003; Paulson et al., 1999; Pfitzner et al., 1998; Zhang et al., 1996); STAT2 recruits in addition the HAT protein general control nonrepressed 5 (GCN5) through its TAD (Paulson et al., 2002). Recruitment of HATs results in localized transient acetylation of histones and the enhancement of activator-dependent transcriptional activity. The association of Stats and CBP/p300 is further enhanced by the N-Myc interactor (Nmi) protein, which interacts with all STATs except Stat2. Nmi amplifies STAT-mediated transcription in cells stimulated with IL-2 and IFNγ (Zhu et al., 1999). In addition, associations have been shown between: Stat1 and mini-chromosome

maintenance5 (MCM5), ubiquitous factor1 (USF1), SP1, BRCA1, PU.1 and glucocorticoid receptor (GR) (Aittomaki et al., 2000; Look et al., 1995; Muhlethaler-Mottet et al., 1998; Ouchi et al., 2000; Zhang et al., 1998); Stat5 and GR, Sp1, and C/EBPβ (Martino et al., 2001; Stocklin et al., 1996; Wyszomierski and Rosen, 2001); and Stat6 with C/EBPβ, p100, and NCoA-1 (Litterst and Pfitzner, 2001; Mikita et al., 1998; Yang et al., 2002b).

Stat3's transcriptional activity is enhanced by CBP/p300, Nmi, forkhead transcription factor (FKHR), c-Jun, SP1, and EZI and repressed by Tip 60 (Cantwell et al., 1998; Kortylewski et al., 2002; Schaefer et al., 1995; Xiao et al., 2003; Zhang et al., 1999b).

Biological function of STATs

Gene-targeting studies indicate that some STAT proteins are highly specific in their function and that they are responsible for mediating the immune response in mammals (Table 8).

Gene	Phenotype of null mice
Stat1	Viable and fertile, defective IFNα/β and IFNγ signaling, defective immune response, increased tumorigenicity
Stat2	Viable and fertile, defective IFNα/β signaling
Stat3	Embryonic lethal, impaired cell survival, impaired response to pathogens
Stat4	Viable and fertile, defective IL-12 signaling, impaired Th1 differentiation
Stat5a	Viable and fertile, defective prolactin signaling, impaired mammary gland development
Stat5b	Viable and fertile, defective growth hormone signaling, impaired growth
Stat6	Viable and fertile, defective IL-4 signaling, impaired Th2 differentiation

Table 8: Phenotype of STAT knockout mice. Adapted from (O'Shea et al., 2002).

Stat1

Stat1 knockout mice are viable and fertile and display no developmental defects. However, Stat1$^{-/-}$ mice failed to induce transcription of target genes after simulation with IFNα/β and IFNγ; these mice are highly susceptible to microbial and viral infections (Durbin et al., 1996; Meraz et al., 1996). Atypical susceptibility to mycobacterial but not to viral infection has also been identified in patients suffering with a natural heterozygous germline Stat1 mutation, further strengthening the importance of Stat1 in contributing to immune responses (Dupuis et al., 2001). Interestingly, Stat1 deficient mice also showed sensitivity for both spontaneous tumor development and tumor development after methylcholanthrene treatment (Kaplan et al., 1998; Shankaran et al., 2001). This suggests that Stat1 has a proapoptotic function.

Stat2

Stat2 is an exceptional member of the STAT family, since it does not bind to GAS elements and it does not homodimerize. Instead, upon activation by IFNα Stat2 forms, the transcription factor complex ISGF3, together with Stat1 and p48/ISGF3gamma. Therefore, it is no surprising that the Stat2 knockout exhibits similarities with the Stat1 knockout. The mice are viable, fertile and display no developmental defects. Nevertheless, they are also susceptible to viral infections due to their impaired ability to respond to IFNα/β signaling. Additionally, Stat1 tyrosine phosphorylation and activation after IFNα stimulation is weakened in the Stat2 deficient mice, arguing for an enhancer effect of Stat2 in binding to the receptor complex in the Stat1/Stat2 heterodimers (Park et al., 2000a).

Stat3

Will be discussed in detail in the section Stat3.

Stat4

Stat4 is only expressed in natural killer cells (NK cells), dentritic cells and T lymphocytes and is mainly activated by IL-12. $CD4^+$ T cells differentiate with the help of IL-12 to Th1 cells, which in turn leads to the expression of IFNγ. Th1 cells are involved in host defense against intracellular pathogens and tumors, and in the development of autoimmune diseases, for example, rheumatoid arthritis, diabetes and multiple sclerosis. Consequently it was no surprise that the Stat4 and IL-12 deficient mice show a similar phenotype, i.e., loss of Th1 differentiation, loss of IFNγ expression and enhanced tendency towards Th2 differentiation, which is usually inhibited by Th1 cells (Kaplan et al., 1996b; Thierfelder et al., 1996). Recent data indicate that Stat4 deficient mice show a resistance to autoimmune diseases like rheumatoid arthritis, diabetes and experimental, allergic encephalomyelitis (EAE) (Chitnis et al., 2001). In humans, it was shown that IFNα/β can stimulate T cells to drive Th1 development, bypassing the need for IL-12-induced signaling, in contrast IFNα does not cause Th1 differentiation in mice (Rogge et al., 1998). The reason for this is that IFNα/β is able to activate Stat4, by recruitment of Stat4 to the IFNα receptor complex exclusively via the C-terminus of Stat2 in human cells. The difference between mouse and human signaling is that the mouse Stat2 gene harbors a minisatellite insertion, which change the C-terminus sequence and selectively disrupte its ability to activate Stat4, but not other STATs (Farrar et al., 2000).

Stat5

Stat5 was originally characterized as a prolactin-responsive transcription factor in sheep and exists as two closely related genes, Stat5a and Stat5b, which share 95 % identity at the N-terminus and some variability at the C-terminus (Azam et al., 1995; Mui et al., 1995). Both genes are expressed in all tissues and are activated by a wide variety of cytokines. Given the broad range of activation one might think that the knockout of these proteins would have many effects. However, the Stat5 specific knockouts exhibited a very precise phenotype for the individual gene. Both of the Stat5a and Stat5b deficient mice are viable, fertile and displaye no developmental defects. Stat5a $^{-/-}$ mice are impaired in prolactin activated mammary gland development, necessary for lactation (Liu

et al., 1997; Teglund et al., 1998), while Stat5b $^{-/-}$ mice have impaired GH signaling effects, similar to GH deficient mice (Teglund et al., 1998; Udy et al., 1997). Since high GH levels are mainly observed in males, female Stat5b $^{-/-}$ showed almost no phenotype while males were smaller and revealed the loss of male-specific liver genes (as MUP and CYP2D9). Interestingly the Stat5a/b double knockout mice are infertile, small, die after a few weeks of birth, and are defective in the development of the mammary gland and the corpus luteum (Teglund et al., 1998).

Stat6

Stat6 is ubiquitously expressed and is mainly activated by IL-4 and IL-13 (Kisseleva et al., 2002). IL-4 is expressed by activated T and B cells and regulates differentiation of $CD4^+$ T cells to Th2 cells and class switching of B cells resulting in secretion of IgE. Th2 helper cells are essential for host defense against helminthes and further allergic responses. Stat6 and IL-4 deficient mice show similar phenotypes; neither they are able to differentiate into Th2 cells and they are inoperative to class switch to IgE; otherwise they are viable, fertile and display no developmental defects (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). In addition, Stat6 deficient mice, have a predominantly Th1 phenotype, experience a severe clinical course of EAE, are inhibited in host defense against helminthes, are resistant to septic peritonitis because of enhanced local bacterial clearance, show resistance to tumor recurrence and deregulated activation of NF-κB leading to reduced expression of proinflammatory cytokines and chemokines induced by endotoxins (Chitnis et al., 2001; Lentsch et al., 2001; Matsukawa et al., 2001; Terabe et al., 2000).

STATs and cancer

Constitutively activated Stat1, Stat3 and Stat5 proteins have been observed in a wide variety of human cancer cell lines as well as primary tumors, which is probably due to dysregulation of STAT activators or mutations and loss of proteins that negatively regulate STAT proteins, since mutations in STATs leading to constitutively active proteins have not been reported yet (Table 9).

Tumor Type	Activated STAT
Breast Cancer	Stat1, Stat3, Stat5, Stat6
Multiple myeloma	Stat1, Stat3
Head and neck cancer	Stat1, Stat3
Acute lymphocytic leukemia (ALL)	Stat1, Stat5
Chronic lymphocytic leukemia (CLL)	Stat1, Stat3, Stat5
Acute myelogenous leukemia (AML)	Stat1, Stat3, Stat5
Chronic myelogenous leukemia (CML)	Stat1, Stat3, Stat5
Lymphoma	Stat3
Lung cancer	Stat1, Stat3
Renal cell carcinoma	Stat3
Prostate carcinoma	Stat3
Melanoma	Stat3
Pancreatic adenocarcinoma	Stat3
Ovarian carcinoma	Stat3

Table 9: Activation of STATs in human cancers. Adapted from (Bowman et al., 2000).

As discussed earlier Stat1 is considered a tumor suppressor, since mice lacking Stat1 are more susceptible to chemically induced primary tumors and to transplanted tumors (Kaplan et al., 1998; Lee et al., 2000a; Lee et al., 2000b; Shankaran et al., 2001). Furthermore, Stat1 is essential for cell growth suppression in response to IFN-gamma and EGF, partly by inducing cyclin dependent kinase (cdk) inhibitor p21 WAF1/CIP1 in response to cytokines, this involves BRCA1, which is often lost in breast cancer (Bromberg et al., 1998a; Bromberg et al., 1996; Chin et al., 1996; Ouchi et al., 2000). The fact that several tumors show constitutively active Stat1 suggest that Stat1 activation

is part of the host's tumor surveillance capability and it will be of interest to study if Stat1 is mutated in some human tumors.

Stat5 is mainly activated in leukemias and lymphomas and it was shown by expression of a dominant negative form of Stat5 that active Stat5 is necessary for the growth of hematopoietic cells, myeloid precursor cells and leukemia cells transformed by the BCR-Abl oncogene (de Groot et al., 1999; Nieborowska-Skorska et al., 1999; Sillaber et al., 2000). However, recent data, which was obtained with Stat5 deficient bone marrow derived B cells, shows that Stat5 is essential for TEL-JAK2 induced tumors, but not for v-Abl or BCR-ABL dependent transformation (Schwaller et al., 2000; Sexl et al., 2000). It therefore plays a critical role in the development of TEL-JAK2 induced leukemias and lymphomas but not, as suggested before, in v-Abl or BCR-ABL dependent transformation. In addition, Stat5 is able to induce expression of antiapoptotic signaling proteins for example Bcl-xl (Calo et al., 2003). The role of Stat3 in cancer will be discussed in the next section.

Stat3

Stat3 was initially identified as the acute-phase response factor (APRF), activated by IL-6 (Wegenka et al., 1993). The authors further showed that Stat3 activation occurred in the cytoplasm, that Stat3 phosphorylation is essential and that Stat3 binds to IL-6 response elements of various acute-phase protein genes (e.g., the alpha 2-macroglobulin, fibrinogen, and alpha 1-acid glycoprotein genes) (Wegenka et al., 1993). The Stat3 cDNA was cloned one year later and encodes an open reading frame of 770 amino acids resulting in a protein of 88 kDa (Akira et al., 1994). Stat3 is ubiquitously expressed and expression starts very early during post-implantation development in the mouse. Stat3 can be activated by many different cytokines, growth factors and oncogenes (Fig.4). The IL-6 family of cytokines has many biological functions and Stat3 plays a major role in these processes (Hirano et al., 2000). IL-6 activation in mouse myeloid leukemia M1 cells has been shown to lead to growth arrest and terminal differentiation into macrophages. Overexpression of Stat3DN abrogated the IL-6 induced effects and resulted in inhibition of IL-6-induced repression of c-myb and c-myc (Minami et al.,

1996; Nakajima et al., 1996). These experiments demonstrated for the first time that Stat3 activation is essential for IL-6 mediated growth arrest. Conversely, Fukada et al. found that Stat3 is involved in anti-apoptosis, proliferation and upregulation of Bcl-2 by overexpression of Stat3DN in mouse pro-B (BAF/B03) cells (Fukada et al., 1996). The authors demonstrated in the following years that Stat3 activation was not only essential for cell survival but also required for cell cycle transition, via Stat3 mediated upregulation of cyclins D2, D3 and A, and cdc25A, and the associated downregulation of p21 and p27 (Fukada et al., 1998).

OSM and ciliary neurotrophic factor (CNTF) promote differentiation of cerebral cortical precursor cells or cultured fetal mouse neuroepithelial cells into astrocytes. Overexpression of Stat3DN completely blocked OSM induced promoter activation of the gene for an astrocyte marker, glial fibrillary acidic protein (GFAP) (Bonni et al., 1997; Yanagisawa et al., 1999). Moreover, expression of GFAP was severely reduced in the brain of gp130 $^{-/-}$ mice as well as in mice expressing a mutant gp130, which is defective in Stat3 signaling but not in SHP2 signaling (Nakashima et al., 1999; Ohtani et al., 2000). The mice that express a mutant gp130 helped also to understand the mechanism of B cell differentiation into antibody-forming plasma cells, since mice harboring a construct lacking the tyrosines of gp130 important for Stat3 activation, showed decreased IgG2a and IgG2b production (Ohtani et al., 2000). Another receptor that is capable of inducing B cell differentiation is CD40, which lacks intrinsic tyrosine kinase activity, but is still able to induce tyrosine phosphorylation and activation of constitutively associated JAK3, as well as of Stat3. Mutation of the JAK3 binding domain inhibits B cell differentiation (Hanissian and Geha, 1997). Taken these results into consideration one has to argue that Stat3 is involved in astrocyte and B cell differentiation.

Interestingly, in a few cases Stat3 is also able to inhibit cell differentiation, following its activation with IL-6 or LIF. IL-6 was shown to induce differentiation of PC12 cells that have been pretreated with nerve growth factor (NGF). Stimulation of the MAPK pathway is important for neurite outgrowth, since cells overexpressing gp130 mutants incapable of activating the MAPK cascade or cells treated with the MEK inhibitor PD98059 treatment with IL-6 failes to induce differentiation of PC12. Conversely, overexpression of a

mutant gp130, which is defective in Stat3 signaling but not in SHP2 signaling, did not inhibit, but rather stimulated neurite outgrowth. NGF pretreatment inhibited the IL-6-induced activation of Stat3 and overexpression of Stat3DN did not require NGF pretreatment for neurite outgrowth. These facts indicate that Stat3 is negatively involved in PC12 differentiation (Ihara et al., 1997). Likewise, Stat3 is essential for self renewal of embryonic stem (ES) cells, that are continuously propagated in an undifferentiated pluripotent state with LIF, since overexpression of a dominant negative Stat3 is able to abrogate LIF mediated self-renewal and promotes differentiation (Boeuf et al., 1997; Niwa et al., 1998; Raz et al., 1999). Concordant with this finding is a study of ES cells expressing a fusion protein composed of the entire coding region of STAT3 and the ligand binding domain of the estrogen receptor (Stat3ER) that can be activated by the synthetic ligand 4-hydroxytamoxifen (4HT), maintained an undifferentiated state upon stimulation with 4HT (Matsuda et al., 1999).

Stat3 conditional gene targeting in mice

Stat3 is the only STAT family member whose knockout leads to embryonic lethality. Stat3 deficient mice develop into the egg cylinder stage but show a rapid degeneration between embryonic days 6.5 and 7.5. This is probably due to nutritional insufficiency, since Stat3 is expressed at day 7.5 in the embryonic visceral endoderm, which is important for nutrient exchange between the maternal and embryonic environment (Takeda et al., 1997). Some knockouts of components of Stat3 activating pathways also lead to embryonic lethality, such as gp130 and LIFRβ. However, none of the known activating cytokines or growth hormones or JAKs function so early in development. So, the early lethality as well as the Stat3 activating cascade during early development remains an enigma up to date. Nevertheless, with the use of the Cre-lox technology, which allows targeting of Stat3 $^{-/-}$ to any desired embryonic or adult tissue, it has became possible to generate tissue specific knockouts (Table 10).

Stat3-deficient T cells showed a loss of proliferative response due to a defect in IL-6 mediated prevention of apoptosis. The antiapoptotic protein, Bcl-2, is normally upregulated in response to IL-6 even in Stat3-deficient T cells (Takeda et al., 1998). This suggests that Stat3 has a Bcl-2 independent anti-apoptotic function

Target tissue	Phenotype of null mice
Skin	Impaired second hair cycle, wound repair and keratinocyte migration
Liver	Impaired acute-phase response
Neurons	Enhanced neuronal apoptosis, impaired survival after nerve damage
T lymphocytes	Impaired IL-6 dependent survival, impaired IL-2Rα expression
Thymic epithelium	Age-dependent thymic hypoplasia, hypersensitivity to stress
Granulocytes	Viable and fertile, defective growth hormone signaling, impaired growth
Mammary epithelium	Decrease in apoptosis, delayed mammary involution
Cardiomyocyte	Higher sensitivity to inflammation, cardiac fibrosis and heart failure with advanced age
Monocytes /Neutrophils	Enhanced inflammatory response, chronic colitis and Th1 differentiation

Table 10: Phenotypes of tissue-specific knockout of Stat3. Adapted from (Levy and Darnell, 2002).

Targeted Stat3 knockout in macrophages and neutrophils results in mutant mice that are highly susceptible to endotoxin shock and develop chronic enterocolitis with age, due to abolished inhibitory effects of IL-10 on inflammatory cytokine production (Takeda et al., 1999). In addition, Stat3 $^{-/-}$ plus IL-10 $^{-/-}$ mice show similar phenotypes and it has been shown that IL-10 suppresses expression of TNF-α via Stat3 (Riley et al., 1999).

Stat3 phosphorylation occurs at the onset of mammary gland involution and Stat3 null mammary glands show a decrease in apoptosis and a dramatic delay of involution, a stage of mammary gland development that is characterized by extensive apoptosis of the epithelial cells. In normal glands involution is accompanied by increase in insulin-like growth factor binding protein (IGFBP5) levels, which binds the survival factor insulin-like growth factor (IGF1) and inhibits IGF1. There is evidence to suggest that IGFBP-5 is a direct or indirect target for Stat3 since IGFBP5 upregulation is not observed in Stat3 null mammary glands (Chapman et al., 1999). These data showed for the first time that Stat3 is important for apoptosis in vivo.

Mice with Stat3 deficient epidermal and follicular keratinocytes were viable and did not have any defects in the development of epidermis and hair follicles. However, the mice had sparse hair, wound-healing processes were severely impaired and they spontaneously developed ulcers with age. Considering that migration and proliferation are essential for wound healing, motility and growth of keratinocytes was examined. Results illustrated that migration of Stat3-disrupted keratinocytes in response to growth factor stimulation, in vitro, was impaired while proliferation was not disturbed. Furthermore, the mice exhibited a normal first hair cycle (morphogenesis), but an impaired second hair cycle (skin remodeling) (Sano et al., 1999). This analysis provided the first in vivo data showing that Stat3 has a function in cell migration and that Stat3 is necessary for skin remodeling, including hair cycle and wound healing.

Activation of Stat3 in the brain functions via CNTF and leptin and inactivation of Stat3 in cytokine-dependent sensory neurons of the nodose ganglion reduced the response of these neurons to CNTF and LIF and enhanced their death after nerve lesion (Alonzi et al., 2001b; Schweizer et al., 2002). Knockout of Stat3 in cardiomyocytes showed that Stat3 is involved in regulation of inflammatory responses and survival (Jacoby et al., 2003). Stat3 deficient hepatocytes severely impaired IL-6 induced acute-phase response in the liver during inflammation (Alonzi et al., 2001a). Interestingly, Stat3β deficient mice exhibited reduced recovery from endotoxic shock and showed hyperresponsiveness to a subset of endotoxin-inducible genes in liver. These findings reveal a critical negative regulatory role for Stat3β in the control of systemic inflammation (Yoo et al., 2002). Considering all

knockout data together it is surprising that Stat3 has such mild phenotypes in the conditional knockout mice compared to the early embryonic lethality in the full knockout. The data suggests that the Stat3 activators in early development are different from the known activators in the adult and it will be of interest to identify them. Moreover, Stat3 elicits diverse functions in various cell types since the readout of the Stat3 tissue deficient mice is diverse and ranges from apoptosis, survival, and effects on migration up to proliferation. It will be a challenge to solve the riddle why Stat3 activation has diverse biological functions and the more information we are able to gain about the different sets of genes that are regulated by Stat3, in different cell types, the more we will be able to understand the function of the Stat3 molecule.

Stat3 specific negative regulator GRIM-19

Gene associated with retinoid-interferon-induced mortality-19, (GRIM-19) is essential for tumor cell death induced by interferon-beta (IFN-beta) and retinoic acid (RA) (Angell et al., 2000). Since GRIM-19 is localized on human chromosome 19p13.2., a region that is essential for prostate tumor suppression, it was speculated that GRIM-19 might be a novel tumor suppressor (Chidambaram et al., 2000). The cellular localization as well as the domain structure of GRIM-19 is unclear. The interaction of GRIM-19 and Stat3 was first observed via yeast two-hybrid screening and is specific for Stat3, since GRIM-19 did not interact with Stat1 and Stat5. GRIM-19 inhibits Stat3 transcriptional activation stimulated by EGF or IL-6 (Lufei et al., 2003; Zhang et al., 2003b). Lufei et al. demonstrated that the coiled-coil region of Stat3 is able to bind to GRIM-19 and is involved in the formation of aggregates with GRIM-19. It was also suggested that GRIM-19 inhibits Stat3 by blocking nuclear translocation of Stat3 (Lufei et al., 2003). Zhang et al. however, showed that the TAD domain of Stat3 is important for GRIM-19 binding and inhibition. They did not see any change in Stat3 nuclear localization and DNA-binding in cells overexpressing GRIM19 and therefore argue against the model of Lufei et al. (Zhang et al., 2003b). The mechanism of inhibition of Stat3 by GRIM19 has therefore to be investigated further to draw a final conclusion.

Stat3 specific negative regulator TIP60

Tat-interactive protein 60kDa (Tip 60) belongs to the MYST family of HAT proteins, a family that is highly conserved from yeast to mammals. Expression of Tip60 can be observed in a wide variety of tissues and cell lines. Tip 60 is mainly localized to the nucleus where it forms stable nuclear complexes possessing ATPase and HAT activity for histones H4, H3 and H2A. The Tip 60 structure bears two important domains a chromatin organization modifier (CHROMO) domain at the N-terminus and a MYST ('MOZ, Ybf2/Sas3, SAS2 and Tip60', where MOZ stands for male absent on the first, SAS for something about silencing and Ybf2 for identical with SAS2) region at the C-terminus (Sterner and Berger, 2000). Recent results indicate that Tip60 interacts with Stat3 and represses Stat3 transcriptional activity, since overexpression of Tip60 repressed Stat3 reporter gene expression. The authors showed further that Tip60 interacts as well with HDAC7 and that HDAC7 activity is necessary to inhibit Stat3 activity (Xiao et al., 2003).

Stat3 function in human disease

Crohn's disease is a chronic inflammatory bowel disease of the gastrointestinal tract of poorly known etiology. Through the studies of patients and mouse models, it was shown that the disease occurs as the consequence of a deregulated immune response to normal components of the intestinal flora in a genetically predisposed host. Two recent publications demonstrate that Stat3 might have critical roles in the development and regulation of innate immunity leading to Crohn's disease. First, tissue-specific disruption of STAT3 in bone marrow cells during hematopoiesis, causes death of the mice within 4-6 weeks after birth with Crohn's disease-like pathogenesis (Welte et al., 2003). Second, in a study of intestinal T cells from Crohn's disease and healthy volunteers it was demonstrated that STAT3 and STAT4 are constitutively activated in Crohn's patients, but not in healthy volunteers. (Lovato et al., 2003). Nevertheless, the mechanism whereby Stat3 contributes to Crohn's disease pathology is not known.

Stat3 in cancer

As mentioned earlier Stat3 is constitutively activated in a wide variety of human tumors (Table 9), this activity is attributed to dysregultion of cytokine receptors, growth factors receptors and aberrant JAK activity. For example, the upregulation of TGFα and EGFR activates Stat3 in squamous cell carcinoma of the head and neck (SCCHN) (Song and Grandis, 2000). Furthermore, Stat3 is also constitutively activated by oncogenes such as v-abl, v-src, v-fps, v-sis, v-ros, and v-eyk (Bowman et al., 2000; Yu et al., 1995). Overexpression of Stat3DN, Stat3β, the use of Stat3 antisense oligonucleotides or a Stat3 decoy oligonucleotide has proven to be valid tools to examine the function of constitutively activated Stat3 in a large number of cancer cell lines or tumors. For example, it has been demonstrated by overexpression of Stat3DN that Stat3 activity is essential for oncogenic transformation of cells by v-src (Bromberg et al., 1998b; Cao et al., 1996; Turkson et al., 1998). Furthermore, inhibiting Stat3 signaling leads to suppression of the transformed phenotype, growth arrest and apoptosis of non-small lung cancer cells, melanoma cells, breast carcinoma cells, head and neck cancer cells, prostate cancer cells, ovarian cancer cells, glioblastoma multiforme cells and melanoma B16 tumors (Burke et al., 2001; Catlett-Falcone et al., 1999; Garcia et al., 2001; Grandis et al., 2000; Leong et al., 2003; Mora et al., 2002; Ni et al., 2000; Nielsen et al., 1999; Niu et al., 2002a; Niu et al., 1999; Rahaman et al., 2002; Song et al., 2003). Further evidence for Stat3 mediated transformation arises from experiments with a Stat3 molecule (Stat3-C) that dimerizes spontaneously due to substitution of two cysteine residues within the C-terminal loop of the SH2 domain, binds to DNA and activates transcription Overexpression of this construct in immortalized fibroblasts lead to transformation and tumor formation in nude mice (Bromberg et al., 1999).

Taken together, these experiments suggest that Stat3 activation prevents apoptosis, stimulates proliferation and is involved in malignant transformation. However there are also examples were Stat3 activation has pro-apoptotic and growth inhibitory functions. Thus, as discussed earlier, Stat3 activation is an important step in IL-6 induced growth arrest of M1 leukemia cells (Minami et al., 1996; Nakajima et al., 1996). Additional, overexpression of Stat3DN reduces inhibition of cell proliferation in T47D breast cancer

and A375 melanoma cells, stimulated with IL-6 and OSM (Badache and Hynes, 2001; Kortylewski et al., 1999), blocks LIF induced growth arrest in medullary thyroid cancer (MTC) cells (Park et al., 2003) and IL-6 induced growth inhibition in LNCaP prostate cancer cells (Spiotto and Chung, 2000). IL-10 inhibits proliferation of a mouse macrophage cell line (J774) and expression of Stat3DN blocks this inhibition. Furthermore, expression of an inducibly active Stat3-gyraseB chimera (Stat3-GyrB) is able to induce growth inhibition without IL-10 stimulation (O'Farrell et al., 1998). Recent results show a correlation with Stat3 activity and better survival. For example, node-negative breast cancer patients with positive Phospho-Stat3 nuclear expression have a significantly improved survival at both short- (5-year) and long-term (20-year) (Dolled-Filhart et al., 2003). Similarly nasopharyngeal carcinoma (NPC) patients with constitutive Stat5 activation, or activation of both Stat3 and Stat5, had better disease-free survival and overall survival, following radiotherapy, than those without activated STATs (Hsiao et al., 2003).

Stat3 target genes

The answer as to why activated Stat3 has different functions in different cell types is not known but it is suggested that this is due to the expression of different Stat3 target genes. Genes that are regulated by Stat3 are very important since they trigger signaling cascades that lead to the final observed biological effects (Table 11).

Stat3 target genes, upregulated by Stat3	Cells	Reference
angiotensinogen II	HepG2	(Sherman and Brasier, 2001)
Bcl-xl	Stat3C transformed NIH3T3, U266 myeloma cells, head and neck squamous cell carcinomas	(Bromberg et al., 1999; Catlett-Falcone et al., 1999; Grandis et al., 2000)
cdc25A	BAF/B03 pro-B cells	(Fukada et al., 1998)
C/EBP δ	MCF-7	(Zhang et al., 2003a)
c-myc	Stat3C transformed NIH3T3, BAF/B03, murine pro-B cells, MCF-7, HepG2	(Bowman et al., 2001; Bromberg et al., 1999; Kiuchi et al., 1999; Zhang et al., 2003a)
cyclin A	BAF/B03 pro-B cells	(Fukada et al., 1998)
cyclin D1	Stat3C transformed NIH3T3, MCF-7, v-src transformed NIH3T3 and BALB/c 3T3	(Bromberg et al., 1999; Sinibaldi et al., 2000; Zhang et al., 2003a)
cyclin D2	BAF/B03 pro-B cell	(Fukada et al., 1998)
cyclin D3	BAF/B03 pro-B cells	(Fukada et al., 1998)
cyclin E	v-src transformed NIH3T3 and BALB/c 3T3	(Sinibaldi et al., 2000)
fibronectin	MCF-7, T47D	(Zhang et al., 2003a)
mcl-1	Large granular lymphocyte (LGL) leukemia cells	(Epling-Burnette et al., 2001)
p21	v-src transformed NIH3T3 and BALB/c 3T3	(Sinibaldi et al., 2000)
p27	A375	(Kortylewski et al., 2001)
p53	MCF-7	(Zhang et al., 2003a)
pim-1 and pim-2	BAF/B03 pro-B cell	(Shirogane et al., 1999)
VEGF	NIH3T3, B16 tumor cells, human pancreatic cancer cell lines	(Niu et al., 2002b; Wei et al., 2003)
Stat3 target genes, downregulated by Stat3	**Cells**	**Reference**
cyclin D1	fetal hepatocytes	(Matsui et al., 2002)
cyclin D2	fetal hepatocytes	(Matsui et al., 2002)
p21	BAF/B03 pro-B cells	(Fukada et al., 1998)
p27	BAF/B03 pro-B cells	(Fukada et al., 1998)

Table 11: Stat3 target genes

Stat3 could induced effects on malignant transformation; through the upregulation of antiapoptotic genes such as Bcl-xl, and Pim, the upregulation of genes that are important for cell cycle transition, for example, cyclin D1 and c-myc and the activation of roangiogenic factors, such as VEGF (Fig.17).

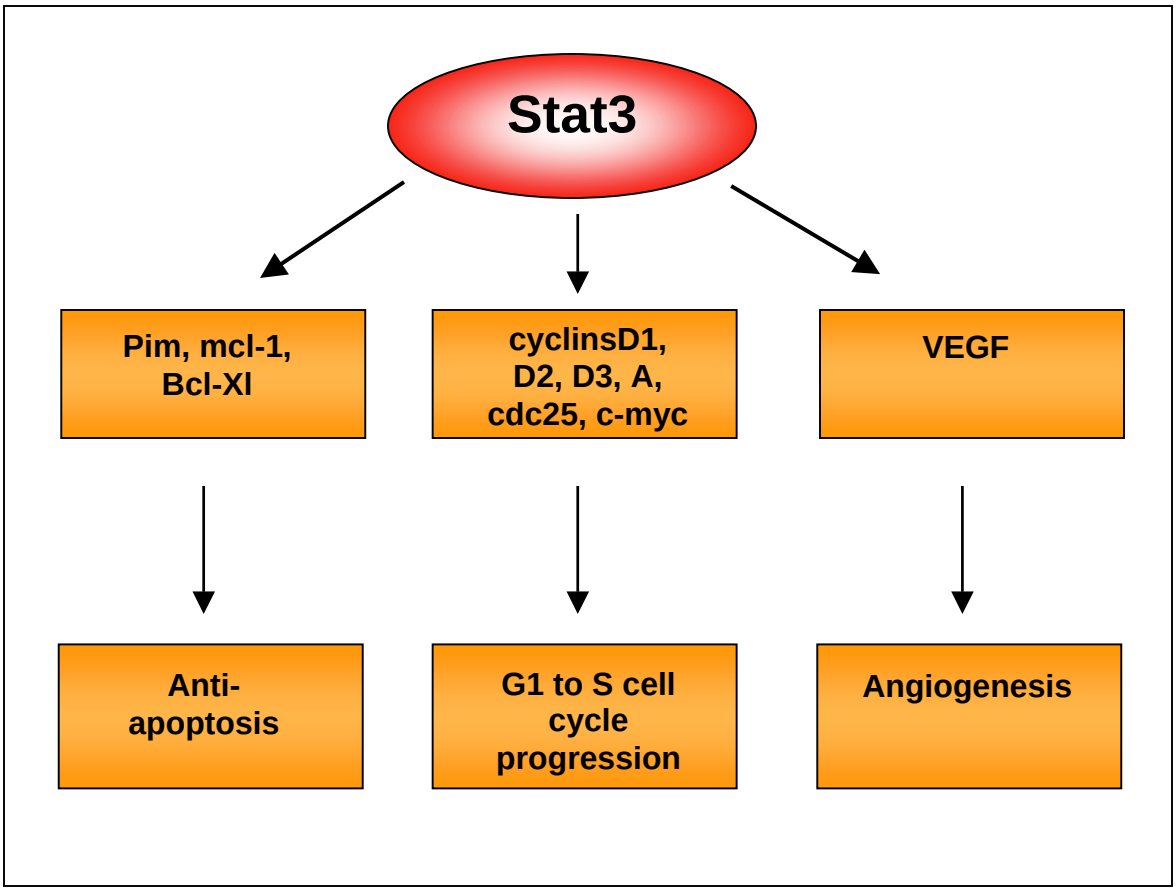

Fig. 17: Stat3 regulated genes important for cancer progression

With the exception of p27 and C/EBPδ, which are upregulated by Stat3, targets that are involved in Stat3 mediated growth inhibition in cancer cells have not been identified yet. Interestingly, p27 is downregulated by Stat3 in BAF/B03 pro-B cells (Kortylewski et al., 2001). This discrepancy might be due to the different genetic background of the cells.

The transcription factor TEL

TEL, a member of the E-Twenty-Six (ETS) family of transcription factors is one of the few ETS genes associated with human malignancies and with transcriptional repression. TEL is ubiquitously expressed (Poirel et al., 1997) and is essential during embryonic development: TEL knockout leads to embryonic lethality at day E10.5–11.5 due to severe defects in the developing vascular network of the yolk sac (Wang et al., 1997). Through alternative translation initiation at two successive AUG initiation codons in the TEL mRNA two nuclear proteins of 452 (TEL-M1) and 409 (TEL M43) amino-acids residues are encoded, TEL-M1 being the most abundantly expressed (Poirel et al., 1997). TEL contains several functional domains: the N-terminal pointed (PNT) homodimerization domain (also called B-domain, helix-loop-helix or SAM domain), which mediates dimerization and protein-protein interaction (Kwiatkowski et al., 1998), and an ETS DNA-binding domain in the C-terminus. The ETS domain is also important for protein-protein interactions in addition to directing protein-DNA interactions. As mentioned before, TEL is a sequence specific transcriptional repressor of natural and model promoters and this repressive activity was shown to be mediated by several functional domains of TEL; the PNT domain, the ETS domain and in addition by a repression domain composed of amino acids 268-333 (Figure 18) (Chakrabarti and Nucifora, 1999; Fenrick et al., 1999; Lopez et al., 1999; Wang and Hiebert, 2001).

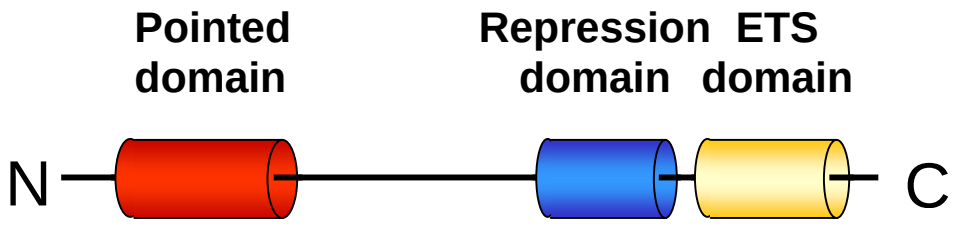

Figure 18: Domain structure of TEL protein.

TEL mediated repression was shown to be dependent upon the recruitment of co-repressors including mSin3A, SMRT, N-CoR, L(3)MBT and Tip 60 and is dependent on HDACs, since repression is inhibited by TSA, a general deacetylase inhibitor (Boccuni et al., 2003; Chakrabarti and Nucifora, 1999; Fenrick et al., 1999; Guidez et al., 2000; Nordentoft and Jorgensen, 2003; Wang and Hiebert, 2001).

TEL was first identified through its implication in chromosomal translocations in leukemia and solid tumors (Rubnitz et al., 1999). Chromosomal translocations are initiated by double-strand DNA breaks, the main repair mechanism underlying the resultant illegitimate recombination is probably non-homologous end-joining. The products of chromosomal translocations are fusion genes, generated by fusion of TEL to unrelated genes leading to the expression of oncogenes with either altered transcriptional regulation or constitutive kinase activity. The N-terminal PNT of TEL is usually fused to tyrosine kinases including platelet-derived growth factor β (PDGFRβ), c-ABL, and JAK2 (Golub et al., 1994; Golub et al., 1996; Hiebert et al., 1996; Lacronique et al., 1997). However, the most prominent chromosomal translocation fuses the PNT of TEL to the acute myeloid leukemia (AML) transcription factor (Golub et al., 1995; Romana et al., 1995). Interestingly, the second allele of TEL was deleted in some of the described cases above, suggesting that TEL is a tumor suppressor (Stegmaier et al., 1995). Further evidence for the role of TEL as tumor suppressor is supported by three studies; first, overexpression of TEL, in Ras-transformed NIH3T3 cells, inhibits cell growth and tumor formation in nude mice (Rompaey et al., 2000) second, TEL represses the expression of stromelysin-1, a metalloproteinase that is important in tissue remodeling and tumor cell invasion (Fenrick et al., 2000), third, mutational inactivation of TEL might occur in prostate carcinoma and ovarian cancer (Hatta et al., 1997; Kibel et al., 2002). Not much is known about the regulation of TEL. However, some evidence indicates that TEL is regulated at translational and post-translational levels. So far two types of post-translational modifications have been identified, phosphorylation and sumoylation. Both isoforms of TEL are subject to phosphorylation events, although, the function of phosphorylation is still unknown (Poirel et al., 1997). Sumoylation of TEL at lysine 99, within the PNT domain, by the ubiquitin-conjugating enzyme (UBC9) leads to nuclear export of TEL and therefore regulates TEL's repressive ability (Wood et al., 2003).

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V. Results

TEL/ETV6 is a Stat3-induced repressor of Stat3 transcriptional activity*

Nicole Schick, Edward J. Oakeley, Nancy E. Hynes[¶] and Ali Badache

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Data not shown and unpublished observations that are refereed to in the paper will be shown in the appendix, numbered the order as they appear in the paper.

SUMMARY

The Stat3 transcription factor is required for the anti-proliferative effects induced by cytokines, such as interleukin-6-type cytokines. In order to investigate the role of Stat3 in inhibition of cell proliferation, we have used an inducible Stat3 construct in A375 melanoma cells. We found that activation of Stat3 to moderate levels was sufficient to repress A375 proliferation, by slowing cell transit through the cell cycle. Enhanced and prolonged Stat3 activity led to cell cycle arrest and apoptosis. Genes whose expression was altered by Stat3 activation were identified by oligonucleotide microarray analysis. One of the Stat3 targets, C/EBPδ, is involved in Stat3 anti-proliferative activity, as revealed by small interfering (si) RNA targeting. Interestingly, TEL (ETV6), a novel Stat3 target identified in this study, is a negative regulator of Stat3 activity. SiRNA-mediated inhibition of TEL expression resulted in increased Stat3-dependent transcriptional activity and stronger Stat3 anti-proliferative activity. Confirming these results, overexpression of TEL repressed Stat3 transcriptional activity; intriguingly, this effect did not require the TEL DNA-binding domain. Inhibition of Stat3 activity by TEL represents a novel mechanism regulating the Stat3 signaling pathway.

Introduction

Signal transducer and activator of transcription 3 (Stat3)[1] is a latent cytoplasmic transcription factor that can be activated by a variety of kinases, including receptor tyrosine kinases and cytoplasmic kinases associated with cytokine receptors. Stat3 appears to play a critical role in the control of many biological processes during development (1-3) and is associated with cell proliferation, differentiation and survival (4). However, constitutive activation of Stat3 has been associated with oncogenesis. Indeed, there is increasing evidence that Stat3 is activated in a wide variety of human tumors, including head and neck, prostate and breast carcinomas, multiple myelomas and lymphomas (5,6). Moreover, experimental studies revealed that Stat3 is required for transformation by oncogenic tyrosine kinases such as v-Src, TEL-ABL and TEL-JAK (7-9), and that activation of Stat3 is sufficient for transformation of fibroblasts and Ba/F3 cells (9,10). On the other hand, members of the interleukin (IL)-6 family of cytokines which include IL-6, oncostatin M (OSM), leukemia inhibitory factor, and ciliary neurothrophic factor have been shown to inhibit proliferation of some leukemia, melanoma, prostate and breast cancer cells (11-14). Inhibition of cell proliferation by IL6-type cytokines was shown to be mediated by Stat3 (15-17). Moreover, recent observations indicate that, active, nuclear Stat3 correlates with a better prognosis in node-negative breast tumors and nasopharyngeal tumors (18,19). These results indicate that Stat3 contributes to anti-proliferative, and maybe anti-oncogenic effects, in some cellular contexts.

Binding of IL-6-type cytokines to their receptors leads to hetero-oligomerization with the common signal transducer gp130, resulting in activation of the receptor

associated Janus kinases (JAKs) and phosphorylation of specific tyrosine residues in the cytoplasmic tail of gp130. The phosphorylated tyrosines serve as docking sites for two major signal-transducing molecules: SHP2 (Src homology domain 2 protein tyrosine phosphatase) which interacts with adaptor molecules such as Gab1/2, p85 and Grb2 leading to activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) intracellular signaling pathways (20); and Stat3, which will in turn be phosphorylated by the JAKs (21). Tyrosine phosphorylation allows Stat3 dimerization, translocation to the nucleus, binding to specific DNA sequences and activation of transcription (10,22). Considering the critical role of Stat3 in controlling cell growth and differentiation, it is not surprising that negative regulators of Stat3 that control Stat3 activity at multiple levels, have been identified (22): *e.g.* control of JAK activity by SOCS (23), dephosphorylation of Stat3 by nuclear phosphatases (24), regulation of Stat3 nuclear translocation by GRIM-19 (25) and prevention of DNA binding by PIAS3 (26) .

We have further investigated the mechanism underlying Stat3 anti-proliferative effects. Using a conditionally active Stat3 construct, we found that activation of Stat3 was sufficient to inhibit A375 melanoma cell proliferation. Transcriptional targets of Stat3 in A375 cells were determined by oligonucleotide microarray analysis. We found that the leucine zipper transcription factor C/EBPδ, one of the targets identified in our screen, is a mediator of Stat3-induced inhibition of melanoma cell proliferation. Moreover, we identified Tel/ETV6, a member of the ETS family of transcription factors, as a Stat3 target and, interestingly, discovered that TEL is a novel repressor of Stat3 transcriptional and biological activity.

MATERIALS AND METHODS

Reagents and plasmids-Recombinant human IL-6 and OSM were from PeproTechEC (London, UK). 4-hydroxytamoxifen (4HT) was from Sigma (St. Louis, MO). Human erythropoietin (EPO) was from R&D Systems (Minneapolis, MN). The plasmid containing the mutated ligand-binding domain of the murine estrogen receptor (ER^{TM}) was kindly provided by T. Littlewood (Imperial Cancer Research Fund, London, UK). ER^{TM} was cloned into pcDNA3.1 as a BamH1-EcoR1 fragment. The Stat3 sequence with an additional BamH1 site was obtained by PCR amplification from pRc/CMVStat3 (kindly provided by J. E. Bromberg and J. F. Darnell, Rockefeller University, NY, NY) and cloned into pcDNA3.1-ER^{TM}. The chimeric receptors consisting of the extracellular part of the murine EPO receptor fused to various regions of gp130 cytoplasmic domain (16) were a kind gift from M. Kortylewski and I. Behrmann (RWTH, Aachen, Germany). The pCMV-TEL, pCMV-TELΔP, pCMV-TELΔ122-176, pCMV-TELΔ122-217, pCMV-TELΔ268-333 and pCMV-TELΔ303-333 constructs, generously provided by S. W. Hiebert (Vanderbilt University School of Medicine, Nashville, TN), were previously described (27). TEL DBDM construct (28), kindly provided by G. Grosveld (St. Jude Children's Research Hospital, Memphis, TN), was subcloned into pCMV.

Cell Culture and cell transfection-A375 melanoma cells were maintained in RPMI supplemented with 5% fetal calf serum (FCS; Invitrogen, Inc., Grand Island, NY). HEK293T cells were maintained in DMEM supplemented with 10% FCS (Invitrogen). For transient expression, plasmids were introduced into cells using the Effectene reagent

(Qiagen, Hilden, Germany) according to the manufacturer's protocol. Stable ASER cells were obtained by transfection of A375 cells with pcDNA3.1-Stat-ER. Transfected cells were selected using 1 mg/ml G418 (Invitrogen) and several clones were picked, expanded and analyzed for STAT3-ER expression. Similar results were obtained with two independent clones.

For siRNA transfection, cells were plated at a density of 5 x 10^4 cells/well in six-well dishes, 24 h before transfection. The RNA oligonucleotides were obtained from XERAGON Inc. (Huntsville, AL). SiRNA duplexes (0.2 nmole/well) were introduced into the cells using 3 µl OligofectAMINE reagent (Invitrogen) according to the manufacturer's protocol. The following 21-mer oligoribonucleotide pairs were used: for C/EBPδ (accession number NM005195) nucleotide 844 to 864, for TEL (accession number NM001987) nucleotide 540 to 560, for control LacZ (accession number M55068) nucleotide 4277 to 4297 (obtained from D. Cappellen, FMI, Basel). SiRNA sequences were 'blasted' against the GenBank®/EMBL databank to ensure gene specificity. Transfection with expression vectors was carried out using Effectene 24h after introduction of siRNA.

Immunoprecipitation and western blots-Cells were harvested and lysed in NP40 extraction buffer [50 mM Tris (pH 7.5), 1 mM EGTA, 5 mM EDTA, 120 mM NaCl, 1% NP40, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM ß-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride] for 5 min on ice. The lysates were clarified by centrifugation and protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories GmbH, Munich, Germany). For immunoprecipitation,

equal amounts of proteins were incubated with an anti-Stat3 antibody (F-2; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Immune complexes were collected with protein G-Sepharose (Sigma) and washed three times with lysis buffer. Precipitated proteins were released by boiling in loading buffer and subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Boehringer Mannheim, Mannheim, Germany). After blocking with 10 % horse serum (Invitrogen) in 50 mM Tris pH 7.5, 150 mM NaCl, 0.05 % Tween20, filters were probed with specific antibodies: c-Tel (kindly provided by G. Grosveld), Stat3 (C-20), cyclin D2, cyclin D3 and cyclin E from Santa Cruz Biotechnology, phospho-Stat3 (Tyr 705) and p27 Kip1 from Cell Signaling Technology (Beverly, MA), cyclin D1 from Novocastra Laboratories Ltd, Newcastle upon Tyne, UK. Proteins were visualized with peroxidase-coupled secondary antibodies using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

DNA-binding assay-Nuclear extracts from cells treated with 4HT (1 μM) or OSM (100 ng/ml) were prepared as described (29). To measure ETS DNA binding, nuclear extracts were pre-cleared with immobilized NeutrAvidin beads (Pierce, Rockford, IL). 5'-biotinylated double-stranded oligonucleotide, corresponding to the ETS-binding sequence 5'-ATAAACAGGAAGTGG-3' (30), was pre-incubated with NeutrAvidin beads. Immobilized ETS oligonucleotides or Stat3 TransCruz oligonucleotide agarose conjugate (Santa Cruz Biotechnology) were added to 80 μg of nuclear extracts in binding buffer (20 mM HEPES, 0.5 mM EDTA, 1 mM DTT, 2 μg/ml poly dI-dC) and rotated for 2 h at 4°C. Samples were centrifuged and the pellets were washed three more times with

binding buffer. Proteins were eluted from beads by boiling in loading buffer and SDS-PAGE was performed as described above.

Flow Cytometric Analyses-Cells were harvested after treatment with 4HT or OSM/4HT, washed three times with ice-cold phosphate-buffered-saline (PBS) and resuspended in propidium iodide buffer [1mM sodium citrate (pH 4.0), 1.5 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 % NP40, 4 mg/ml propidium iodide and 80 mg/ml RNaseA]. After 30 min incubation on ice, cell cycle distribution was measured with a Becton Dickinson FACScan flow cytometer. In some cases, the cells were arrested in the G1 phase of the cell cycle, by treating cultures with UO126 (25 μM; Promega Corporation, Madison, WI) for 24 h either with or without the addition of 4HT. Cells were then washed in PBS before addition of control or 4HT-containing medium. Cells were harvested at the times indicated in Fig. 3B and treated as described above.

Luciferase Assays-Cells (2 x 10^5 cells in 6-well plates) were transfected, as described above, with a luciferase reporter containing four copies of the acute phase response element (APRE) from the α2-macroglobulin gene in front of the minimal junB promoter (14), a generous gift from Dr M. Hibi and T. Hirano (Osaka University, Osaka, Japan), together with the *Renilla* control plasmid pRL-SV40 from Promega. Twenty-four hours after transfection, cells were either left unstimulated or stimulated with OSM, 4HT or the combination of 4HT/OSM for 24 hours. In some instances, Trichostatin A (Sigma) was added to the medium at 250 nM. The preparation of cell extracts and measurement of luciferase activity were carried out using the Dual-Luciferase reporter assay system (from Promega) in an Autolumat LB953 (Berthold Technologies, Wildbad, Germany). Changes

in firefly luciferase activity were expressed relative to *Renilla* luciferase activity in the same sample.

Microarray analysis and quantitative radioactive RT-PCR-Microarray analysis was performed using HG U95A GeneChips™ (Affymetrix, Santa Clara, USA). Biotin-labeled cRNA probes were generated from each sample starting from 10 μg of total cellular RNA, which was extracted using TRIzol (Invitrogen) and further purified with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The cRNA probes were hybridized on the arrays and signals detected according to the manufacturer's instructions. Chip analysis was performed using the Affymetrix Microarray Suitev5 (target intensity 500 used for chip scaling) and GeneSpring 4.2.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test (as recommended by Affymetrix). The "change" p-value threshold was < 0.003 for increase and > 0.997 for decrease. After concordance analysis these values become $< 9 \times 10^{-6}$ and > 0.999991 respectively. Any gene whose detection p-value was > 0.05 was discarded from the analysis.

For quantitative PCR, cells were transfected with siRNA duplexes 48 h before treatment with 4HT. Cells were then harvested in a guanidinium isothiocyanate-containing buffer and total RNA was purified using RNeasy mini kit (Qiagen). The quantitative radioactive RT-PCR for C/EBPδ was performed as described (31) using the following primers: forward 5'-GCCGACCTCTTCAACAGCAA-3' and reverse 5'-CAAGCTCACCACGGTCTGTC-3' (30 cycles).

RESULTS

Stat3-ER is a tool to investigate biological functions of Stat3.

Stat3 is required for inhibition of breast carcinoma and melanoma cell proliferation by IL-6-type cytokines. In order to investigate the role of Stat3 in this process, independently of other IL-6-induced signaling pathways, we designed a conditionally active Stat3. It has been shown that dimerization of Stats is sufficient for nuclear translocation, even in the absence of Tyr phosphorylation (10,32). Therefore, the entire Stat3 coding sequence was fused to the estrogen receptor ligand-binding domain (ER-LBD), which contains a dimerization domain. In the presence of the ER-LBD ligand 4-hydroxytamoxifen (4HT), similar chimeric constructs were shown to translocate to the nucleus and stimulate Stat-dependent transcription (32).

The Stat3ER construct was transfected into A375 melanoma cells, whose proliferation has been shown to be reduced by IL-6 and OSM (16), and A375 clones expressing high levels of Stat3-ER (ASER clones) were selected. Using a consensus Stat3-binding oligonucleotide as an affinity probe, Stat3-ER DNA binding activity was measured in nuclear extracts obtained from ASER cells, treated with 4HT for different times (Fig. 1A). While low Stat3ER-DNA binding, as measured by western blotting analysis of the oligonucleotide-bound proteins, was detected as early as 20 min after stimulation, binding increased strongly after two hours to reach a maximum 24 h after 4HT addition (Fig. 1A). This increase in DNA binding was accompanied by an increase in Stat3-ER protein levels, probably due to ligand-dependent stabilization of the Stat3-ER fusion protein (data not shown).

Transcriptional activity of Stat3-ER in ASER cells was verified by introduction of a luciferase reporter gene driven by the acute-phase response element (APRE) of the α_2-macroglobulin gene, a Stat3 target. 1 μM 4HT, which we have determined to be the optimal concentration, stimulated APRE reporter activity as efficiently as 100 ng/ml OSM (Fig.1B). Thus, Stat3-ER appears to be an appropriate tool to study the mechanism underlying Stat3's biological activity.

In order to evaluate the role of Stat3 in A375 cell proliferation, ASER cells were grown for 3 days in the presence of 4HT or OSM. Treatment with 1 μM 4HT reduced ASER cell number by about 40 %, which is equivalent to the effect of 100 ng/ml OSM (Fig. 1C). 4HT, at 1 μM, does not affect proliferation of A375 cells (data not shown), confirming that the anti-proliferative effects of 4HT are due to Stat3-ER activation. Thus, while Stat3 was previously shown to be required for OSM-induced inhibition of cell proliferation (15,16), our results show that it is also sufficient for this effect. Interestingly, the combination of 4HT and OSM had a dramatic effect on proliferation, reducing cell number by more than 90% over the 3-day time course (Fig. 1C). This observation raised the possibility that other pathways induced by OSM might reinforce Stat3's biological activity.

OSM enhances Stat3-ER transcriptional activity and DNA binding.

The mechanism by which 4HT and OSM cooperate to repress cell proliferation was further investigated. With respect to the APRE-driven reporter assay, the combination of 4HT and OSM dramatically increased luciferase activity, compared to the activity induced by 4HT or OSM alone (Fig. 1B). For instance, transcription induced by combination of 1 μM 4HT with 100 ng/ml OSM was more than 100-fold stronger than

the transcriptional activity induced by the single agents. IL-6 showed the same cooperative effect with 4HT (data not shown). Thus, in ASER cells, 4HT and gp130 signaling synergize to increase Stat3 transcriptional activity.

In A375 cells, OSM and IL-6 binding to their receptors trigger multiple signaling pathways including the JAK/Stat, the MAPK and PI3K pathways (data not shown). We have investigated whether these pathways are responsible for enhancing Stat3-ER transcriptional activity, by using chimeric receptors composed of the extracellular domain of the erythropoietin (EPO) receptor fused to various regions of the gp130 cytoplasmic domain (Fig. 2A), in order to specifically activate individual pathways (33). EPO was used to activate chimeric gp130 signaling, but not endogenous gp130 (34). A375 cells were transfected with the EG chimera, which contains the transmembrane and the entire cytoplasmic domain of gp130 (Fig. 2A), together with the Stat3-ER construct and the APRE-driven luciferase reporter. Stat3 transcriptional activity was evaluated after treatment with EPO and/or 4HT. While EPO and 4HT added individually had only a modest effect on Stat3 activity, the combined addition of EPO and 4HT induced strong luciferase activity in EG expressing cells (Fig. 2B). A chimera containing only the membrane-proximal box1/box2 region of gp130, which mediates association with the JAKs, but has none of the Tyr residues required for Stat3 or SHP2 recruitment (ΔB; Fig. 2A), failed to respond to the concomitant addition of 4HT and EPO in comparison to 4HT alone. These results show that the chimeric receptors are an appropriate tool for studying the synergistic effect between the 4HT and gp130 signals. Cells were also transfected with chimeric receptor constructs containing the Stat3 (Y814) or SHP2 (Y759) tyrosine modules attached to the ΔB receptor, resulting in receptors capable of

specifically activating Stat3 or the MAPK/PI3K pathways, respectively (35). Treatment of the Y814-transfected cells with EPO and 4HT strongly increased transcriptional activity when compared to the response observed when the ligands were added individually (Fig.2B). In contrast, in cells expressing the receptor with the SHP2 module (Y759), no synergy was observed between EPO and 4HT (Fig. 2B). These results indicate that the gp130/4HT synergy involves only Stat3 signaling and not the SHP2-dependent pathways.

Next, we examined specific DNA binding of Stat3-ER to a Stat3 consensus oligonucleotide. ASER cells were treated with 4HT to induce Stat3-ER binding. OSM was then added for the indicated times and DNA binding of Stat3-ER and endogenous Stat3 was monitored as described above. Binding of endogenous Stat3 to the DNA probe could be detected 15 min after addition of OSM, peaked at 30 min and returned to low levels after 1 h of treatment (Fig. 2C, upper panel). In the presence of OSM, Stat3-ER DNA binding was enhanced and, unlike the endogenous Stat3, was maintained for several hours (Fig 2C, upper panel). Levels of Tyr705 phosphorylation of Stat3/Sta3-ER followed the kinetics of DNA binding (Fig. 2C, lower panel). These results indicate that OSM-induced gp130 activation leads to phosphorylation of both Stat3 and Stat3-ER; while Stat3 gets rapidly dephosphorylated, Stat3-ER displays prolonged phosphorylation and DNA-binding. This result could explain the synergy between 4HT and OSM for Stat3-dependent transcriptional activity and Stat3-induced inhibition of cell proliferation.

Stat3 activity inhibits A375 cell proliferation.

In order to understand the mechanism by which Stat3 activity leads to decreased cell number, cell cycle analyses were performed after treatment of ASER cells with 4HT

and/or OSM for up to 3 days. Flow cytometry revealed that neither 4HT (Fig. 3A) nor the physiological activator OSM (data not shown) significantly affected the cell cycle profile, *i.e.*, there was no obvious accumulation of cells in a particular phase of the cell cycle. A375 cells can be arrested in the G1 phase of the cell cycle by blocking the Ras/MAPK pathway (35). ASER cells that had first been synchronized in the G1 phase of the cell cycle and then released in 4HT-containing medium showed delayed entry into the S phase compared to cells released in control medium (Fig. 3B). These results suggest that Stat3 activity negatively influences proliferation by increasing transit time of the cells through the cell cycle, without influencing a particular phase of the cell cycle.

In contrast to treatment with a single compound, ASER cells treated for 24 hrs with a combination of 4HT and OSM accumulated in the G1 phase of the cell cycle (+ 24%; Fig. 3C). After 3 days, a large proportion of these cells underwent apoptosis, as indicated by the appearance of a sub-G1 peak and PARP cleavage (not shown). Thus, the striking reduction in cell number induced by the combined action of 4HT and OSM is largely due to cell death.

We next analyzed nuclear cell cycle regulators known to be involved in the G1-S transition. There were no detectable changes in the protein levels of cyclin D1, D2 or D3, or the cyclin-dependent kinase inhibitor $p27^{Kip1}$, in cells treated with 4HT or OSM for 24 h (Fig 3D); $p21^{Cip1}$ was not detected in any of the ASER lysates (data not shown). In contrast, cells treated with the combination of 4HT and OSM had lower amounts of cyclin D2, increased expression of $p27^{Kip1}$ (Fig. 3D) and increased levels of cdk2/$p27^{Kip1}$ complexes (data not shown), which is the likely cause of the accumulation of cells in G1 (Fig. 3C).

Transcriptional profiling of Stat3 target genes.

We used oligonucleotide microarrays to identify Stat3 target genes that might contribute to the anti-proliferative effects of Stat3. Experiments were carried out on two independent ASER clones expressing similar amounts of Stat3-ER and showing a similar response to 4HT with respect to DNA binding, reporter gene assays and inhibition of cell proliferation (data not shown). Stat3 DNA binding (Fig. 1A) and reporter gene activity (data not shown) were strongly increased after 2 to 4 h of 4HT treatment. Accordingly, RNA was collected from Stat3-ER expressing cells after 4 and 24 h of 4HT treatment. RNAs from three independent experiments were pooled and the resulting biotinylated cRNAs were hybridized to the Affymetrix U95A oligonucleotide array containing probe sets for over 12,000 transcripts. Genes that were significantly increased or decreased in both clones, relative to untreated cells (change $p<0.00009$) were identified. Genes that were significantly increased or decreased upon 4HT treatment of parental, non-transfected A375 cells were excluded from the study.

Several known direct targets of Stat3 were induced upon 4HT treatment of ASER cells, validating the experimental approach. For example, 4 h after addition of 4HT, the JAK inhibitor SOCS3, α-antichymotrypsin (serpin A3) and the transcription factors C/EBPδ and JunB were significantly increased (data not shown). Some genes, not previously described as Stat3 targets, were also activated within 4 h; these include the transcription factor TEL (ETV6), the death-associated protein kinase 1 (DAPK1) and the serine protease inhibitor serpin B3.

After 24 h, 4HT-induced Stat3 activation resulted in altered expression of a number of genes; 154 genes were increased and 23 were decreased. Genes whose

expression was increased more than 1.5 fold (64 genes) or decreased more than 1.3 fold (11 genes) are listed (Fig. 4). These genes belong to various functional groups encoding: transcriptional regulators that could mediate the Stat3 effect (TEL, C/EBPδ, FOS); receptors, *e.g.* OSM receptor; intracellular signaling molecules; adhesion proteins such as osteopontin (secreted phosphoprotein 1) and genes involved in various aspects of cellular metabolism.

Most of these genes were also activated by OSM, however, to a lower extent, most likely because OSM triggered a more moderate transcriptional response. It is noteworthy that the majority of these genes is sensitive to the level of Stat3 activity, as they are very highly induced by the combined 4HT/OSM treatment (Fig. 4). However, 4HT/OSM triggered the activation of many more genes, which were not induced by 4HT alone (data not shown), which might be involved in specific program leading to G1 arrest and cell death.

The function of some Stat3 target genes (induced by 4HT treatment and by OSM treatment) identified in this screen was investigated further. We focused on two transcription factors whose expression is increased at early time points, since each of these could in turn control expression of secondary target genes contributing to Stat3's biological effects. C/EBPδ, a previously described Stat3 target, has been implicated in growth arrest of mammary epithelial cells (36). TEL, a newly identified Stat3 target, which belongs to the ETS family of transcription factors, is known to repress transcription of some genes that have an ETS consensus site in their promoter (37).

C/EBPδ mediates the anti-proliferative effect of Stat3.

The contribution of C/EBPδ to the anti-proliferative activity of Stat3 was evaluated using siRNA to knock-down its expression. When examined by quantitative PCR, C/EBPδ mRNA was increased following 4HT treatment (Fig 5A), confirming the data obtained by microarray analysis. Specific siRNA significantly reduced the expression of C/EBPδ, both basal and in response to 4HT (Fig. 5A, right lanes). The knock-down of C/EBPδ expression decreased Stat3's anti-proliferative activity in ASER cells by ~ 40 % (Fig. 5B; average of three independent experiments). Thus, C/EBPδ plays an important role in the anti-proliferative effects of Stat3.

TEL is a negative regulator of Stat3 activity.

The role of the newly identified Stat3 target, TEL, in Stat3's anti-proliferative effect was further evaluated. Microarray data indicate that TEL mRNA is increased within 4 h of Stat3 activation**.** Two TEL protein isoforms are found in most cells corresponding to initiation of TEL mRNA translation at ATG codon 1 and 43. Protein levels of both isoforms, TEL and TEL-43, steadily increased from 8 h after addition of 4HT to ASER cells (Fig. 6A). We also examined a panel of cancer cell lines and determined TEL levels in response to IL-6-type cytokines. TEL protein levels were significantly increased after IL-6 or OSM treatment of T47D and SKBr-3 breast carcinoma cells, DU145 prostate carcinoma cells and HepG2 hepatoma-derived cells (Fig. 6B), suggesting that TEL is a fairly common Stat3 target. However, TEL protein level was unchanged in other cell lines, especially those with constitutively elevated TEL levels, such as LNCaP prostate carcinoma and MDA-MB-468 breast carcinoma cells.

The role of TEL in Stat3 signaling was explored by knocking down its expression. Transfection of TEL siRNA strongly reduced TEL expression relative to the levels observed in cells transfected with the control LacZ siRNA (Fig. 7A). In contrast to the results observed after C/EBPδ knock-down, in the presence of TEL siRNA, Stat3-mediated inhibition of ASER cell proliferation was stronger (Fig. 7B), suggesting that TEL might be a Stat3-induced negative regulator of Stat3 signaling.

Since TEL is a transcriptional repressor (38), we tested whether siRNA-mediated knock-down of TEL affected Stat3 transcriptional activity. Stat3-dependent transcription induced by 4HT or OSM was significantly increased when TEL expression was reduced (Fig. 7C). Conversely, overexpression of TEL in ASER cells (Fig. 8A) and in HEK-293T cells (Fig. 8C) resulted in decreased Stat3 activity induced by 4HT and OSM, respectively. These results demonstrate that TEL is a negative regulator of Stat3 transcriptional activity.

Mechanism of TEL-dependent repression of Stat3 activity.

Our results show that TEL is a repressor of Stat3 transcriptional activity. A number of negative regulators acting at different levels of the Stat3 signaling cascade have been identified. The SOCS proteins inhibit JAK activity leading to decreased Stat3 Tyr phosphorylation; GRIM-19 interferes with Stat3 nuclear shuttling and PIAS3 prevents Stat3 from binding to DNA (25,26,39). Overexpression of TEL in A375 cells did not affect the Stat3 expression levels or tyrosine phosphorylation in response to OSM (data not shown). Moreover, Stat3 nuclear translocation and DNA binding were not changed when TEL was over-expressed in A375 cells (data not shown). Thus, TEL functions differently from the previously identified negative regulators of Stat3.

TEL repressor activity is dependent on the recruitment of a co-repressor complex, including molecules such as mSin3A, NcoR and SMRT (27,40). Since these three proteins are known to interact with histone deacetylases (HDACs), we tested the possibility that HDAC recruitment plays a role in TEL-dependent repression of Stat3 activity using Trichostatin A (TSA), a general HDAC inhibitor. Addition of TSA to 4HT-stimulated ASER cells prevented the repression of Stat3 activity by TEL (Fig. 8A), suggesting that TEL repressor activity toward Stat3 is dependent on the recruitment of HDACs.

The co-repressor, HDAC-containing complex has been shown to interact with at least two different domains of TEL: the pointed domain and a central repression domain (27,40). To identify the domains of TEL essential for repression of Stat3 transcriptional activity, various TEL mutants (Fig. 8B) were overexpressed in ASER cells or HEK-293T cells and 4HT- or OSM-induced Stat3 transcriptional activity was evaluated (Fig. 8C and D). Among the TEL mutants tested, only TEL ΔP, lacking the pointed domain, failed to repress Stat3 activity. Mutants with deletions in the central domain were still able to block Stat3 activity. Thus, repression of Stat3 activity by TEL requires an intact pointed domain. Intriguingly, a TEL DNA-binding domain mutant (TEL DBDM) retained the ability to repress 4HT- or OSM-induced Stat3 activity in ASER and HEK-293T cells respectively (Fig. 8C and D, right panels), showing that TEL represses Stat3 activity independently of its DNA-binding domain.

We next investigated the possibility that Tel and Stat3 form a complex in the nucleus of A375 cells. As expected, there was an increase in nuclear Stat3 levels following OSM addition, while TEL levels in the nucleus were unaffected by cytokine

treatment (Fig 9, right panels). TEL was detected in immunoprecipitates of Stat3, with increased levels in the OSM treated lysates, reflecting increased nuclear Stat3 (Fig 9, left panels). Pull-downs of TEL using an immobilized ETS consensus site-containing oligonucleotide confirmed this association, showing that there was more of the TEL/Stat3 complex in the nuclei of OSM treated cells (Fig. 9, middle panels). Taken together, these results lead us to suggest that TEL represses Stat3 transcriptional activity by interacting with Stat3 and recruiting HDACs to the Stat3 transcriptional complex.

DISCUSSION

Stat3 mediates the effects of multiple growth factors and cytokines and, as such, Stat3 is involved in many physiological processes, but also in disease, such as cancer. The identification of Stat3 effectors and regulators should improve our understanding of the multiple and sometimes contradictory biological roles attributed to this transcription factor. Indeed, some studies describe a role of Stat3 in oncogenesis, while others demonstrate its ability, acting downstream of cytokine receptors, to inhibit tumor cell proliferation. We have evaluated the anti-proliferative effect of Stat3 by introducing a conditionally active Stat3 into A375 melanoma cells. Our results show that depending on the extent of Stat3 activity, the cancer cells show decreased proliferation, or growth arrest accompanied by apoptosis. Stat3 targets in the melanoma cells were identified using oligonucleotide microarray analyses. Functional studies were carried out on two transcription factors whose expression was rapidly induced by Stat3: C/EBPδ, a previously described Stat3 target; and TEL, a novel Stat3 target, which is a member of

the ETS family of transcription factors. We report here that, in accordance with studies performed in mammary epithelial cells (41), C/EBPδ has an important role in mediating Stat3's anti-proliferative effect on A375 melanoma cells. In contrast, TEL is a novel repressor of Stat3 activity: the overexpression of TEL leads to decreased Stat3-driven transcription and the loss of TEL results in stronger Stat3-dependent transcription and anti-proliferative effects.

Stat3 activation caused a general decrease in the progression of A375 cells through all phases of the cell cycle, without affecting the levels of the major regulators of the G1-S transition, including p21, p27, D-type cyclins or cyclin E. Our results are in contrast to previous studies showing accumulation of cells in G1 and an increase in p27 levels upon cytokine-triggered Stat3 activation (16,42). This discrepancy might reflect differences in levels of Stat3 activity achieved or differences in the sensitivity of the cells to active Stat3. Indeed, we observed that further enhancing Stat3 activity in A375 cells, by combining 4HT with OSM, led to an increase in p27 levels, an accumulation of cell in G1 and massive apoptosis.

While a number of previously described Stat3 target genes, *e.g.* JunB, serpin A3, fibronectin or VEGF (5,43,44) were detected in our analysis, others, including Pim 1 or angiotensinogen (5,45) were only detected when Stat3 activity was synergistically enhanced by 4HT and OSM^2. However, some genes, such as Bcl-X_L, cyclin D1 and Myc, which were strongly up-regulated by Stat3 activation in rat fibroblasts (10) were not increased even after combined treatment of A375 cells with 4HT and OSM. Thus, Stat3's ability to affect specific target genes is dependent upon the cellular context.

Considering the importance of Stat3 in different biological processes, tight regulation of the Stat3 signaling pathway is essential and is achieved via diverse mechanisms. We have identified TEL, a novel Stat3 target, and have shown that it plays a role in a Stat3 negative feed-back loop. In contrast to other negative regulators of Stat3, *e.g.* SOCS3, GRIM-19 and PIAS3 (25,26,46), which act at various levels of the JAK/Stat3 pathway causing an overall reduction in DNA-bound Stat3, TEL does not affect Stat3 DNA binding, but represses its transcriptional activity. Our finding that TEL levels are induced in many different cancer cell lines after IL6 or OSM treatment suggests that TEL might be a general Stat3-induced negative regulator.

The repressive activity of TEL has previously been shown to depend on recruitment of a co-repressor complex comprising mSin3A, NcoR and histone deacetylases (HDACs) to distinct TEL domains (27,40,47,48): the C-terminal pointed domain (also called helix-loop-helix domain or B-domain), which is necessary for TEL oligomerization and association with other proteins (38,48,49); the N-terminal ETS domain, which interacts with specific DNA elements, but also mediates protein-protein interactions (27,49,50); and the central TEL region spanning amino-acid residues 268-333, which has been found to associate with HDAC3 (27). We show here that TEL-mediated repression of Stat3 is dependent on HDAC activity and found that the TEL pointed domain, but not the central region, is required for this repression.

Interestingly, neither point mutations in the ETS domain, which prevent TEL DNA-binding, nor complete deletion of the ETS domain[3] prevented repression of Stat3 activity. To our knowledge, this is the first study showing that TEL repressor function does not require the ETS DNA binding domain. The fact that TEL and Stat3 could be co-

immunoprecipitated from nuclear extracts suggests that TEL can be recruited to the Stat3 transcriptional complex, even in the absence of DNA-binding, through protein-protein interaction.

By knocking-down TEL expression levels, we show here that TEL regulates Stat3's anti-proliferative effects. Recent studies suggest that TEL might also control Stat3's oncogenic activity. Indeed, it has been shown that TEL-43 inhibits transformation of NIH-3T3 fibroblasts by Src (51), which is known to depend upon Stat3 for its oncogenic activity (7,8). Our data suggest that TEL might inhibit Src-induced transformation through its ability to repress Stat3 activity.

The fact that inhibition of TEL nuclear function is required for Src to induce cellular transformation (51) suggests that the balance between Stat3-activating signals and Stat3-repressing signals determines the biological outcome. Excessive signaling to Stat3, which is known to occur in many tumors, has been associated with abnormal activity of intracellular kinases, growth factor and cytokine receptors (9,52-54). Recent studies demonstrate that persistent Stat3 signaling can also result from the silencing of negative regulators, such as SOCS1 or PIAS3 (55,56). The absence of TEL might have a similar role in some tumors and especially leukemias.

The *TEL* gene is often disrupted by chromosomal translocation in leukemia, most commonly with AML-1 (t (12;21)), resulting in production of a TEL-AML fusion proteins. This is often associated with loss a of heterozygosity (57,58). Fusions of TEL with the ABL and JAK2 tyrosine kinases, leading to constitutively active kinases, have also been reported (58-61). Interestingly, TEL-JAK2 and TEL-ABL fusion proteins induce constitutive activation of Stat3, which was shown to cause hematopoietic

precursor cell transformation (9). Our results suggest that chromosomal translocations involving TEL could promote transformation, not only through JAK- or ABL-mediated activation of Stat3, but also through attenuation of TEL-mediated negative regulation of Stat3 due to the loss of one *TEL* allele, especially if the non-rearranged *TEL* allele is also lost or mutated (58,59,61). Our data showing that TEL is a novel type of negative regulator of Stat3 activity call for further analysis of the impact of TEL on Stat3 biology.

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FOOTNOTES

[1] The abbreviations used are: Stat3, signal transducer and activator of transcription 3; IL-6, interleukin-6; OSM, oncostatin M; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; SOCS, suppressor of cytokine signaling; C/EBPδ, CCAAT/enhancer-binding protein delta; 4HT, 4-hydroxytamoxifen; HDAC, histone deacetylase; TSA, Trichostatin A; FACS, fluorescence-activated cell sorter

[2] N. Schick and A. Badache, unpublished observations.

[3] N. Schick and A. Badache, unpublished observations.

FIGURE LEGENDS

Fig. 1. Stat3 activation inhibits A375 melanoma cell proliferation (A) Nuclear extracts from ASER cells stimulated with 1 μM 4HT for the indicated times were incubated with an immobilized Stat3 consensus oligonucleotide and the amount of bound Stat3ER was analyzed by western blotting. (B) ASER cells transfected with an APRE-luciferase reporter and the *Renilla* plasmid were treated with OSM, 4HT or the combination of OSM and 4HT for 24h. Luciferase activity was determined and normalized to the *Renilla* internal control. (C) ASER cells were stimulated with 100 ng/ml OSM, 1 μM 4HT or the combination of OSM and 4HT and cell number was determined at the times indicated. The experiments were performed a minimum of three times and a typical result is shown. Bars represent averages of three determinations +/- SD.

Fig. 2. OSM and 4HT synergize to induce Stat3 activity. (A) Schematic representation of chimeric receptors containing the extracellular and transmembrane region of the mouse erythropoietin (EPO) receptor fused to various portions of the gp130 cytoplasmic domain. EG: chimera with the full length gp130 cytoplasmic tail; ΔB: truncated gp130 construct containing only the membrane proximal box1/box2 region; Y759 and Y814: ΔB with a fusion of 'tyrosine modules' mediating SHP2 or Stat3 respectively. (B) A375 cells were co-transfected with constructs coding for the indicated chimeric receptor and the Stat3-ER together with an APRE-luciferase reporter plasmid and a *Renilla* plasmid, before stimulation with EPO (3.5 U/ml), 1 μM 4HT or EPO/4HT for 24 h. Luciferase activity was normalized to the *Renilla* internal control. (C) ASER cells were treated with 4HT or the combination of 4HT and OSM for the indicated times. Binding of Stat3ER

and Stat3 to an immobilized Stat3 specific consensus DNA probe was analyzed as described in Fig. 1. The blot was stripped and re-probed for phosphorylation of Stat3 Tyr705 (PY-Stat3).

Fig. 3. Stat3-mediated effects on cell cycle distribution of ASER cells. Cultures were treated with 1 μM 4HT (A) or OSM/4HT (C) for 24 h. Cells were harvested and nuclei were stained with propidium iodide, before flow cytometry was performed. Representative histograms are shown. Changes in the percentage of cells in each phase of the cell cycle is indicated. (B) Cells were synchronized in G1 phase then released into control or 4HT-containing medium and cell cycle distribution was analyzed at the indicated times. (D) Western blotting analysis of regulators of the G1-S transition in lysates from ASER cells treated with 4HT, OSM or the combination of 4HT and OSM for 24 h.

Fig. 4. Stat3 target genes. Genes whose expression was changed after 24 h 4HT-treatment of ASER cells were identified and hierachichally clustered, based on their expression levels after 4 h 4HT, 24 h OSM, 24 h 4HT and 24 h OSM/4HT treatments. Data are presented in a matrix format: each row represents a single gene, and each column an experimental sample. Red represents expression above and blue represents expression below the median value. Experiments are indicated at the top and genes are listed on the right.

Fig. 5. C/EBPδ mediates anti-proliferative effects of Stat3. ASER cells were transfected with control siRNA (LacZ) or C/EBPδ siRNA for 48 h prior to 4HT treatment. (A) The levels of C/EBPδ mRNA after stimulation with 4HT for 24 h were analyzed by quantitative radioactive RT-PCR. The radioactive products were separated by polyacrylamide gel electrophoresis and imaged using a PhosphorImager. (B) Cell numbers were determined after stimulation with 4HT for 72 h. Percentage of inhibition induced by 4HT relative to unstimulated cells is shown.

Fig. 6. TEL protein level is increased following Stat3 activation in human cancer cells. (A) ASER cells were stimulated with 4HT for the indicated times, nuclear extracts were prepared and the abundance of TEL protein was determined by immunoblot analysis. (B) Various human cancer cell lines were stimulated for 24 h with OSM or IL-6, nuclear extracts were prepared and the protein level of TEL and active Stat3 (PY-Stat3) was determined by western blotting.

Fig. 7. TEL is a negative regulator of Stat3 activity. Cells were transfected with LacZ siRNA or TEL siRNA for 48 h prior to further treatments. (A) TEL protein levels were evaluated in nuclear lysates from cells treated with 4HT for 24 h. (B) ASER cell number was determined after 72 h 4HT or OSM treatment and expressed as percentage of inhibition relative to unstimulated cells. (C) ASER cells were transfected with an APRE-luciferase reporter plasmid and a *Renilla* plasmid and Stat3 activity was assessed after addition of 4HT or OSM for 24 h. Luciferase activity was normalized to the *Renilla* internal control.

Fig. 8. TEL-induced repression of Stat3 transcriptional activity does not require the TEL DNA binding domain but depends on the TEL pointed domain. (A) ASER cells were transfected with the TEL construct along with an APRE-luciferase reporter plasmid and a *Renilla* plasmid 24 h prior to stimulated with 4HT alone or 4HT and TSA (250nM) for 24 h. Luciferase activity was normalized to the *Renilla* internal control. (B) Schematic diagram of TEL mutants. DBDM: DNA-binding domain mutant, P: Pointed domain. (C and D) ASER cells and HEK-293T cells were transfected with the different TEL mutants, an APRE-luciferase reporter plasmid and a *Renilla* plasmid. Cells were treated with 4HT or OSM for 24 h, before determination of luciferase activity.

Fig. 9. TEL interacts with Stat3. Nuclear extracts were prepared from control or OSM-treated ASER cells. Left panel: extracts were subjected to immunoprecipitation (IP) with anti-Stat3 antibody and analyzed by western blotting using anti-TEL and anti-Stat3 antibodies. Middle panel: TEL was pulled down using an immoblized ETS consensus site-containing oligonucleotide before western blotting analysis with anti-TEL and anti-Stat3 antibodies. Right panel: levels of Stat3 and TEL in total nuclear lysates were determined by western blotting.

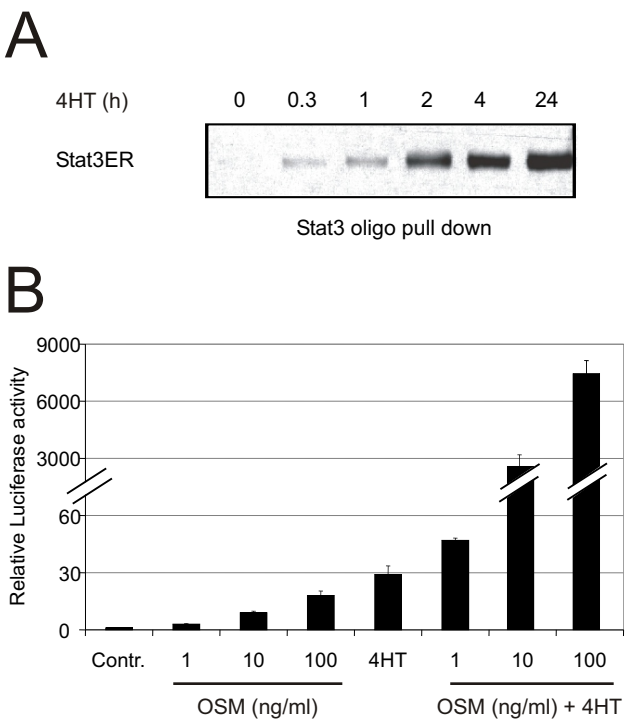
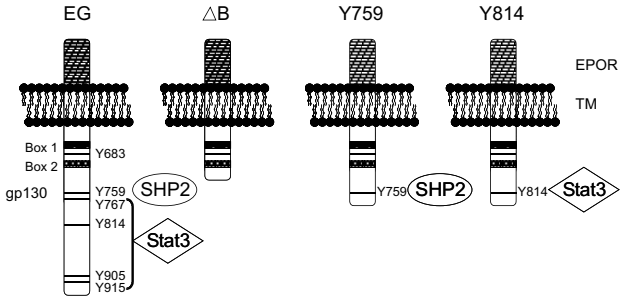


Figure 1 Nicole Schick, Edward J. Oakeley, Nancy E. Hynes and Ali Badache

A



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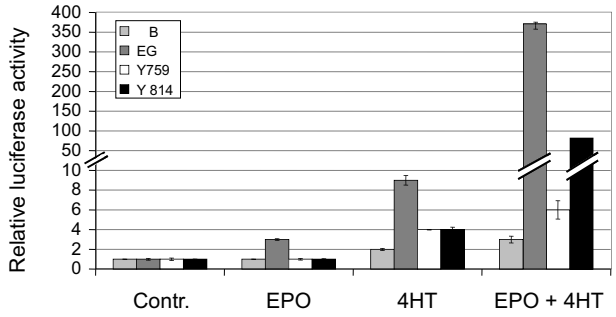

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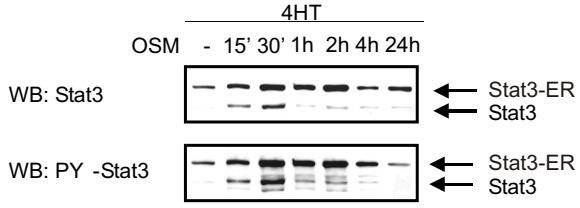

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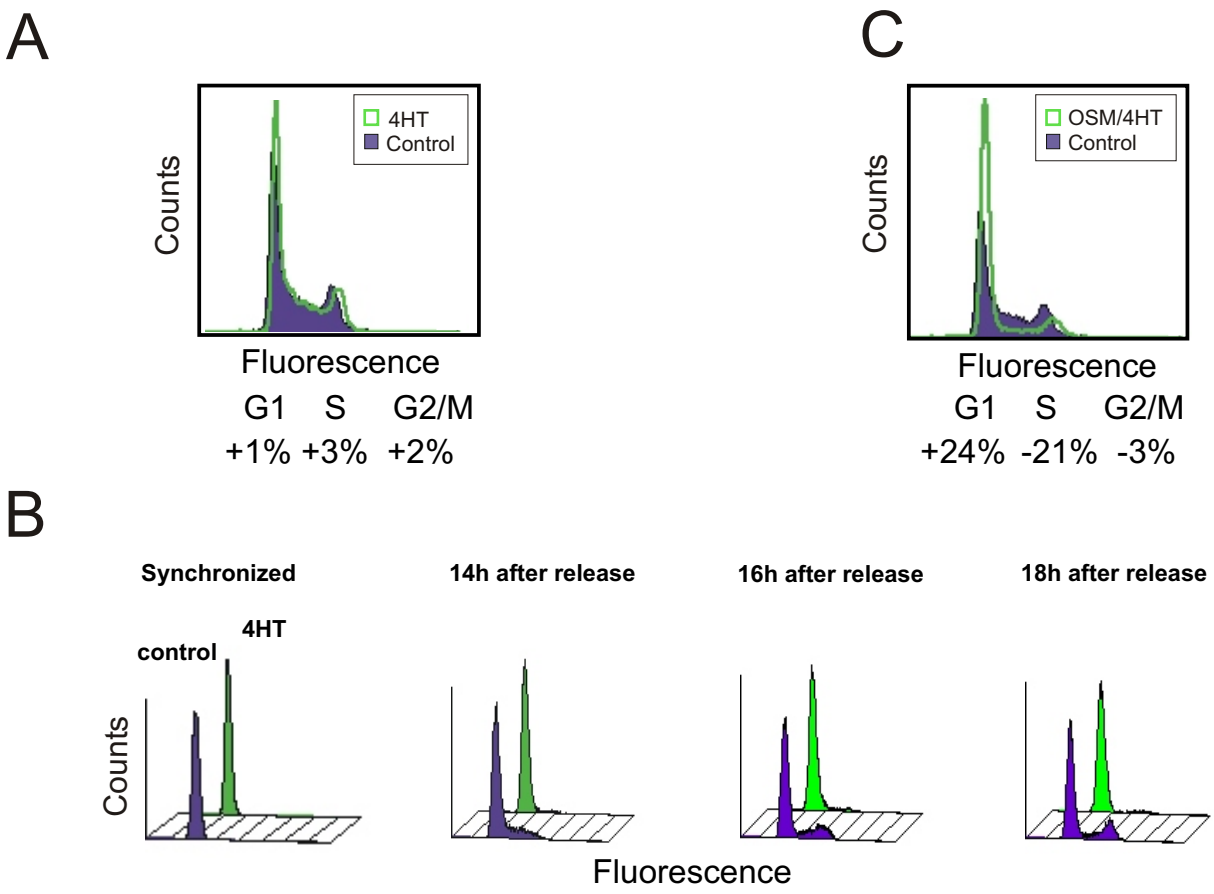
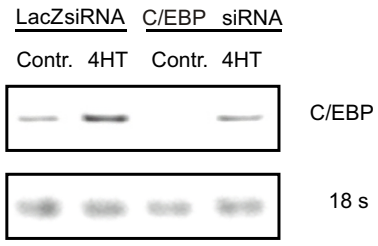


Figure 3 Nicole Schick, Edward J. Oakeley, Nancy E. Hynes and Ali Badache

Figure 4 Nicole Schick, Edward J. Oakeley, Nancy E. Hynes and Ali Badache

A



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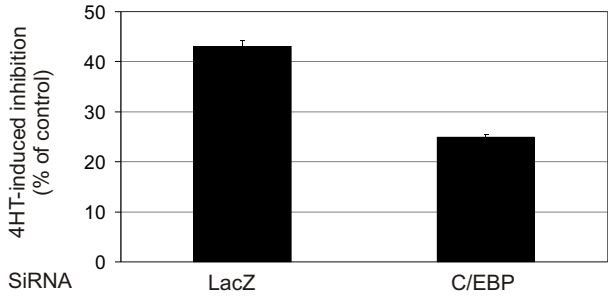

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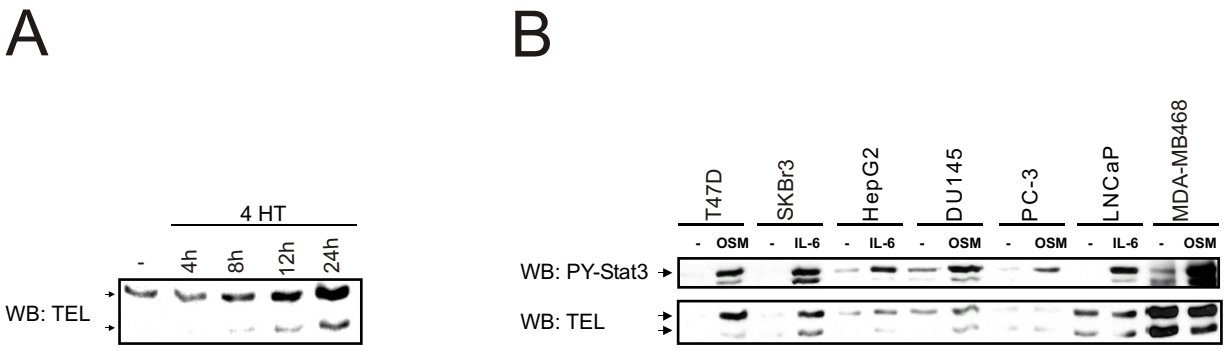

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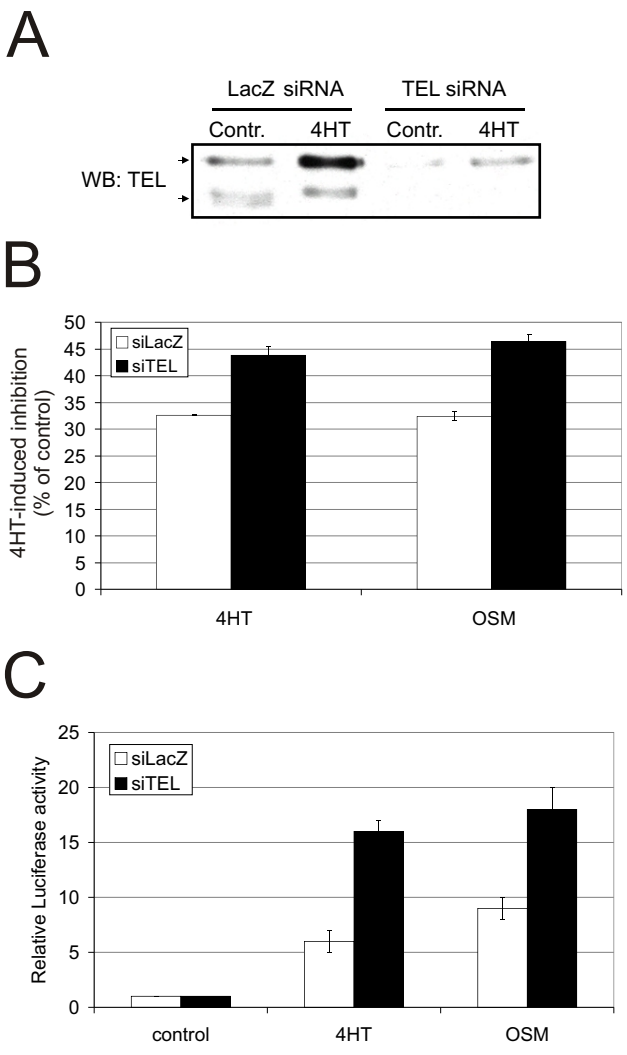

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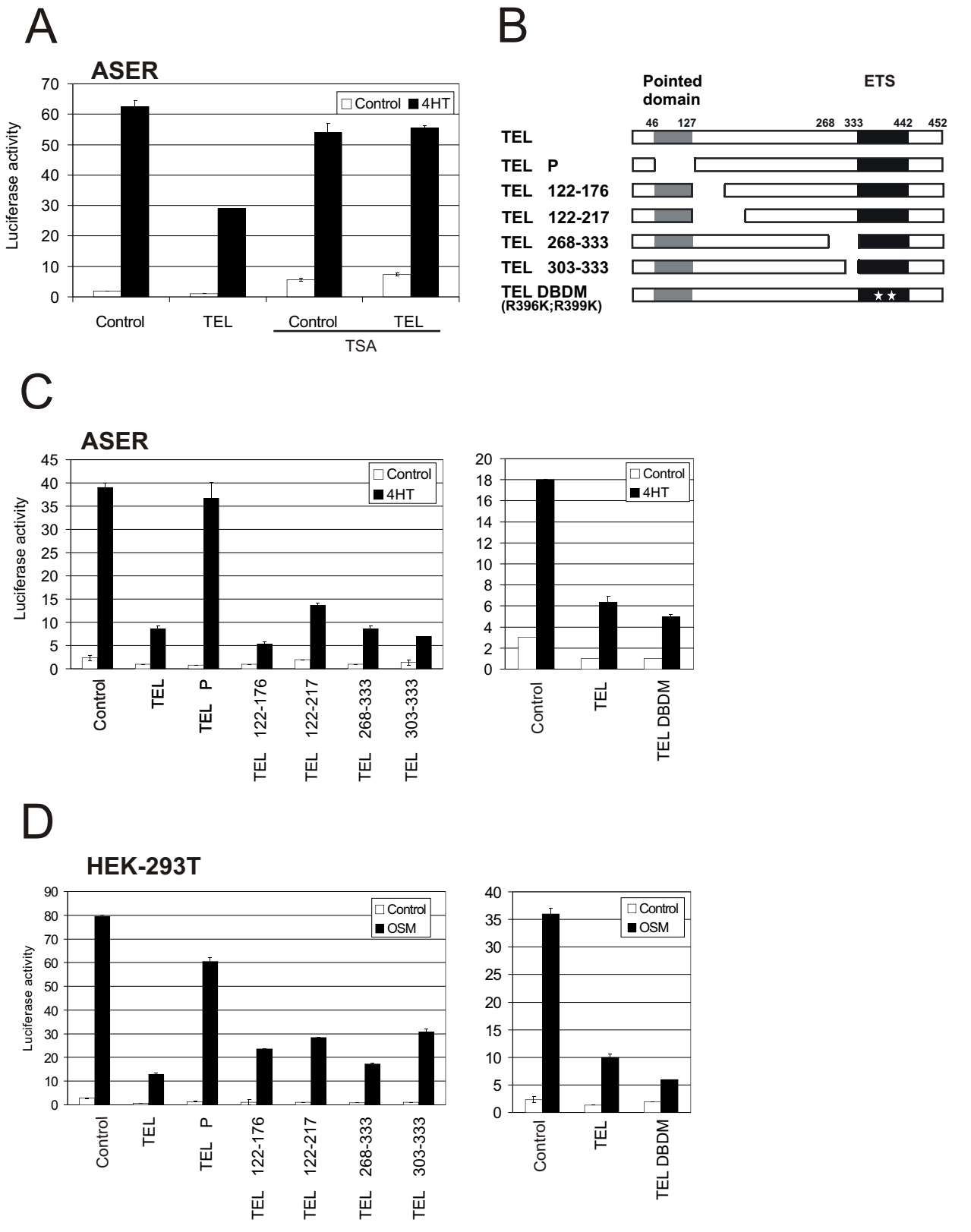

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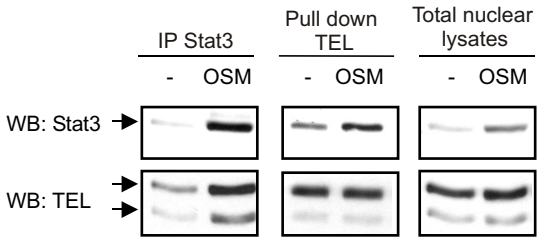


Figure 9 Nicole Schick, Edward J. Oakeley, Nancy E. Hynes and Ali Badache

IV. Appendix

Figure 1: Stat3ER protein levels increase after 24 h treatment of ASER cells with 4HT

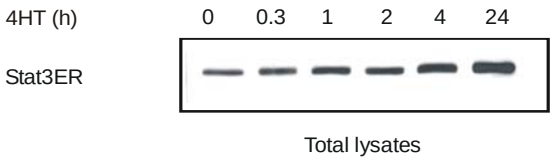


Figure 2: 1 μM 4HT is the optimal concentration to stimulate APRE reporter activity in ASER cells

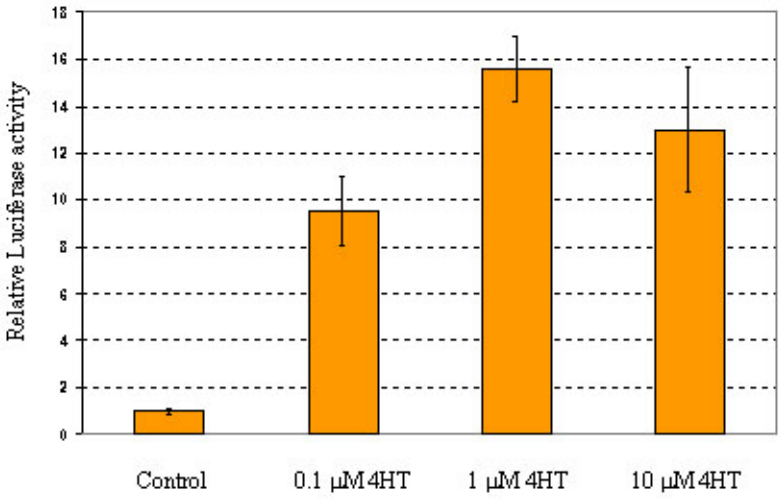


Figure 3: A375 cell proliferation is not reduced after stimulation with 1 μM 4HT

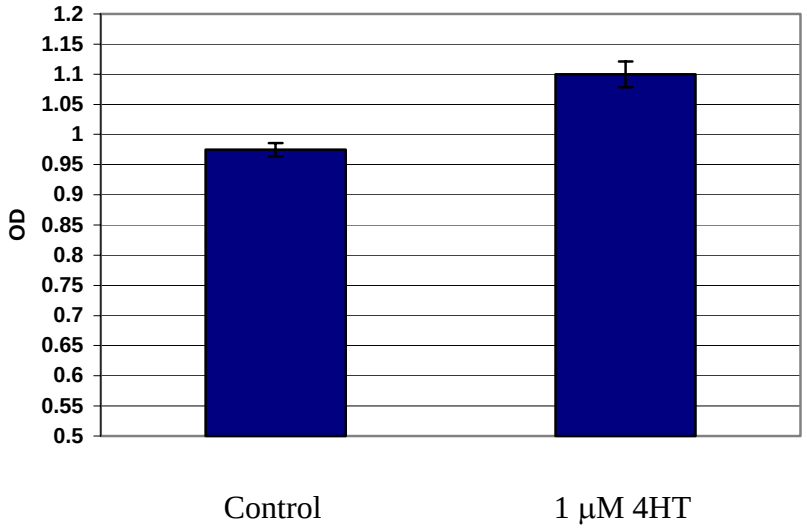


Figure 4: Stimulation of ASER cells with IL-6 (10 ng/ml) and 4HT (1 μM) shows the same cooperative effect that was observed with OSM and 4HT.

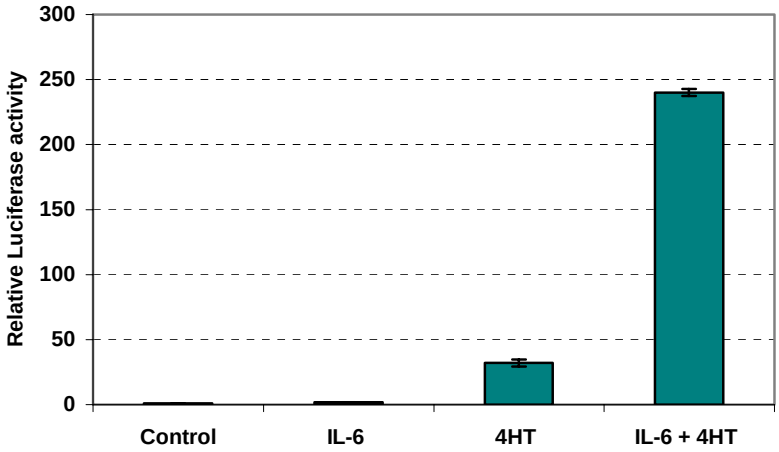


Figure 5: Stimulation of A375 cells with IL-6 and OSM leads to a dose dependent increase in the activity of the JAK/STAT, MAPK and PI3K signaling pathways.

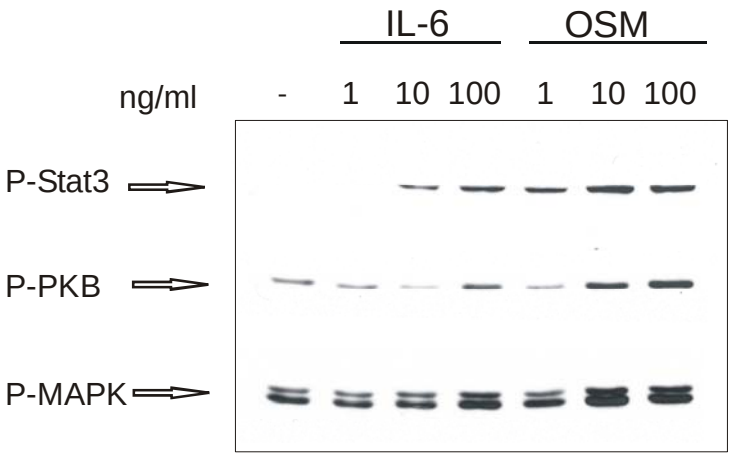


Figure 6: Stimulation of ASER cells with 100 ng/ml OSM does not affect the cell cycle profile after 48h and 72h of treatment.

- Cells were harvested after times indicated and nuclei were stained with propidium iodide, before flow cytometry was performed. Changes in the percentage of cells in each phase of the cell cycle are indicated.

OSM (100ng/ml)	Δ G1	Δ S	ΔG2/M
24h	+ 7%	- 7%	+ 1%
48h	+ 1%	+ 4%	- 5%
72h	- 4%	+ 3%	+ 1%

Figure 7: Simultaneous treatment of ASER cells with 100 ng/ml OSM and 1 μM 4HT leads to apoptosis as demonstrated by a sub-G1 peak.

	Δ G1	Δ S	ΔG2/M	Apoptotic cells
24h	+24%	-21%	- 3%	<1%
48h	+17%	- 7%	-17%	8%
72h	- 9%	- 5%	- 7%	21%

Figure 8: Simultaneous treatment of ASER cells with 100 ng/ml OSM and 1 μM 4HT for 24 h leads to increased association of $p27^{Kip1}$.with cdk2.

- Nuclear extracts were prepared from ASER cells treated with the stimuli indicated. Extracts were subjected to immunoprecipitation (IP) with anti-cdk2 antibody and analyzed by western blotting using anti-p27 antibody.

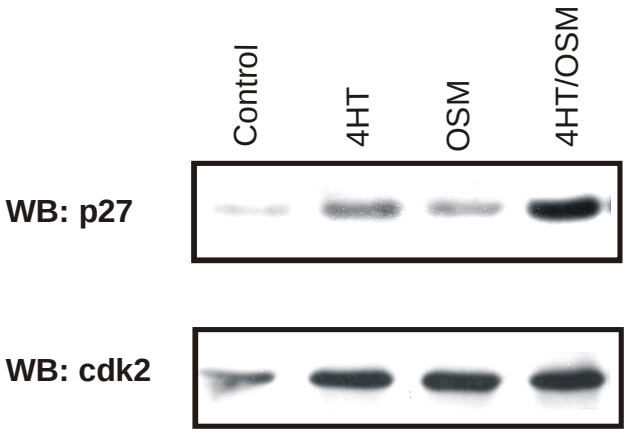
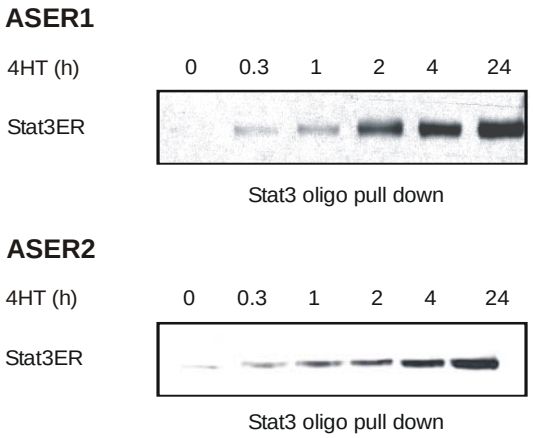
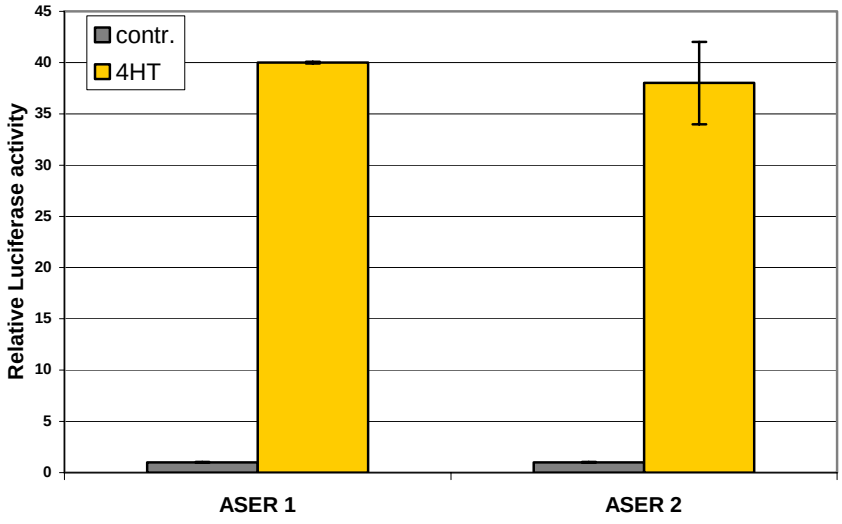


Figure 9: Two independent ASER clones show a similar response to 4HT treatment with respect to DNA binding, reporter gene assays and inhibition of cell proliferation.

- Stat3 DNA binding assay of two ASER clones stimulated with 4HT for the times indicated.



- Stat3 reporter assay of two ASER clones stimulated with 4HT for 24 h.



- cell proliferation assay of two ASER clones stimulated with 4HT or OSM for 72 h.

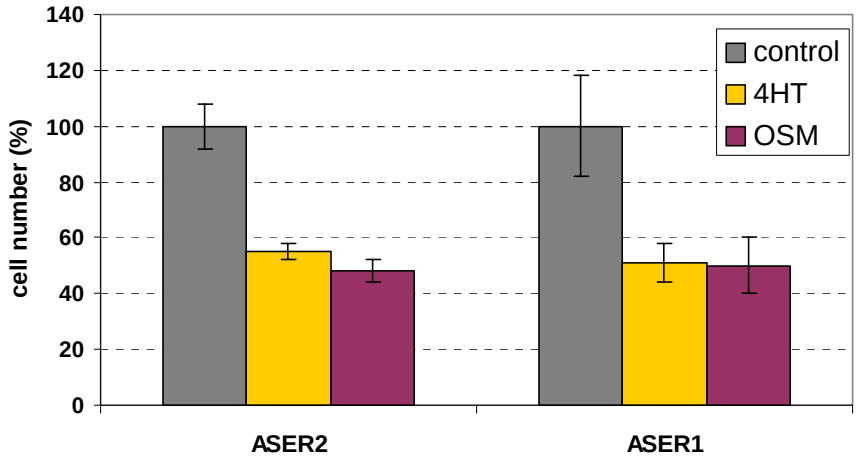


Figure 10: Stat3 reporter gene activity was strongly increased in ASER cells between 2 & 4 h of 4HT treatment.

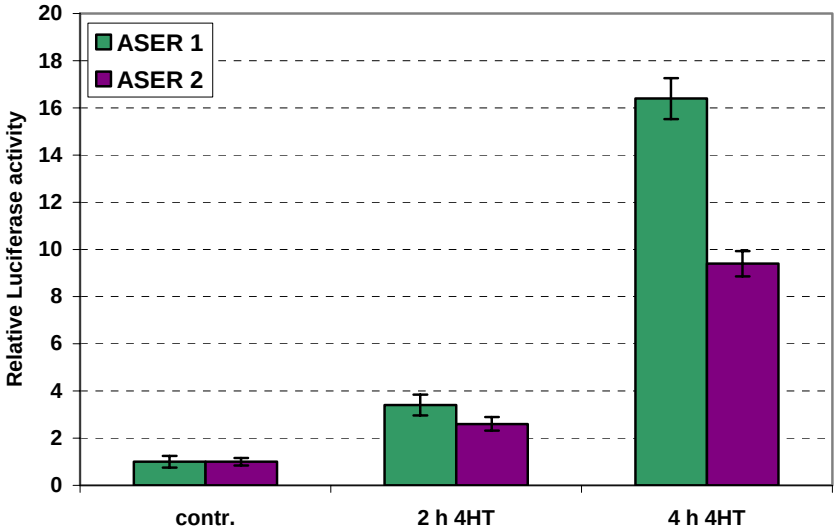


Figure 11: Microarray data showing that expression of several known Stat3 targets was increased after 4 h of 4HT treatment of ASER cells.

Gene	Fold increase (relative to control)
SOCS3	9.9
C/EBP δ	3.92
JunB	2.40
Serpin A3	2.27

Figure 12: Stimulation of ASER cells with 4HT and OSM triggeres the activation or repression of many more genes than stimulation of ASER cell with 4HT alone.

- Venn diagram of genes whose expression was increased more than 1.5 fold or decreased more than 1.3 fold after treatment of ASER cells with 4HT and OSM or 4HT alone.

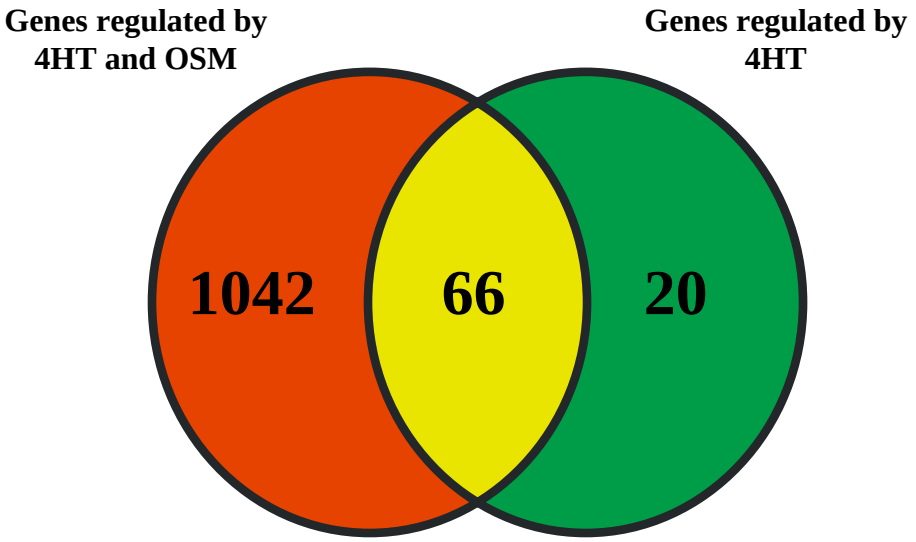
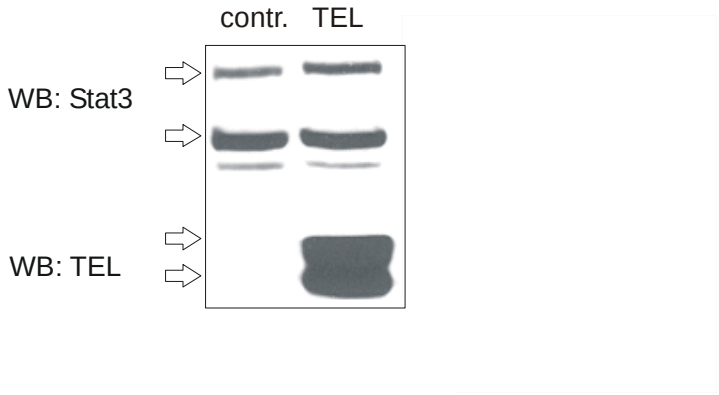


Figure 13: Overexpression of TEL did not affect Stat3 expression levels or nuclear translocation and DNA binding in response to treatment with OSM.

- Stat3 and TEL western blot of whole cell extracts of ASER cells transfected with control or TEL plasmids.



- Stat3 DNA binding assay of nuclear extracts of ASER cells transfected with TEL, followed by stimulation with OSM for 30 min.

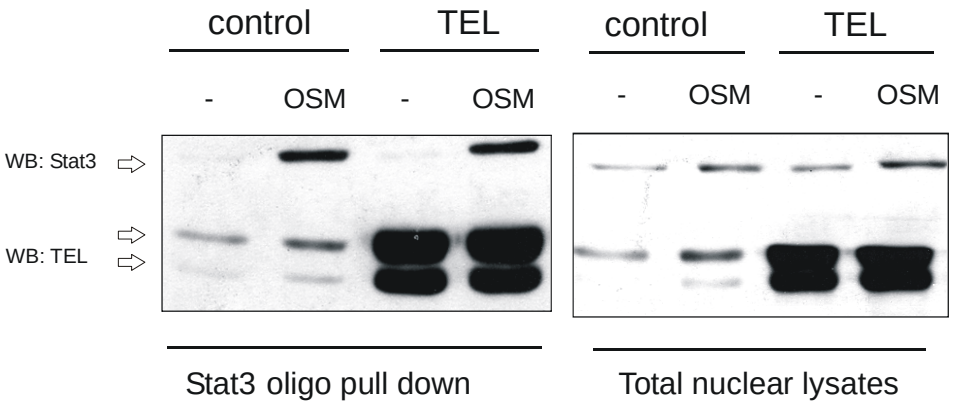
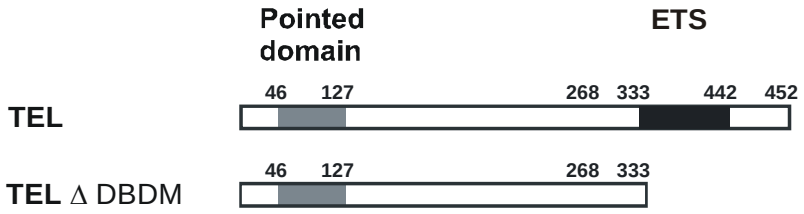


Figure 14: Microarray data showing that two known Stat3 target genes, angiotensinogen and PIM 1, were only upregulated after 24 h stimulation of ASER cells with 4HT and OSM together.

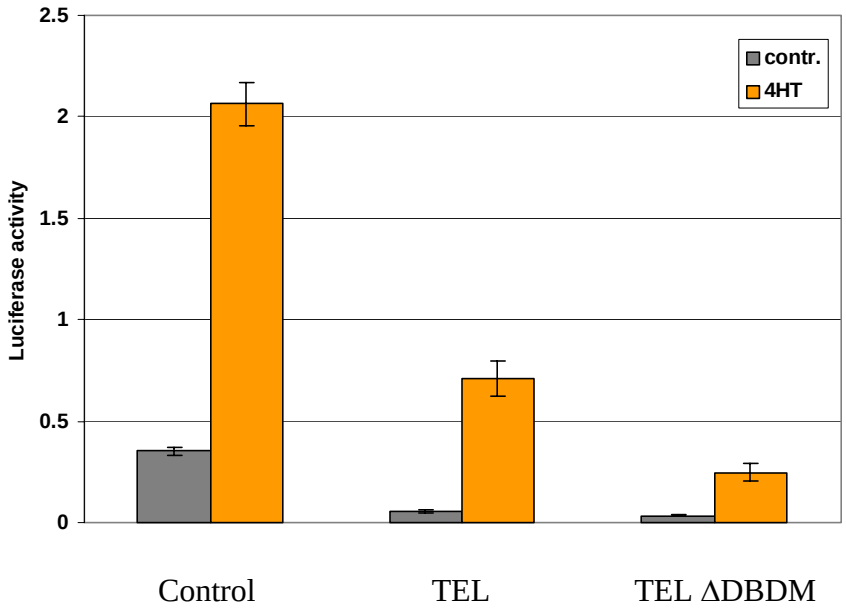
Gene	Fold increase (relative to control)
angiotensinogen	11.59
Pim 1	6.96

Figure 15: The TEL DBD is not essential for repression of Stat3 transcriptional activity.

- Schematic diagram of TEL ΔDBDM mutant.



- Stat3 reporter assay of ASER cell transfected with control, TEL or TEL ΔDBDM plasmids followed by 24 h stimulation with 4HT.



VII. Discussion

Cancer is a disease of deregulated cell proliferation and suppressed apoptosis, resulting from changes in gene expression that allow the cell to overcome normal growth inhibitory signals. In contrast to untransformed cells, which double only a limited time in culture and in vivo, most malignant tumor cells have the potential to grow indefinitely. It became clear in recent years that cancer arises through stepwise mutations, be it through exposure to some environmental factor (e.g. tobacco smoke) or because of a genetic predisposition, or both, that usually occur over a long timeframe. These mutations can be either as subtle as point mutations, or as obvious as changes in chromosome complement and they can cause either transformation of normal genes into oncogenes (gain-of-function) or loss of a tumor suppressor gene. Taken together, cancer development occurs via a stepwise acquisition of mutations, which lead to six essential features of a cancerous cell including; self-sufficiency in growth signals, insensitivity to growth -inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Traditionally, cancer drug discovery was pointed towards targeting DNA synthesis and cell division, resulting in drugs such as antimetabolites and alkylating agents that are efficient but often show severe side effects, since they lack selectivity for tumor cells compared to normal cells. From the 1980s onwards, a more targeted drug discovery approach has been developed, which focuses on specific key proteins in cell proliferation, such as tyrosine kinases. An advantage of the new model for anticancer drug discovery is likely to be targeted specificity leading to limited side effects. There are different possibilities to design a cancer drug; two successful options are the use of antibodies that block cell-surface receptors or small molecular drugs. Antibodies have the disadvantage that they are limited to proteins that span the plasma membrane since they cannot gain access to the interior of the cell. This weakness is balanced by the fact that they are more specific compared to small molecular drugs due to the unlikeliness that they inhibit other proteins. Herceptin, a monoclonal antibody against the Her2/neu receptor tyrosine kinase, is one of the best-studied antibodies and prolongs the survival of women with Her2/neu positive metastatic breast cancer in combination with chemotherapy. One example for a

small molecular kinase inhibitor is Gleevec, a drug that inhibits BCR–ABL, c-KIT and PDGFR and has shown remarkable success for the treatment of CML (chronic myeloid leukemia), a pluripotent stem cell disorder characterized by the Philadelphia chromosome translocation, generating Bcr-Abl (Sawyers, 2002). In recent years it has become apparent that transcription factors are good targets for cancer drug discovery. Two reasons speak for this; first, it is known that a limited set of transcription factors is constitutively active in most human cancers; second, there are many more human oncogenes in signaling pathways than there are oncogenic transcription factors, so blocking one transcription factor might inhibit multiple upstream oncoproteins as well. Three groups of transcription factors seem to play a role in cancer: The nuclear receptors, for example oestrogen receptors in breast cancer, androgen receptors in prostate cancer and retinoic-acid receptors (RAR) in acute promyelocytic leukemia (APL); the second group of transcription factors are resident nuclear proteins that are activated by serine kinase cascades, such as c-jun; the third group are latent cytoplasmic factors, which are normally activated by receptor ligand interaction at the cell surface, for instance NF-κB, β-catenin, notch intracellular domain and STATs. However, the inhibition of specific transcription factors or inhibition of their interactions with coactivators or accessory proteins is not an easy task and so far the only drugs available that target transcription factors are tamoxifen, bicalutamide or retinoids, which target oestrogen-, androgen- and retinoic-acid receptors (Altucci and Gronemeyer, 2001; Darnell, 2002).

As mentioned above STATs might be important for cancer progression since two members of the family, Stat3 and Stat5, are constitutively activated in a wide variety of human malignancies. Stat5 is mainly associated with various types of lymphomas and leukemias and it clearly implicated in cancer progression, whereas there are conflicting observations reported for Stat3. It is thought that Stat3 plays a critical role in cancer, given that constitutively active Stat3 is present in various cancers such as squamous cell carcinoma of the head and neck (SCCHN), breast, melanoma and prostate cancer cells (Bowman et al., 2000; Grandis et al., 2000; Leong et al., 2003; Mora et al., 2002) as well as in cells transformed by oncogenes, such as v-src and v-abl (Cao et al., 1996; Danial et al., 1995; Yu et al., 1995). The critical role of Stat3 was proven by experiments

performed with dominant negative Stat3, since this blocked the transformation induced by v-src (Bromberg et al., 1998; Turkson et al., 1998) and induced growth arrest and apoptosis in a wide variety of cancer cells (Darnell, 2002). Furthermore, transfection of cultured cells with Stat3-C, a constitutively active Stat3 mutant, can transform cells (Bromberg et al., 1999). These are all examples, in which constitutively Stat3 activity was induced via oncogenes or aberrant signaling of cytokines or growth factors associated JAKs. However, there is also a vast amount of data showing that controlled Stat3 activation via cytokine stimulation does not lead to transformation, and even inhibits cell proliferation and/or induces apoptosis of normal and cancerous cells (Kortylewski et al., 1999; Minami et al., 1996). Our findings indicate that Stat3 activation alone is sufficient to inhibit proliferation of cells that are inhibited in cell proliferation by IL-6 type cytokine stimulation. Furthermore, strong and prolonged activation of Stat3 leads to growth arrest and apoptosis. Taken together, the results in the literature and ours suggest that it might be desirable to inhibit Stat3 signaling in cancer cells showing constitutively active Stat3 signaling, and to activate Stat3 signaling in tumor cells that are inhibited in cell proliferation upon cytokine signaling, to induce in both cases growth arrest and apoptosis. Considering all this, it might be important to check the Stat3 status and the status of known Stat3 target genes to conclude if Stat3 activity leads to either a growth advantage or to a disadvantage. Our data will be very valuable to assess this issue, since we identified Stat3 target genes in cancer cells that are growth inhibited after Stat3 activation, an approach that was not undertaken so far. It is therefore essential to identify the Stat3- and the Stat3 target genes status in the cancerous tissue in order to achieve effective patient tailored cancer therapy.

Given the fact that the biological readout of activated Stat3 in the cases mentioned above can be growth stimulation or inhibition, suggests that the genes, which are transcriptionaly activated by Stat3 might be different in both circumstances. In fact, in our experiments we were not able to detect the majority of the published Stat3 target genes, which is not surprising since these genes have been mainly identified in cells that respond to Stat3 activation with accelerated cell proliferation. Among the known target genes, our analyses has revealed only genes that do not seem to have a function in

stimulating cell proliferation, such as SOCS3, serpin A3, and VEGF. Interestingly only C/EBPδ, a protein that we have identified as being a mediator of the Stat3 induced inhibition of cell proliferation, had previously been identified as a Stat3 target. Other interesting targets, we identified, have not been found in cells that are growth promoted by Stat3 (discussed below). The dissimilarity in identified Stat3 target genes between our results and published results could have cell specific origins including differences in transcription factors, coactivators, corepressors, chromatin remodelers, histone acetylases, deacetylases, kinases and methylases. These are all required for the regulation of gene expression and play important roles in generating the promoter and tissue specific responses of a transcription factor (Orphanides and Reinberg, 2002). The interesting Stat3 targets identified in our studies after short treatment (4h) with 4HT are discussed below.

Several reports have demonstrated that C/EBPδ is a downstream target of Stat3. The C/EBPδ promoter contains a Stat3 binding site and C/EBPδ is up-regulated after OSM treatment of mammary cells (Hutt et al., 2000; O'Rourke et al., 1997). Our results showing the importance of C/EBPδ as a Stat3 target and mediator of Stat3 induced growth inhibition of melanoma cells are in agreement with these studies and with another study showing that C/EBPδ is important for G_0 growth arrest of mouse mammary epithelial cells (Hutt and DeWille, 2002). It is important to note, however, that the C/EBPδ mediated suppression of melanoma cell proliferation, described in this thesis, does not lead to an observed reduction of cells in S phase and increase of cells in G_0-G_1 phase of the cell cycle described by Hutt et al. (Hutt and DeWille, 2002). This might be due to different cell types used in the experiments. Interestingly, while siRNA mediated knockout of C/EBPδ had no effect on the G1 block induced by combined stimulation with 4HT and OSM (data not shown) it partially rescued 4HT induced inhibition in cell proliferation. We propose, that C/EBPδ is a mediator of Stat3 induced inhibition of melanoma cells proliferation, but not of Stat3 induced cell cycle arrest and apoptosis.

Osteopontin (OPN), a ligand for CD44 and most integrin receptors (Furger et al., 2001) is another Stat3 target gene we identified. Analysis of OPN-null mice revealed its important

function in would healing, which is in accordance with the data of a tissue-specific knockout of Stat3 in keratinocytes in which wound healing processes are severely compromised (Liaw et al., 1998; Sano et al., 1999). It could, therefore, be speculated that the observed phenotype in Stat3-null mice in keratinocytes might be due to insufficient up-regulation of OPN; further investigations will be necessary to prove this. Osteopontin has also been shown to be a marker for cancer progression, since elevated levels of osteopontin have been detected in various cancers, especially head and neck cancer, squamous cell carcinomas, hepatocellular and breast carcinomas (Le et al., 2003; Pan et al., 2003; Wang-Rodriguez et al., 2003). Interestingly, Stat3 is frequently activated in these types of tumors and contributes to cellular transformation (Garcia et al., 2001; Grandis et al., 2000; Yoshikawa et al., 2001). It will be of interest to characterize whether elevated levels of osteopontin might be a consequence of Stat3 activity.

Death-associated protein kinase 1 (DAPK1), a pro-apoptotic, Ca^{2+}/calmodulin (CaM)-regulated serine/threonine kinase, which functions as a positive mediator of apoptosis (Shohat et al., 2002), was rapidly increased upon Stat3 activation in our studies. While this up-regulation was not accompanied by increased apoptosis, synergisitic enhancement of Stat3 activity by 4HT and OSM, led to strongly increased expression of DAPK1 and cell death. The possible contribution of DAPK1 to Stat3-mediated apoptosis remains to be determined. We did not observe any signs of apoptosis in melanoma cells after limited DAPK1 up-regulation by 4HT stimulation. This might be due the insufficient upregulation or inactivation by posttranslational modifications e.g. phosphorylation in the CaM regulatory domain, which inactivates the kinase. Interestingly, DAPK1 expression is frequently lost in various tumor cell lines, which gives a selective advantage to cancer cells and might play a causative role in tumor progression (Raveh and Kimchi, 2001). Inactivation of tumor-suppressor genes such as DAPK1 occurs in many cases through mutation or loss of large portion of their genetic sequence or through hypermethylation of CpG islands located in the promoter region. Indeed, DAPK1 has been found to be silenced by methylation in ~ 15 % of human tumors, for example in gastric, lung, bladder, breast, and head and neck cancer, (Chan et al., 2002; Esteller et al., 1999; Lee et al., 2002; Rosas et al., 2001; Sanchez-Cespedes et al., 2000).

It might further be of great interest to analyze tumors, which are positive for phosphorylated Stat3, for mutations in some of the genes mentioned above. Loss of proteins that are important for inhibition of cell proliferation or for induction of apoptosis might be a reason that in some cases, tumor cells demonstrate a growth advantage with constitutively activated Stat3. In addition, these genes might be important markers for tumors with activated Stat3, where Stat3 activation is necessary for inhibition of cell proliferation, which should not be targeted by Stat3 blocking reagents.

The TEL transcription factor a novel negative regulator of Stat3

In this thesis I describe a novel Stat3 target, TEL (ETV6), that is induced after Stat3 activation in a panel of cell lines. We also show that TEL represses Stat3 transcriptional activity as well as Stat3's biological effects. Therefore, TEL is involved in a Stat3 negative feedback loop. The known negative regulators of Stat3 signaling inhibit Stat3 activity by various means. Nuclear and cytoplasmic phosphatases dephosphorylate Stat3 (ten Hoeve et al., 2002; Yamamoto et al., 2002); SOCS3 inhibits JAK activity, therefore abolishing Stat3 tyrosine phosphorylation and activation (Croker et al., 2003; Krebs and Hilton, 2000); GRIM-19 inhibits Stat3 activity by blocking its nuclear translocation and thus preventing Stat3 DNA-binding (Lufei et al., 2003; Zhang et al., 2003); and PIAS3, a nuclear inhibitor, blocks the DNA-binding activity of Stat3 via interaction with Stat3 (Figure 19)(Chung et al., 1997). In contrast, TEL does not inhibit Stat3 nuclear translocation and DNA binding after OSM stimulation. Based on these observations it appears that the mechanism of repression of Stat3 by TEL is different from the known inhibitors of Stat3. TEL does not inhibit Stat3 DNA binding but rather its transcriptional activity. We also addressed the mechanism whereby TEL represses Stat3 activity in our work

Transcription in eukaryotic cells is influenced by the way in which DNA is packaged into chromatin, which is a dynamic macromolecular complex that consists of DNA, histones, and non-histone proteins. The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of four core histones –an H3-H4 tetramer and two H2A-H2B dimers surrounded by 146 bp of DNA. This compact, inaccessible DNA is

transcriptionally repressed, whereas transcriptionally active genes are found in areas of open chromatin, which results from modifications of the nucleosomes. The charged amino-terminal “tails” of histones extend out of the nucleosomes and are targeted for various post-translational modifications, including acetylation, phosphorylation and methylation. Acetylation, the best understood modification, occurs at the ε amino groups of conserved lysine residues located at the N-terminus of all core histones. In general, increased levels of histone acteylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased acetylation (hypoacetylation) is associated with repression of gene expression. The balance between the opposing activities of histone acetyltransferases (HATs) and histone deactylases (HDACs) controls the steady-state level of histone acetylation. Three classes of HDACs have been identified that include 17 proven or putative HDACs. Class I human HDACs include HDAC1, HDAC2, HDAC3 and HDAC8 and seem to be nuclear localized. They bind to numerous transcription factors, either directly or indirectly, through other proteins such as the nuclear-receptor corepressor (N-CoR), the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) protein and through complexes that include mSin3A and NuRD (nucleosomes remodeling and deacetylating). Class II human HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10, and they shuttle between the cytoplasm and the nucleus. Class II HDACs can also bind to SMRT and N-CoR, however, they do not form complexes with mSin3A and NuRD (de Ruijter et al., 2003). Previous studies demonstrated that the recruitment of a repressor complex comprising mSin3A, N-CoR and HDACs is necessary for TEL-induced repression (Chakrabarti and Nucifora, 1999; Fenrick et al., 1999; Guidez et al., 2000; Wang and Hiebert, 2001). In particular, HDAC3 was shown to associate with TEL’s central region (Wang and Hiebert, 2001). Even though we could show with Trichostatin A (TSA), a general HDAC inhibitor, that TEL-mediated repression of Stat3 is also dependent on HDAC activity, the central domain is not essential for the effect, suggesting an alternative mechanism for repression of Stat3. Furthermore, recent studies suggest that the tat-interactive protein, 60kDa (Tip60), a histone acetyltransferase, could be an element of the TEL co-repressor complex. Indeed, it was shown that Tip60 interacts with and acts as a transcriptional co-repressor of TEL (Nordentoft and Jorgensen, 2003). Moreover Tip60, together with

HDAC7, was found to interact with Stat3 and to repress Stat3 activity (Xiao et al., 2003) suggesting that Tip60 might be part of the TEL co-repressor complex for Stat3 activity. However, that study describes that the ETS domain is the interaction domain for TEL and Tip60 and we have evidence that the ETS domain is not essential for repression of Stat3 activity. In fact, we have been able to demonstrate that TEL interacts with Stat3. This suggests that TEL might be recruited to Stat3 via protein-protein interaction and not by direct binding to the DNA. It would be of great interest to identify the components of the protein complex that are involved in repression of Stat3 activity.

Several recent reports highlight the importance of the known negative regulators of Stat3 in cancer. SOCS 1 expression was found to be silenced by aberrant methylation of CpG islands located in the SOCS1 promoter region in 65% of 26 human primary hepatocellular carcinoma (HCC) tumor samples. In cells where SOCS-1 was silenced by methylation and JAK2 was constitutively activated, re-introduction of the SOCS-1 protein suppressed growth and induced apoptosis (Yoshikawa et al., 2001). Hypermethylation of the SOCS1 promoter was also observed in 63 % of human multiple myelomas, 60 % of acute myeloid leukemia, and in 67 % of chronic myeloid leukemia (Chen et al., 2003; Galm et al., 2003; Liu et al., 2003). Moreover a new study showed that SOCS3 is frequently silenced by hypermethylation in breast- and lung cancer cell lines and in primary lung cancer tissue. Silencing was associated with higher levels of phosphorylated Stat3; restoration of SOCS3 lead to suppression of cell growth and apoptosis (He et al., 2003). Constitutively active Stat3 has also been shown in anaplastic large cell lymphoma where PIAS3 expression was silenced (Zhang et al., 2002). Considering these results, the loss of a negative regulator of Stat3 appears to bring growth advantages to cancer cells. There is accumulating evidence that TEL might be lost in cancer as shown in acute lymphoblastic leukemia of childhood, and in ovarian- and prostate cancer (Hatta et al., 1997; Kibel et al., 2002; Stegmaier et al., 1995). Since we found that TEL is a Stat3 inhibitor, loss of TEL could, therefore, contribute to Stat3's oncogenic abilities. Further analysis will be necessary to screen for TEL deletions and/or mutations in cancers that show constitutive active Stat3 signaling and to evaluate whether loss of TEL contributes to cancer development.

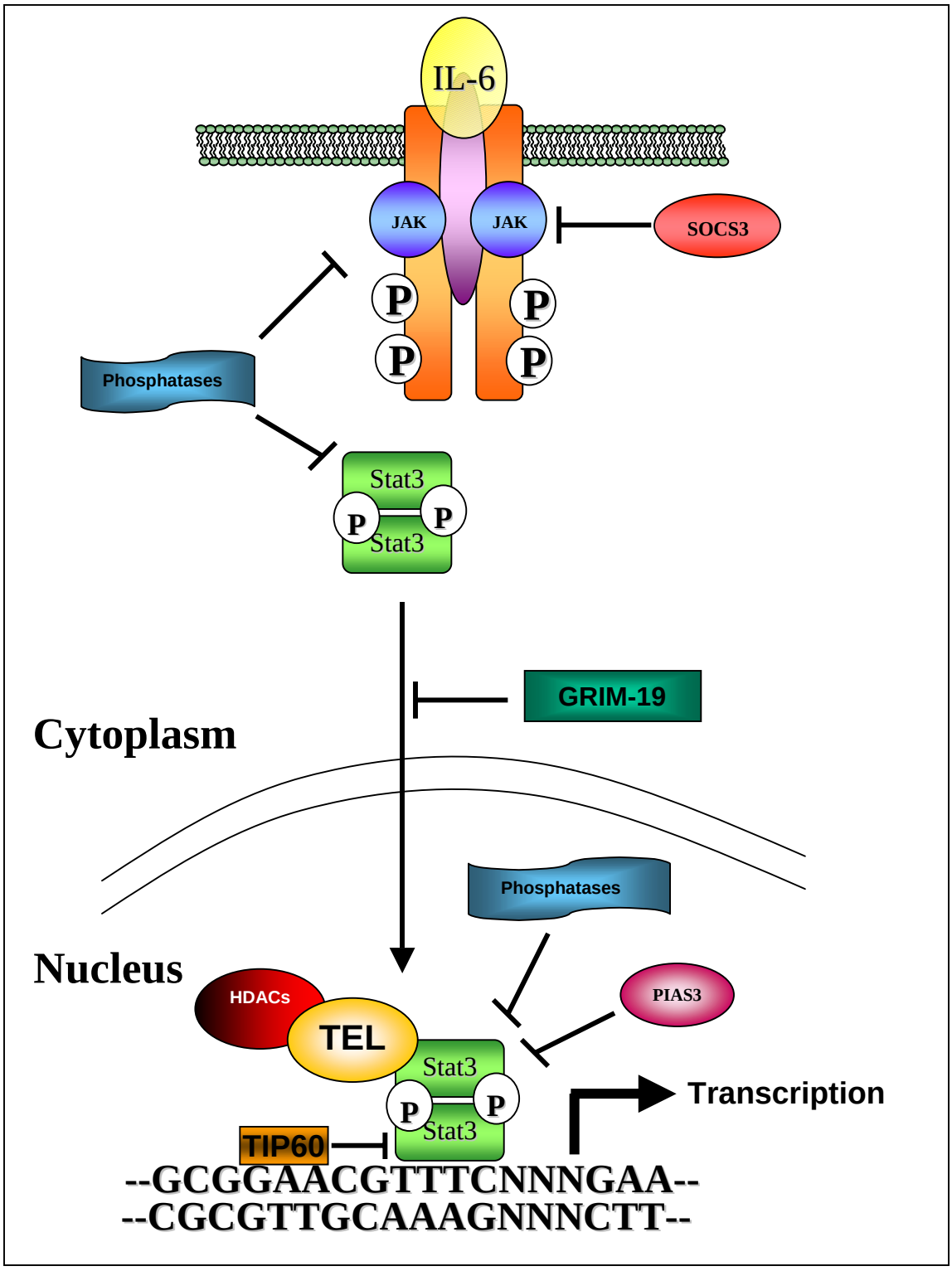


Figure 19: Model of the negative regulation of Stat3 signaling.

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in anaplastic lymphoma kinase-positive T/null-cell lymphoma. *J Immunol*. 168:466-74.

VIII. Curriculum Vitae

Nicole Schick

Address

Friedrich Miescher Institute
Maulbeerstrasse 66
CH 4058 Basel
Switzerland
Tel. +41 61 697-6651
Fax. +41 61 697-3976
nicky@fmi.ch

Home Address

Eimeldingerweg 20
79576 Haltingen
Germany

Tel. +49 7621 668751

Date of Birth:	June 27, 1971
Nationality:	German
Marital Status:	Single
Languages:	English and German

Education

1981-1987	Secondary school, Pestalozzi-Realschule, Mannheim, Germany.
1991-1992	Comprehensive secondary school, Helene-Lange-Schule, Mannheim, Germany.
1992-1997	Master of Science in Food Technology, University of Applied Sciences Trier, Germany,
1999- present	Ph.D. student in the Laboratory of Dr. Nancy Hynes, at the Friedrich Miescher Institute, Basel Switzerland. Research area: Role and regulation of the JAK-STAT signaling pathway in tumor cell proliferation.

Professional Experience

1987-1991	Apprenticeship as a Chemical Laboratory Assistant (Chemielaborant) at Boehringer Mannheim, Mannheim, Germany.
1996-1997	MS work in the Laboratory of Dr. Wolfgang Fischer, department of. Dr. Wylie Vale, the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA, USA.
1997-1999	Research Intern, in the Laboratory of Dr. Wolfgang Fischer, department of Dr. Wylie Vale, the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA, USA.

Patent

TEL/ETV6-mediated inhibition of cell proliferation. Patent pending

Awards and Honors

1987	Outstanding student of the year 1987, Pestalozzi-Realschule, Mannheim, Germany.
1992	Outstanding student of the year 1992, Helene-Lange-Schule, Mannheim, Germany.
1993-1996	Fellowship of the German National Scholarship Foundation, Bonn, Germany.
1996	Stipend for performing research at a foreign institution, German National Scholarship Foundation, Bonn, Germany.
2002	Poster Award, awarded by Nature Reviews Molecular Cell Biology, during the 3rd International Conference of Signal Transduction, Cavtat/Dubrovnik, Croatia.

Meeting Presentation

2001	USGEB Young Investigator Meeting, Université de Lausanne, Lausanne, Switzerland. Poster presented: Stat3 mediates inhibition of melanoma cell proliferation.
2002	4th Swiss Cell Cycle & Signaling Workshop, Switzerland. Oral presentation: Stat3 mediates inhibition of tumor cell proliferation.
2002	International Conference on Signal Transduction, Cavtat/Dubrovnik, Croatia. Poster presented: Stat3 dependent inhibition of proliferation and induction of Apoptosis.
2003	2003 Salk Institute/EMBL meeting on Oncogenes & Growth Control. Poster presented: TEL (ETV6), a Novel Stat3 Target Gene, Represses Stat3 Activity

IX Publications

TEL/ETV6 is a Stat3-induced repressor of Stat3 transcriptional activity.
***Schick, N.**, Oakeley, E.J., Hynes, N.E., Badache, A.*
Submitted to JBC, (2003)